Trapped in the Matrix

Neutrophil Extracellular Traps (NETs) and Fibrin in Wound Healing

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Trapped in the Matrix: Neutrophil Extracellular Traps (NETs) and Fibrin in Wound Healing

Gevangen in de Matrix: Neutrophil Extracellular Traps (NETs) en Fibrine in Wondgenezing

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“I have not failed. I've just found 10,000 ways that won't work.”
-Thomas A. Edison

“The greatest danger for most of us is not that our aim is too high and we miss it, but that it is too low and we reach it.”
-Michaelangelo

To everyone who believed in me
As much as to the people who said I couldn’t do it
Don’t worry, I made wrong hypotheses as well

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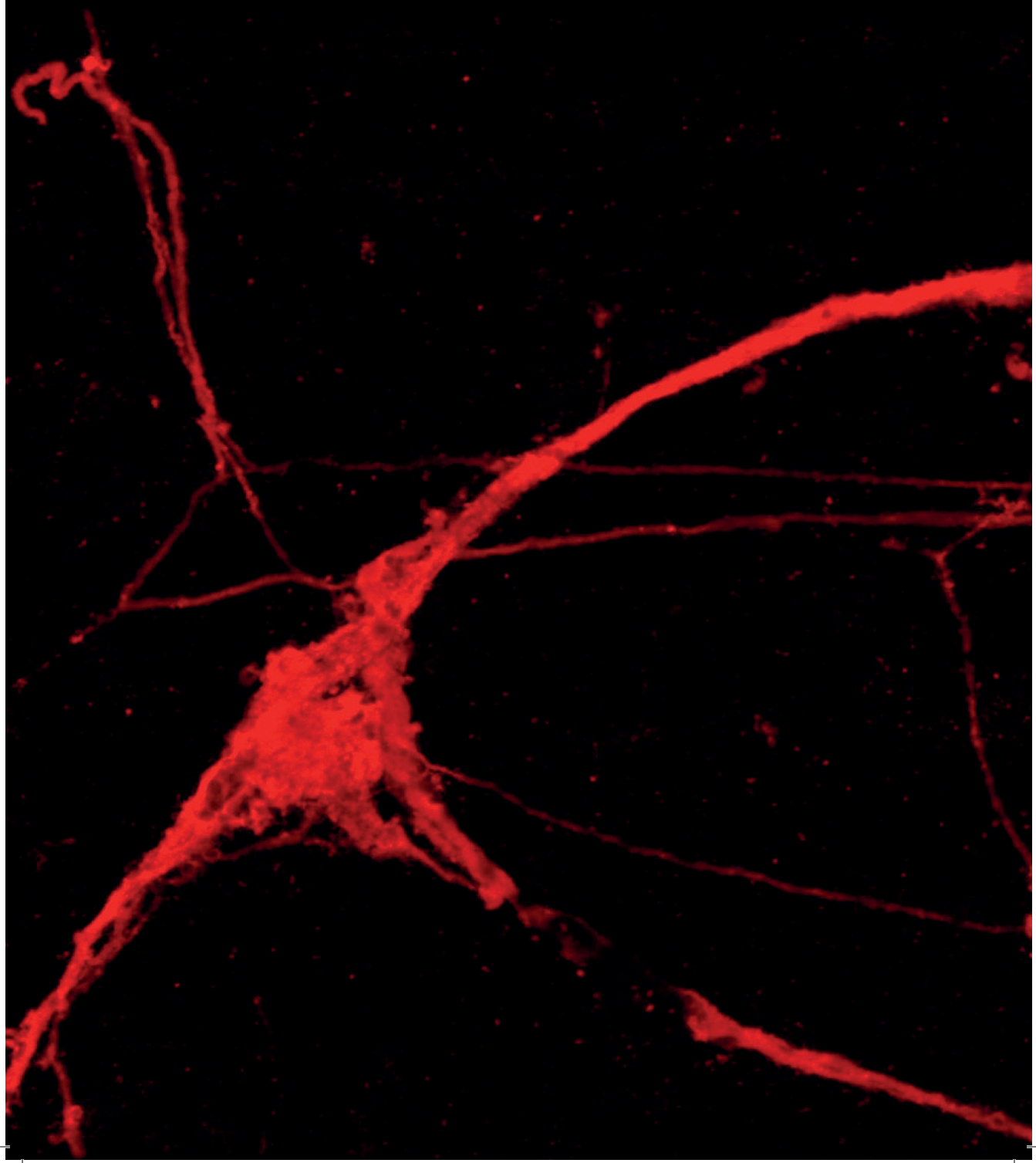
Chapter 1

General introduction and thesis outline



Wound healing: a general outline

Non-healing wounds are a major problem in diseases like venous insufficiency, post-thrombotic syndrome and diabetes mellitus (DM). Especially with an increase in DM in the Western modern population, research in wound healing is getting more and more important. In normal wound healing, after tissue damage, three overlapping healing phases can be distinguished: the inflammatory, proliferation and remodeling phase (1).

Wound healing phase one: inflammation

Clot formation

The inflammatory phase usually takes 4-6 days in normal wound healing. Directly after tissue damage, a thrombus, is formed as a result of activation of the coagulation cascade. The coagulation cascade is activated by the release of Tissue Factor (TF), which is a part of the extrinsic pathway (2, 3). Tissue Factor activates thrombin (FII) formation via activation of several factors, including FIXa and FVIIIa (Fig. 1). In addition, *in vivo*, the pathway intrinsic pathway plays a role, which results in more thrombin formation, which is necessary in hemorrhage of large wounds.

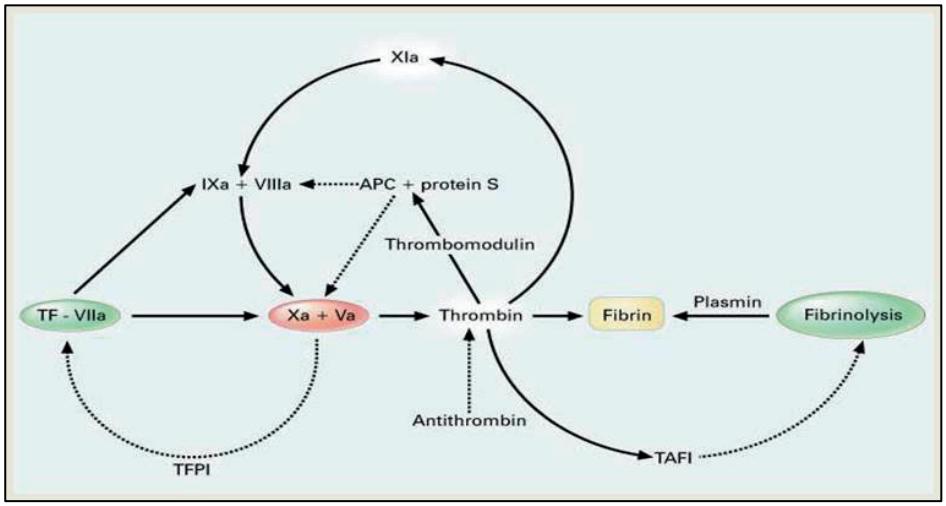


Figure 1: The coagulation cascade. Vanderbroucke et al., 2001. N Engl J Med.

Thrombin cleaves the fibrinopeptides A and B of fibrinogen, which converts fibrinogen into fibrin. Multiple cleaved fibrin monomers bind together, forming a fibrin network, which is stabilized by cross-linking by Factor FXIIIa (Fig. 2). This thrombus, also containing platelets, stops the wound from

bleeding and provides a temporary scaffold for invading cells, such as endothelial cells and inflammatory cells (2, 4, 5). Platelet coagulation in the thrombus is facilitated by Von Willebrand Factor (VWF), a glycoprotein which is released by the endothelium when damaged. Furthermore, VWF stimulates the clotting cascade by binding to FVIII and collagen. VWF is then cleaved by a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13). Lack of VWF leads to bleeding disorders, whilst non-clearance of vWF leads to a high risk of thrombosis.

A few hours after thrombus formation, neutrophils are the first inflammatory cells to invade the wound area. These short-lived cells are a part of the innate immune system and clear the debris by phagocytosis. Additionally, they are also able to phagocytose and kill bacteria by producing toxic components such as reactive oxygen species (ROS), reactive nitrogen species (RNS) and myeloperoxidase (MPO) (6). Recently, it has been found that neutrophils are able to excrete their own DNA and form so-called neutrophil extracellular traps (NETs) (7). NETs will be discussed in detail later. Several hours after neutrophil invasion, monocytes arrive at the wound site, where they convert into macrophages. Both the attraction and conversion of macrophages are modulated by the fibrin matrix (2), which makes it important in the transition from a pro-inflammatory environment into an anti-inflammatory, or wound healing, environment. In the early phase wound healing, macrophages are pro-inflammatory (M1), but soon switch to an anti-inflammatory (M2) phenotype. M2 macrophages secrete growth factors like TGF-β by phagocytosis of apoptotic neutrophils (8), which promote the transition to an anti-inflammatory state and the next phase of wound healing.

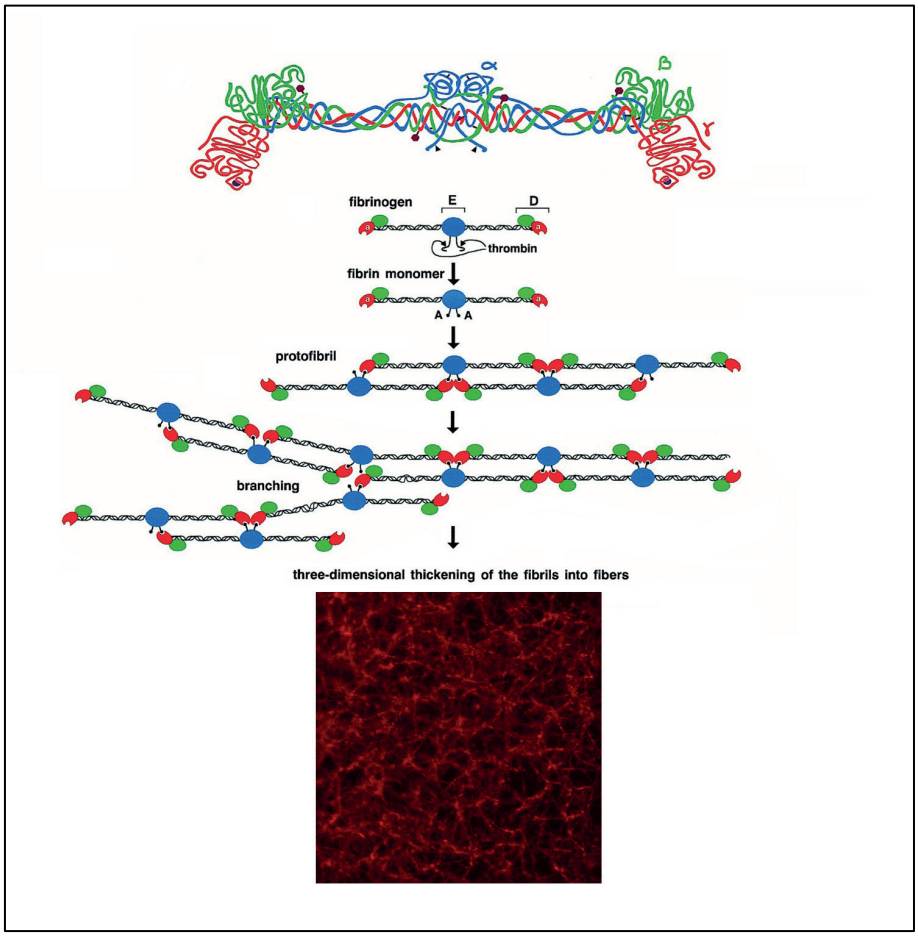


Figure 2: The conversion of fibrinogen to a fibrin network. Adapted image from Côté et al., 1998. Blood.

Wound healing phase two: proliferation

Around 4 days after wounding, when the inflammatory cells in the tissue start producing anti-inflammatory growth factors and cytokines, endothelial cells (EC) infiltrate the wound bed. The formation of new vessels, or angiogenesis, predominantly takes place when these ECs bind to fibrinogen (2, 9). The ingrowth rate of these ECs depends on the fibrin structure, which will be described in more detail later. Fibroblasts are also attracted to the wound bed and contribute to collagen deposition. After approximately 5 days these fibroblast proliferate into myofibroblasts, which causes wound contraction and closure. Furthermore, they synthesize Extracellular Matrix (ECM) components, mainly collagen, that gradually replace the fibrin matrix. The proliferation phase can take around 20 days in normal wound healing.

Wound healing phase three: remodeling

After approximately 21 days in normal wounds, the remodeling phase of wound healing is initiated.

This last phase of wound healing is characterized by the replacement of myofibroblasts by collagen and other extracellular matrix (ECM) proteins. Maturation of the collagen, which in skin occurs in different types (I, III and IV), can take three weeks and may even take a year in the case of a large wound.

A close-up of the coagulation cascade: fibrin(ogen)

As previously mentioned, fibrin is one of the most important ECM proteins in early wound healing, as it provides a scaffold for migrating cells and angiogenesis (10). Fibrin is formed after the cleavage of fibrinopeptide A and B from fibrinogen. Fibrinogen is a soluble glycoprotein produced mainly by hepatocytes. It consists of three chains: Aα, Bβ and γ. All three components, are produced separately in the hepatocytes, encoded by three different genes. Two chains of each component are connected by disulfide bridges to form fibrinogen. All of the chains form a coiled coil (Fig. 3). The molecule has three major domains: one E-domain (middle) and two D-domains (containing carboxy-terminals of Bβ and γ chains and part of the Aα chain) (2, 11, 12). The two αC regions (carboxy-terminals) of the Aα chains are important in fibrin assembly, activation of factor XIII, cell adhesion and modulation of fibrinolysis (4).

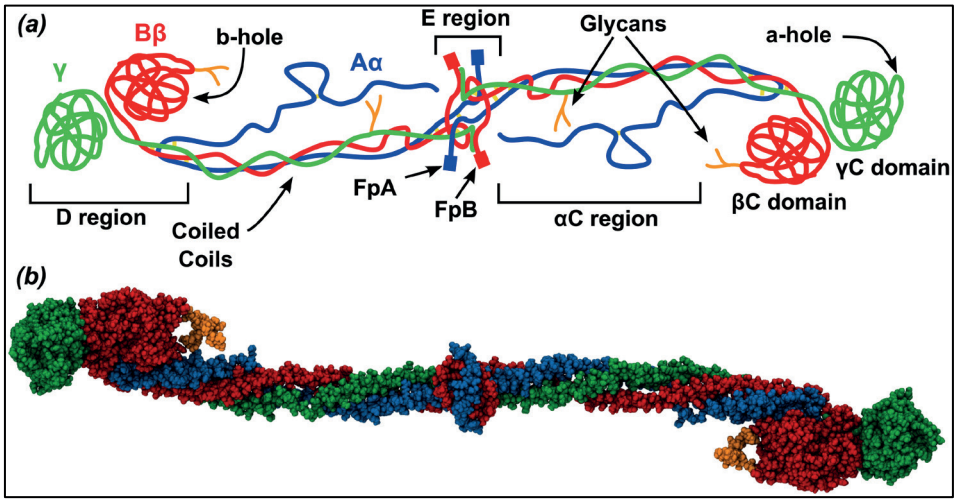


Figure 3: The fibrinogen molecule. Source: Köhler *et al.*, 2015 (13)

In the presence of thrombin, cleavage of fibrinopeptide A and B occurs, exposing two polymerization sites at the N-termini of the α and β chains (14). These sites are able to bind to the αC-termini of other fibrinogen molecules cleaved by thrombin, and are important in mediation of the fibrin matrix assembly and interaction with a large spectrum of cells, including heparin, endothelial cells, fibroblasts and platelets. The fibrin matrix is stabilized by factor XIIIa, formed by thrombin from factor XIII in the presence of calcium, which cross-links the C-termini of γ chains of the fibrinogen molecules (15). The structure of the fibrin matrix depends on many factors such as fibrinogen levels, platelet, thrombin and calcium concentrations, clotting rate, pH, glucose levels and modifications in the fibrinogen molecule (16, 17). These factors can be stimulated by events such as smoking, myocardial infarctions, coronary artery disease, strokes and diabetes. Thrombosis is also associated with inflammatory diseases like rheumatoid arthritis, inflammatory bowel disease and chronic obstructive pulmonary disease. Clot structure is important for processes like fibrinolysis and EC ingrowth. For example, if a clot is dense, EC ingrowth is delayed, leading to inhibited angiogenesis (18). Also, fibrinolysis is inhibited, leading to a persisting fibrin clot which delays the transition from the proliferation phase to the remodeling phase in wound healing.

Fibrinolysis

Regulation of the clotting cascade is a crucial process. Absence of clot formation can cause extensive bleeding after damage to the vessel wall, and a persisting clot can be the cause of thrombosis. Thrombi may have an altered

structure, resulting in resistance to fibrinolysis. Fibrin can be cleaved by plasmin. Plasminogen is a zymogen that is activated to plasmin by plasminogen activators, such as tissue-type plasminogen activator (tPA). tPA is produced by endothelial cells and is able to bind to fibrin, which activates a positive feedback loop (14). Thrombin, on the other hand, is able to activate thrombin-activatable fibrinolysis inhibitor (TAFI), which is a plasma protein that can inhibit fibrinolysis (15, 19).When bleeding is controlled, the pro-inflammatory response weakens and the wounded site is more at equilibrium. Only then, fibrinolysis occurs and the fibrin matrix can be replaced by myofibroblasts and collagen tissue.

A specific process in the inflammatory phase: neutrophils and NETs formation

Neutrophils are short-lived inflammatory cells. Immediately after wounding, they are recruited to the wound site. Neutrophils can be linked to three different antimicrobial mechanisms: (1) Phagocytosis: pathogens are engulfed into a phagosome and destroyed by NADPH-dependent mechanisms or by antimicrobial proteins. (2) Degranulation: pathogens are killed by antimicrobial proteins, that are released into the ECM by neutrophils and (3) NETosis, a process in which neutrophils excrete their DNA, histones and other antimicrobial components into the ECM by degrading their own membranes (20).

NET formation

NETosis, the process of neutrophil extracellular traps (NETs) formation, is the most recently described antimicrobial mechanism of neutrophils. During NETosis, NETs are released. They consist of extracellular fibers of decondensed chromatin and granule proteins, such as antimicrobial factor myeloperoxidase (MPO) and neutrophil elastase (NE). NETs trap and kill microbes efficiently (7).

It is generally believed that MPO plays a very important role in the breakdown of the nuclear envelope. Perforation of the outer membrane of the neutrophil follows after the components are mixed (21). This makes NETosis very different from apoptosis and necrosis, as in these processes the nuclear envelope and granules remain intact (22). However, the full mechanism behind NETosis remains unclear.

During infection, NETosis is activated by the presence of pathogens. Bacteria and fungi are very strong inducers of NETosis (23, 24). One of the strongest inducers of NETosis is *Staphylococcus aureus* (*S. aureus*)(24). *S. aureus* can cause major problems and infections by Methicillin-resistant *Staphylococcus aureus* (MRSA) are very hard to treat and worldwide of medical concern. NETs might be an effective way to limit bacterial spreading and to improve bacterial clearance. However, *S. aureus* has defense mechanisms that conquer NETs. By producing nuclease, they are able to break down NETs (25) and escape, whilst the number of neutrophils decreases, as they die after NETosis.

In addition to pathogens, a variety of other inducers of NETosis has been described. These include PMA (7, 22, 26), LPS (7, 27, 28) and calcium ionophores (29, 30). *In vivo*, NETs play a role in pathophysiological conditions such as tuberculosis, pneumonia, lung fibrosis, sepsis and thrombosis (31-34). Thrombi have been shown to be more persistent when NETs are present (35), and patients with sepsis that have higher NETs levels in plasma are considered to have a worse outcome due to their inflammatory status (36) and contribution to the formation of microthrombi (37).

NETs are able to interact with several factors of the coagulation cascade. Platelets are known to bind neutrophils, which stimulate both neutrophil and platelet activation and platelet aggregation, by the release of multiple inflammatory cytokines and other molecules (35, 38). Also, NETs are able to stimulate fibroblasts to differentiate into myofibroblasts (34) and stimulate fibrin formation and deposition in thrombi by triggering FXII and TF (32, 39). Fibrin clots were shown to have thicker fibrin fibers in the presence of histones, which are abundantly present in NETs (40). This effect can be enlarged in the presence of DNA; as the diameter of the fibers increases, the network itself becomes stiffer, making it harder to lyse. In wound healing, this could be problematic, as clot lysis is necessary for the transition from fibrin to collagen. Also, persisting NETs cause a persisting inflammatory response, as they contain anti-microbial factors such as MPO and NE. Therefore, it we hypothesized that NETs play a role in non-healing wounds.

Disturbed wound healing in diabetes

Patients with diabetes, especially type 2, often suffer from chronic wounds or ulcers, due to their thin skin, vascular insufficiency and neuropathy. The fibrin network of diabetic wounds is denser and less porous (41), due to slower

coagulation and higher fibrinogen levels. A denser fibrin matrix could lead to disturbed EC and fibroblast ingrowth, causing inhibition of angiogenesis and collagen deposition (2, 18). Furthermore, diabetic chronic wounds are considered to be arrested in the pro-inflammatory phase of wound healing. Insulin resistance in type 2 DM has been linked to increased pro-inflammatory factors such as TNF-α, IFN-ɣ and IL-1β (42). This pro-inflammatory state of the immune cells causes insensitivity to actual stimuli, such as bacterial infections. Instead, neutrophils from diabetic patients are more prone to form spontaneous NETs (43). As NETs contain cytotoxic histones and MPO, they have a negative effect wound the progression of wound healing, as their compounds inhibit processes like re-epithelization (44, 45) and angiogenesis (46). Normally, NETs are cleared by macrophages, but in chronic wounds it could be expected that this function is also compromised. If true, NETs could play an important role in the persisting pro-inflammatory phase in diabetic chronic wounds, disturbing the healing process.

Aim and outline of this theses

In this thesis, we studied the induction and the effect of NETs in fibrin in different diseases, with a focus on diabetic wound healing. The aim of this thesis is to obtain more insight in the effect of NETs and the induction of NETosis in chronic wounds.

In the first part we focus on the induction of NETs. In **chapters 2-4** we investigate different stimuli that cause NETosis. Because many different stimuli of NETosis have been described in literature, we made an overview of different stimuli and we test several inducers in a standardized setting in **chapter 2.** Bacteria are very potent inducers of NETosis, so in **chapter 3**, we investigate the role of Protein A, a well-known immunomodulator produced by *S. aureus*, on NETosis. Bacterial biofilms also have been described to influence neutrophil function. Therefore, in **chapter 4** we investigate *S. aureus* formation of biofilm and its association with neutrophils. NETs play a role in different diseases, such as sepsis and thrombosis. In **chapters 5 and 6** we look at the effect of NETs and nucleosomes (extracellular DNA) on children with sepsis and patients with arterial thrombosis at a young age. In **chapter 5**, we study NETs (MPO-DNA) levels in serum from children with Meningococcal sepsis and correlate these levels to patient outcome and inflammatory markers that can cause NETosis. VWF cleavage is stimulated when VWF and

ADAMTS13 are bound to NETs and eDNA. This process is hypothesized to be further affected by FH. Therefore, in **chapter 6**, we studied the effect of Factor H on the cleavage of von Willebrand factor (VWF) by disintegrin and metralloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13) in young patients with arterial thrombosis ('Arterial Thrombosis at a young age: the role of TAFI and other Coagulation factors (ATTAC)' study). Fibrinogen levels in diabetic patients are higher and form clots in diabetic wounds that are inadequate for achieving optimal angiogenesis. Therefore, in **chapter 7** we study the effect of healthy fibrin applied to a diabetic wounds wound in rats.

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Chapter 2

In vitro induction of NETosis: comprehensive live imaging comparison and systematic review

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Abstract

Multiple inducers of *in vitro* Neutrophil Extracellular Trap (NET) formation (NETosis) have been described. Since there is much variation in study design and results, our aim was to create a systematic review of NETosis inducers and perform a standardized *in vitro* study of NETosis inducers important in (cardiac) wound healing. *In vitro* NETosis was studied by incubating neutrophils with PMA, living and dead bacteria (*S. aureus* and *E. coli*), LPS, (activated) platelets (supernatant), glucose and calcium ionophore Ionomycin using 3-hour periods of time-lapse confocal imaging.

PMA is a consistent and potent inducer of NETosis. Ionomycin also consistently resulted in extrusion of DNA, albeit with a process that differs from the NETosis process induced by PMA. In our standardized experiments, living bacteria were also potent inducers of NETosis, but dead bacteria, LPS, (activated) platelets (supernatant) and glucose did not induce NETosis.

Our systematic review confirms that there is much variation in study design and results of NETosis induction. Our experimental results confirm that under standardized conditions, PMA, living bacteria and Ionomycin all strongly induce NETosis, but real-time confocal imaging reveal different courses of events.

Introduction

Neutrophil extracellular traps (NETs) formation, also called NETosis, is considered one of the defense mechanisms against pathogens [1]. During NETosis, the nucleus of a neutrophil decondenses and the nuclear envelope breaks, mixing chromatin, cytoplasmic and granular components. Also, the cell membrane breaks, followed by an extrusion of the neutrophil's DNA, histones and antimicrobial proteins into the extracellular space [1]. Subsequently, pathogens are trapped in the NETs and either killed by the toxicity of the antimicrobial substances of the NETs, or immobilized to facilitate phagocytosis by other neutrophils or macrophages [2]. NETs have been shown to play a role in multiple diseases, such as thrombosis [3-6], fibrotic diseases [7], cardiovascular diseases [8] and sepsis [9-11]. Therefore, elucidation of the mechanism behind NETosis has become an increasingly important topic.

Many stimuli have been reported to induce NETosis [12]. Gram positive [1,13,14] and negative bacteria [1,14] and fungi [15] induce NETosis and subsequently are

trapped in the NETs. Many other inducers have also been described, but their NETosis inducing capabilities are not consistent. These include lipopolysaccharides (LPS) [1,10,16-18], inflammatory cytokines such as IL-6 [19] and IL-8 [1,16,18,20] and the calcium (Ca^{2+}) ionophore A23187 and Ionomycin [21,22]. A difference in experimental methods and definitions of NETosis might contribute to these conflicting results.

For in vitro studies, phorbol 12-myristate 13-acetate (PMA), a plant derived organic compound and well-known activator of the ubiquitous signal transduction enzyme protein kinase C (PKC), is often used as an inducer of NETosis 1,3,12,18. So even though PMA is consistently reported as NETosis inducer, it is not physiologically relevant, since it does not activate physiological processes in vivo. Therefore, it is important to study the effects of other, physiological, NETosis inducers. This is especially relevant in studies on human diseases, such as cardiovascular wound healing, in which NETs also play a role. Results from published studies often cannot be compared because they are derived from a multitude of experimental settings. Therefore, we first made a comprehensive systematic review to make an overview of inducers of interest in cardiovascular wound healing. Subsequently, we selected the most relevant inducers and tested their effect on NETosis induction in a standardized experimental setup using static conditions and imaged using time-lapse confocal imaging.

Material and methods

Systematic Literature Review

A systematic literature review of the Medline-Ovid, Embase, Web of Science and Cochrane databases was conducted, using search and selection criteria according to the PRISMA-2015 criteria for writing a systematic literature review [23]. MeSH-terms for “neutrophil extracellular traps” were not available. We therefore used the search terms “neutrophil extracellular traps” and/or “NET(osis)”. Moreover, we included only journal articles about *in vitro* NET induction, and only inducers that were described as inducer of NETosis by at least two papers. Only journal articles of which the full-text was available to us were included. Reviews were excluded. MEDLINE-OVID: (neutrophil extracellular trap* [TIAB] OR NETosis [TIAB]) NOT Review NOT patient* [TI] Select “journal article”. Articles were included up to January 2017. This review was executed independently by two researchers to prevent bias. Outcomes of

this review were used for creating an overview to perform comparison experiments only.

Neutrophil isolation

Neutrophils were isolated from blood from healthy donors using density gradient medium Lymphoprep™ (Stem cell Technologies, Grenoble, France). All experiments were approved by the Medical Ethics Committee of the Erasmus MC. Blood was diluted 1:1 with PBS (Phosphate Buffered Saline without Ca^{2+}/Mg, 17-516F, Lonza, Walkersville USA), loaded onto the Lymphoprep™ and centrifuged at 830 x g for 15 minutes at room temperature. Erythrocytes were lysed by incubation with erythrolysis buffer (3.1M NH_4Cl, 0.2M $KHCO_3$, 0.02M EDTA, pH 7.4) for 10 minutes at room temperature followed by centrifugation at 690 x g for 8 minutes at room temperature. Cells were washed two times (690 x g for 8 minutes and 560 x g for 5 minutes) with HEPES buffer (0.115M NaCl, 0.012mM $CaCl_2$, 1.51mM $MgCl_2$, 4mM KCl, 0.01M HEPES, pH 7.4) and the concentration of cells was determined using a ABX Micros 80 cell counter (Horiba, Irvine, California).

Neutrophils were transferred to DMEM culture medium containing 10% FCS, L-glutamine and Penicillin/Streptomycin (all from Biowhittaker, Lonza, Walkersville, USA) or DMEM culture medium without any additions for bacterial experiments. Hoechst 34580 (1:10 000, Life Technologies, Landsmeer, The Netherlands) for staining DNA and Propidium Iodide (PI, 1:400, Sigma Aldrich, Zwijndrecht, The Netherlands) for staining extracellular DNA were added and cells were incubated for at least 1 hour at 37^0C on gelatin-coated (Sigma Aldrich, Zwijndrecht, The Netherlands) 24 wells glass-bottom plates.

Selection of NETosis inducers for in vitro experiments

From the inducers we documented in our search we selected inducers that were well described and play a role in (cardiovascular) wound healing. We then selected the best described inducers as well as the inducers with the most variation in reported effect to test in our own standardized experimental setup. As a source for cytokines, we used activated platelets supernatant, containing cytokines such as IL-8, PDGF and VEGF [24,25].

Bacterial strains and culture

Gram-positive (*S. aureus* Newman) and gram-negative (*E. coli* ATCC 25922 (O6:B1)) bacteria were cultured in 100 ml Iscove's Modified Dulbecco's Media

(Gibco® IMDM medium, Life Technologies, Landsmeer, The Netherlands) at 37^0C overnight. The next day the bacteria were diluted to a final concentration of 10^8 bacteria per ml as determined by OD_{600} measurements . For experiments with dead bacteria, the bacteria were either killed by incubation at 90^0C for 10 minutes or by exposure to UV light with 6000 μWs/cm^2 for 66 seconds.

NETosis induction and time-lapse imaging

Isolated neutrophils (10^7 cells/well) were added to a 24 wells plate in a final volume of 500 μl. Stock solutions of PMA and Ionomycin were prepared in dimethyl sulfoxide (DMSO, Sigma Aldrich). Platelets (platelet rich plasma) were isolated from EDTA blood by centrifugation for 7 minutes at 260 x g without brake, and activated for 10 minutes by adding thrombin (1 U/ml). Activated platelets supernatant was collected by centrifuging the activated platelets at 2000 x g for 10 minutes.

To induce NETosis, non-bacterial inducers were individually added to each well. Before addition (t=0), an image was taken and starting directly after addition of the inducers, cells in a random field were imaged every 15 minutes for 3 hours with a 20x 0.7 n.a. lens by using confocal microscopy (Leica SP5 AOBS, Leica Microsystems, Wetzlar, Germany). Excitation with a 405 laser and a BP 420-500 emission filter for Hoechst and a 561 excitation and BP 580-620 emission filter for PI. In this setting, the dish was mechanically moved between fields. We stopped imaging after three hours, since after three hours spontaneous cell death was observed in control neutrophils. In experiments containing bacteria, we imaged continuously for one hour since all neutrophils underwent NETosis within one hour in all bacterial conditions. We defined NETosis as a host defense mechanism in which neutrophils release their nuclear and granular contents to contain and kill pathogens[21]. The NETs that are released form extensive webs of DNA coated with cytotoxic histones and microbicidal proteases. In cells that stained only positive for Hoechst, the cell membrane was still intact. After breakdown of the cell membrane, the DNA became PI positive. Unstimulated cells (in experiments without platelets) and resting platelets were used as negative control and PMA stimulated cells were used as positive control.

Immunofluorescence

To confirm *in vitro* NETosis in the bacteria experiments, we added an immunofluorescent staining with a MPO-Dylight488 complex (1:250) to the

neutrophils immediately before induction. Then, we quantified the positive NETs by using confocal microscopy (Leica SP5 AOBS).

As another measurement for NETosis, cells were stimulated by the described inducers for 3 hours, fixed and stained for myeloperoxidase (MPO, Dako). Briefly, after antigen retrieval with Proteinase K, the slides were blocked with skim milk powder (5%) in PBS Tween 0.1% pH7.4 and incubated overnight with polyclonal rabbit anti-human MPO (1:300) at 4°C. After washing with PBS Tween 0.1% pH7.4 slides were incubated with secondary antibody Dylight goat anti rabbit 488 (1:200) for 30 minutes. Slides were mounted with Prolong Diamond antifade with DAPI (Thermofischer). Images were made by using confocal microscopy (Leica SP5 AOBS) and Structured Illumination Microscopy (Zeiss Elyra PS1 LSM 780 structured illumination microscope, Carl Zeiss, Jena, Germany).

Image Analysis

All images were analyzed using ImageJ (Version 1.49, National Institutes of Health, USA). We quantified the number, area and mean intensity of Hoechst positive and PI positive cells using a macro that includes a segmentation of the nuclei on a Gaussian blurred image (sigma=2px) with a threshold and a watershed segmentation (supplemental file 2). A minimal and maximal size of Hoechst positive and PI positive cells was included in the macro. The Hoechst and PI threshold was kept constant within one experiment. We determined the ratio of PI positive cells and corrected at t=0 for dead cells in the start mixture. To correct for regular cell death during the experiment, conditions were compared to the negative controls: no additions or resting platelets, in which no NETosis was observed.

Statistics

All data are presented as mean±SEM. A repeated measurements ANOVA was used to detect differences in NET ratio with time and inducer as independent parameters. Results were considered statistically significant when $p<0.05$. Data were analyzed using SPSS v22 (IBM, USA).

Results

Systematic Literature Review

Our systematic search strategy resulted in 870 scientific articles, of which 655 were excluded following selection according to the described criteria (Fig. 1).

In the 215 remaining articles we identified 25 different NETosis inducers. These inducers are presented in Table 1.

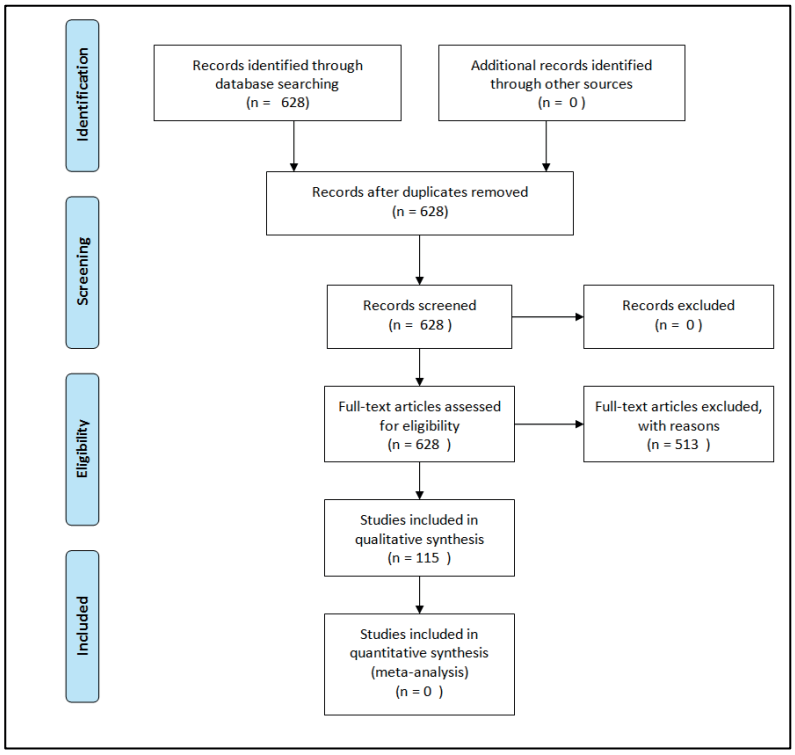

Figure 1: PRISM chart of the systematic literature review.

Table 1: Overview of in-literature described in vitro NETs inducers. MOI: Multiplicity Of Infection (number of bacteria to number of cells). CFU: Colony Forming Units.

Inducer	Concentration	Induction time	NETosis	Reference
PMA	4-50 nM (3-30.8 ng/ml)	10 min-16h	Yes	1-3,12,16,19,20,26-112
	60-100 nM (37-62 ng/ml)	30 min-16h	Yes	7,18,21,113-146
	120-1620 nM (74-1000 ng/ml)	10 min-4h	Yes	147-166
	100000 nM (6168 ng/ml)	10 min-24h	Yes	14,167,168
H_2O_2	0.1 µM	3h	No	116
	100-1000 µM	4h	Yes	61,114
	4000 µM	200 min	Apoptosis	18
	10000 µM	200 min	Necrosis	18
	10000 µM	4-5h	Yes	54
	0.03%	3h	Yes	169
Growth factors/platelets				

IL-8	1-250 ng/ml	10 min-5h	Yes	1,12,27,29,42,64,161,170-172
	10 ng/ml	3h	Little	16
	100-800 ng/ml	4-18h	No	18,76,82
IL-1β	10 ng/ml	6h	Little	62
	50 ng/ml	2h	Yes	27
TNF-α	1 ng/ml	6h	Little	62
	7-20 ng/ml	30 min-5h	Yes	19,20,54
	100 ng/ml	2h	Yes	27
	100 ng/ml	4h	No	76
Platelets	$5x10^7$ /ml		No	10
	$2x10^5 – 5x10^5$	1h	No	44
Activated platelets	$2x10^5 – 5x10^5$ (+ 50 µM TRAP)	1h	Yes	44
	$5x10^5$ (+ 1.3 µg/mL collagen)	2h	Yes	173
	1:400 (+ 0.01 U/mL Thrombin)	4h	Yes	174
	25-100 ml (+ 5 µmol/L PGE1)	20 min	Yes	175
	25-100 ml (+ 25 µmol/l TRAP-6)	20 min	Yes	175
	25-100 ml (+ 5 µmol/l ADP)	20 min	Yes	175
	25-100 ml (+ 1 µg/ml collagen)	20 min	Yes	175
	25-100 ml (+ 0.05 IU/ml recombinant thrombin)	20 min	Yes	175
	(+CoCr)		Yes	176

Calcium

A23187	0.2-25 µM	20 min-4h	Yes	22,130,151,177-179
	1 µM	1h	No	180
	100 µM	1-4h	Little	167
Ionomycin	0.9-7 µM	30 min-4h	Yes	21,29,34,130,132
	100 µM	1-4h	Little	167
MSU crystals	100-200 µg/ml	3-5h	Yes	71,118,181
	1000 µg/ml	2h	Yes	182
			Yes	134
	20 pg/cell	2h	Yes	80

Glucose

Glucose Oxidase	100 mU/ml	1-4h	Yes	12,172
Glucose	5.5-10 nM	2h	No	183
	20-30 nM	2h	Yes	183
	5000000 nM	3h	No	58

	25000000 nM	3h	Yes	58
Bacterial/fungal products				
LPS	0.1 ng/ml	1h	Yes	184
	0.1-10 µg/ml	15 min-18h	Yes	1,19,28,54,61,64,71,76,82,83,147,158,161,185-194
	0.1-25 µg/ml	2,5-3h	Little	16,21
	0.3-5 µg/ml	15 min	No	10,17
	50 µg/ml	30-90 min	Yes	155,195
			No	18
	10 mg/L	30 min	Yes	190
		30 min	Yes	196
LPS + Glucose	2 µg/ml + 30000000 nM	3h	Little	19
	2.5-25 µg/ml	2,5h	Yes	21
LPS + Platelets	1-5 µg/ml + $5x10^7$-$2.4x10^8$ /ml	30 min +	Yes	10,197
	25-100 ml +	20 min	Yes	175
β-glucan	200 µg/ml	15-240 min	Yes	198,199
	1000 µg/ml	1h	Yes	129
Bacteria/fungi				
S. aureus	0.03-50 MOI	30 min-24h	Yes	12,14,34,40,71,116,169,200-202
	$6x10^6$/ml	1h	Yes	180
	25 µl OD 0.5	3h	Yes	86
S. pneumonia	10 MOI	10 min-24h	Yes	14
S. pneumonia (dead)	$2x10^7$/ml	4h	Yes	118
E. coli		4h	No	15
	3-50 MOI	10 min-24h	Yes	14,34,79,124,202
	100 MOI	1-4h	Yes	51,184
	10^6-10^7 CFU	5min-1h	Yes	189,203
	2000 CFU	1-8h	Yes	189
P. aeruginosa	1-50 MOI	5h	Some	204
	10-100 MOI	10 min-24h	Yes	14,34,168,205-207
		8h	Yes	208
	$6x10^6$/ml	1h	Yes	180
A. fumigatus (hyphae)	750 CFU / 50 µl	2h	Yes	209
	0.2-2000 MOI	40-180 min	Yes	64,77
	10^6 conidia	3h	Yes	191
C. albicans (yeast)	0.5 MOI	90 min	Little	125
	2 MOI	15 min	Yes	210
	2 MOI	4h	No	210
	5 MOI	3h	No	148

	10 MOI	3h	Little	120
	10 MOI	2h	Yes	123
C. albicans (hyphae)	0.2-4.2 MOI	5-min–4h	Yes	125,126,210-212
		30 min	Yes	213
	10 MOI	4h	Yes	177
M. bovis	10-1000 MOI	1-4h	No	159

PMA is the most frequently used stimulus with a 100% success rate for inducing NETosis. In literature different concentrations are used ranging from 5 nM to 100 µM. NETosis was observed within a time frame ranging from 10 minutes to 24 hours [1-3,7,12,14,16,18-21,26-69,71-158,160-168,192].

S. aureus has consistently been described to be a potent inducer of NETosis[12,14,34,40,71,75,104,131-133]. In literature search a variety of other bacteria were reported, that also induced NETosis, although most species are weak NETosis inducers compared to *S. aureus* [14]. *E. coli P. aeruginosa, C. albicans* yeast and *M. bovis* have also been described as potent NETosis inducers in most papers, but discrepancies occur [15,148,159,207,210].

The NETosis inducing properties of LPS have been investigated in many papers, but the results are contradicting. For example, in LPS-activated neutrophils multiple papers state to have observed NETosis after 30 min with 100 ng/ml [76,83,158,161,187], whereas other authors did not observe NETosis using a concentration of 10 µg/ml [18].

Results for glucose as an inducer for NETosis indicate that higher concentrations (20-30 mM) of glucose appear to induce NETosis whilst low concentrations (5-10 mM) do not [58,187]. Higher concentrations of glucose are thought to resemble a hyperglycemic environment for neutrophils and may mimic the situation in patients with badly regulated Diabetes Mellitus. NETosis induced by glucose therefore seems concentration dependent.

Studies with A23187 report conflicting results. Six studies reported induction of NETosis after stimulation with 5µg/mL and 0.2-25 µM for 20 minutes to 4 hours [22,130,151,179,181,183]. Two other studies reported little to no NETosis after induction with 1 and 100 µM of A23187for 1-4 hours [167,182]. Ionomycin is also reported to induce NETosis after 30-180 min [21,29,34,130,132,167,195].

Experiments with IL-8 as an inducer of NETosis gave various results. In one study, NETosis was induced between 30-240 min after administration of 10-100ng/ml IL-8 [12]. However, in other studies, after stimulation with 200-800ng/ml IL-8 for 4-18h NETosis was not observed [18,76,82]. TNF-α is reported as an inducer of NETosis in five studies, while two papers report little or no NETosis. NETosis was observed 30 minutes to 6 hours after administration of 7-100ng/ml TNF-α [19,20,27,54,62]. One study used a concentration of 1ng/mL and reported little effect of TNF-α as an inducer, and one study did not observe NETosis at all after 4h with 100ng/ml[76].

Another investigated inducer was H_2O_2. Some experiments including H_2O_2 did not show clear NETosis but showed other forms of cell death such as apoptosis and necrosis [18,116]. However, H_2O_2 also was reported, by other studies, to be a good inducer of NETosis [54,61,114,169].

In summary, the data in literature show that PMA is a well-defined inducer of NETosis with a 100% success rate. Bacterial inducers of NETosis such as *S. aureus* (10:1 - 20:1 bacteria to neutrophils) also seem consistent inducers, but in some strains discrepancies occur and the process is less well described than PMA. Other inducers, such as cytokines IL-8 and activated platelets, different glucose concentrations and especially LPS, display a variable outcome.

Our literature search revealed the observation that numerous experiments have been performed in which it became clear that all inducers, with the exception of PMA, have been studied with experimental conditions that differed between studies, such as time frame, concentration and NETs imaging procedure. This could partly explain the observed differences in NETosis induction.
Hence, there is a need for a well-controlled evaluation of NETosis inducers. We therefore performed a standardized study in which we tested the NETosis capability of different NETs inducers (as defined in Table 2). Bacterial infections, diabetes and calcium influx all influence cardiovascular wound healing differently. Therefore, we selected *S. aureus*, *E. coli*, LPS, Ionomycin, glucose and combinations with (activated) platelets and LPS for our panel. PMA will be taken as a positive control, whilst unstimulated cells are a negative control in experiments without platelets, and resting platelets are a negative control in experiments with platelets. In this study, we use a well-defined experimental setup to test multiple conditions at the same time on the same neutrophils.

Table 2: Concentrations of the potential NETosis inducers in the experiments.

NETosis inducer and final concentrations
PMA (Sigma Aldrich, Saint Louis, Missouri, USA)
• 50 ng/ml
• 250 ng/ml
Platelets (isolated from EDTA blood)
• $5x10^7$ /ml
Supernatant of activated platelets (isolated from EDTA blood)
• $5x10^7$ /ml
D-Glucose (Amresco)
• 25 µM
• 25 mM
Ionomycin (Sigma Aldrich)
• 3 µg/ml
• 5 µg/ml
LPS (Sigma Aldrich): source
E. coli O55:B5
• 10 ng/ml
• 100 ng/ml
• 1000 ng/ml
• 5 µg/ml
E. coli O111:B4
• 10 ng/ml
• 100 ng/ml
• 1000 ng/ml
• 5 µg/ml
P. aeruginosa
• 10 ng/ml
• 100 ng/ml
• 1000 ng/ml
• 5 µg/ml
Platelets + LPS (*E. coli* O111:B4)
• $5x10^7$ /ml + 5 µg/ml
Activated platelets supernatant + LPS (*E. coli* O111:B4)
• $5x10^7$ /ml + 5 µg/ml
Living bacteria
S. aureus (Newman)
• 10^8/ml (±10:1)
E. coli ATCC 25922 (O6:B1)
• 10^8/ml (±10:1)
Dead bacteria
S. aureus (Newman)

- 10^{10}/ml (±1000:1)

E. coli ATCC 25922 (O6:B1)

- 10^{10}/ml (±1000:1)

NETosis experiments

PMA

In our experiments PMA (n=7) consistently and strongly induced NETosis (61.5 ± 9.3 % of PMA stimulated neutrophils vs 4.1 ± 1.3 % of unstimulated neutrophils, p<0.001) (Table 3, Fig. 2 and supplemental Fig. 1). NETosis was observed about 1.5 hours after administration of PMA and observed for both concentrations (50 ng/ml and 250 ng/ml).

Table 3: Percentage of neutrophils that underwent NETosis. % NETosis per time point (hr) is given as mean (SEM). P-value of repeated measures ANOVA with Bonferroni post-hoc test results per NETosis inducer versus unstimulated neutrophils.

	n	Time (hr)				p-value
		0	1	2	3	
None	5	1.94 (0.43)	3.8 (1.35)	3.68 (1.08)	4.10 (1.34)	n.s.
PMA (250 ng/ml)	7	3.89 (0.91)	7.81(1.64)	31.94 (6.17)	61.52 (9.34)	<0.001
LPS (5 µg/ml)	7	2.98 (1.58)	2.62 (1.26)	2.90 (1.08)	3.98 (1.69)	n.s.
Glucose (25 mM)	3	4.71(1.06)	4.92 (1.90)	6.40 (1.98)	6.58 (1.96)	n.s.
Platelets ($5x10^7$)	5	2.53 (0.71)	2.80 (1.12)	1.12 (0.89)	1.16 (0.92)	n.s.
Activated Platelets ($5x10^7$)	7	2.00 (0.60)	3.24 (1.15)	1.45 (0.61)	1.27 (0.47)	n.s.
Activated Platelets Supernatant ($5x10^7$)	7	2.84 (1.12)	4.48 (1.19)	5.68 (2.31)	5.94 (2.45)	n.s.
Platelets and LPS ($5x10^7$ + 5 µg/ml)	7	2.60 (0.79)	3.57 (0.72)	2.43 (1.09)	3.09 (0.98)	n.s.
Activated Platelets and LPS ($5x10^7$ + 5 µg/ml)	7	2.35 (0.77)	2.75 (1.34)	2.65 (0.93)	3.77 (1.09)	n.s.
Activated Platelets Supernatant and LPS ($5x10^7$ + 5 µg/ml)	7	2.87 (1.10)	6.86 (1.84)	7.01 (2.88)	7.88 (3.56)	n.s.

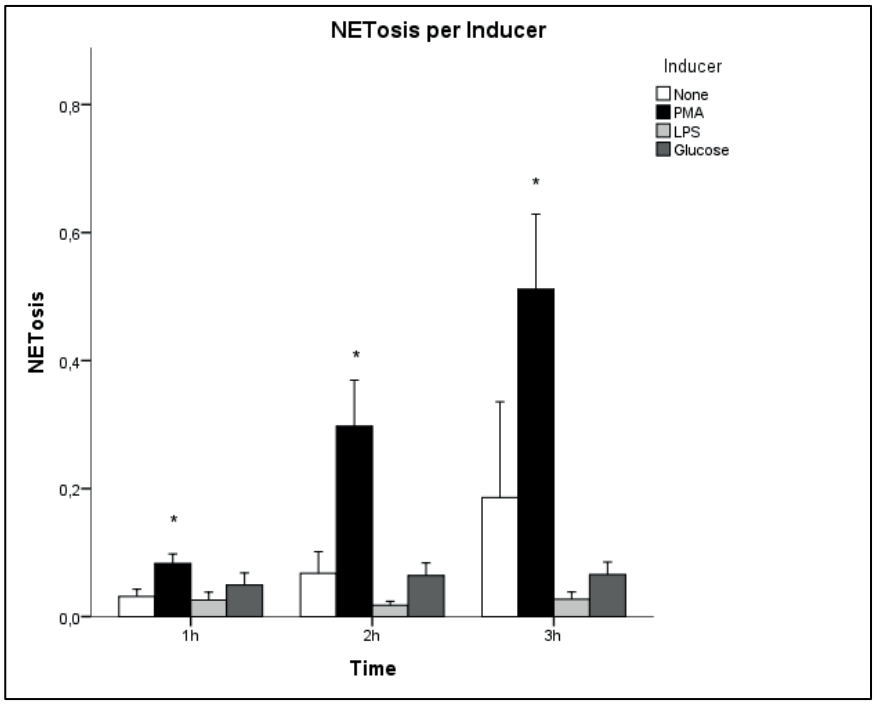

Figure 2: NETosis induction for the different inducers. NETosis was defined as the ratio between the number of Hoechst and PI positive cells. PMA induced NETosis when compared to unstimulated neutrophils, p<0.001 repeated measures ANOVA post-hoc Bonferroni (*) (none n=5, PMA n=7, LPS n=7, glucose n=5). Error Bars +/- SEM.

Living bacteria

In our experiments both gram positive and gram negative bacteria strongly induced NETosis. In *S. aureus* stimulated samples (n=3), NETs were observed after 10-20 minutes and in *E. coli* stimulated samples (n=3), NETs were observed within one hour (Fig. 3), as confirmed by live MPO staining (Supplemental Fig. 2). NETs induction by both bacteria strains differed in the amount of viable (Hoechst positive) neutrophils. After the addition of *S. aureus*, no Hoechst positive neutrophils were observed after 40 minutes. After the addition of *E. coli*, neutrophils remained viable during the total experiment. After the addition of dead *S. aureus* and *E. coli* (n=3), phagocytosis of the bacteria by the neutrophils and no NETosis was observed (Supplemental Fig. 3).

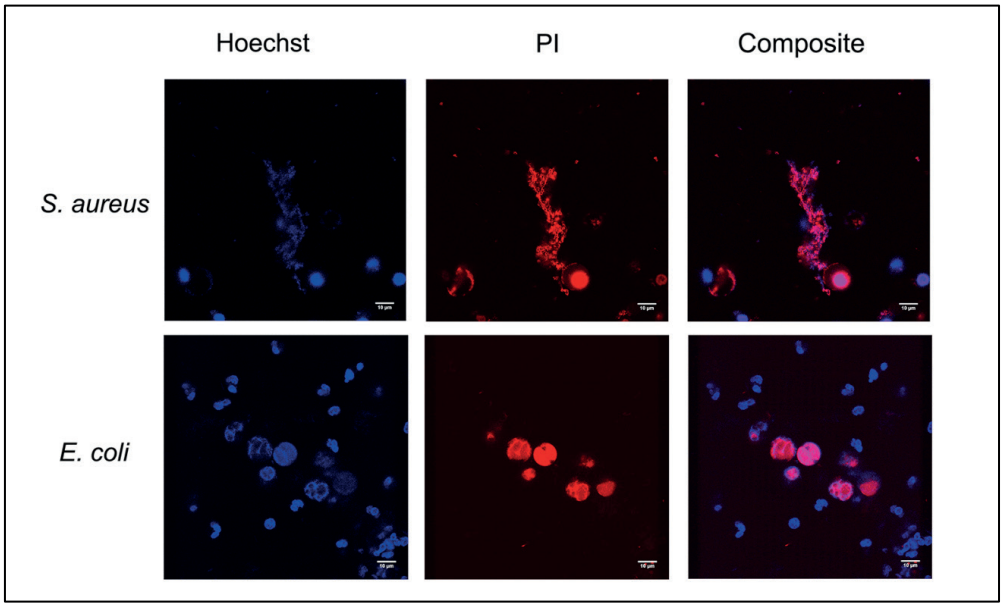

Figure 3: NETs formed by *S. aureus* and *E. coli* 20 minutes after stimulation for one hour. DNA (Hoechst, blue, 405) and Extracelullar DNA (PI, red, 561) were stained.

LPS and glucose

No NETosis was observed when neutrophils were incubated with LPS (n=7) or glucose (n=5). For LPS, multiple concentrations and variants (Table 2) were tested, but none induced NETosis. Also, combinations of LPS with platelets, activated platelets and activated platelets supernatant were unsuccessful in inducing NETosis (Fig. 4) (n=7 for all).

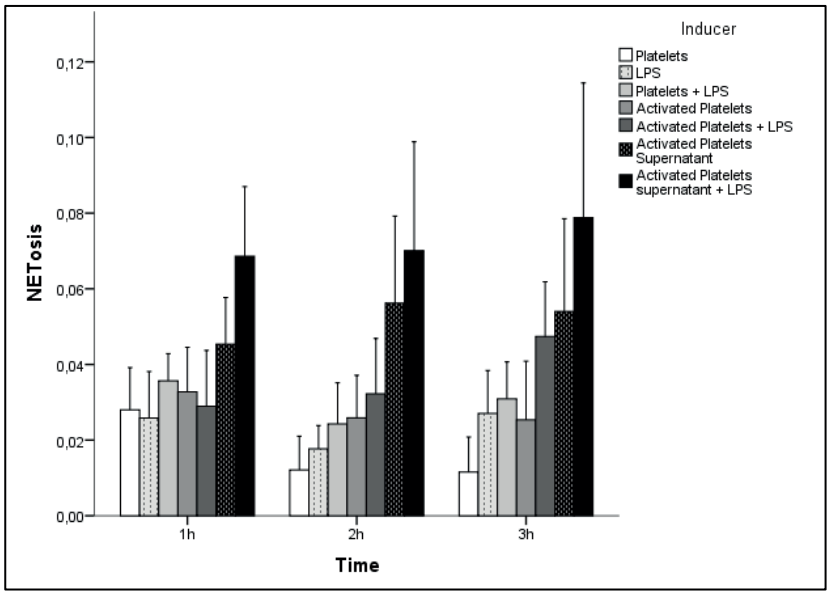

Figure 4: NETosis per inducer comparing the effect of platelets and the effect of activated platelets supernatant (n=5, n=7 respectively). NETosis was defined as the ratio between Hoechst and PI positive cells. Neutrophils stimulated by platelets were compared against neutrophils stimulated by LPS, platelets + LPS, activated platelets, activated platelets + LPS, activated platelets supernatant, activated platelets supernatant + LPS using repeated measures ANOVA post-hoc Bonferroni (*). No significant differences were found (p>0.05 all). Error Bars +/- SEM.

Ionomycin

When neutrophils were incubated with Ionomycin (n=3), the sequence of events leading to DNA extrusion was different from the process we observed after the addition of PMA. Within 15 minutes after the addition of Ionomycin, the membranes of the neutrophils became porous, as shown by staining the nuclei for PI (Fig. 5, supplemental video 1and 2). In our three hour imaging timeframe, DNA was seen to slowly leak out of the cells. This process was not observed in cells that died as a result of necrosis or apoptosis, where the DNA remains within the cells [12]. At the end of the experiment, the neutrophils treated with Ionomycin and the neutrophils that were incubated with PMA looked similar, and, therefore, this difference in the DNA extrusion process may be missed in studies that did not study early time points.

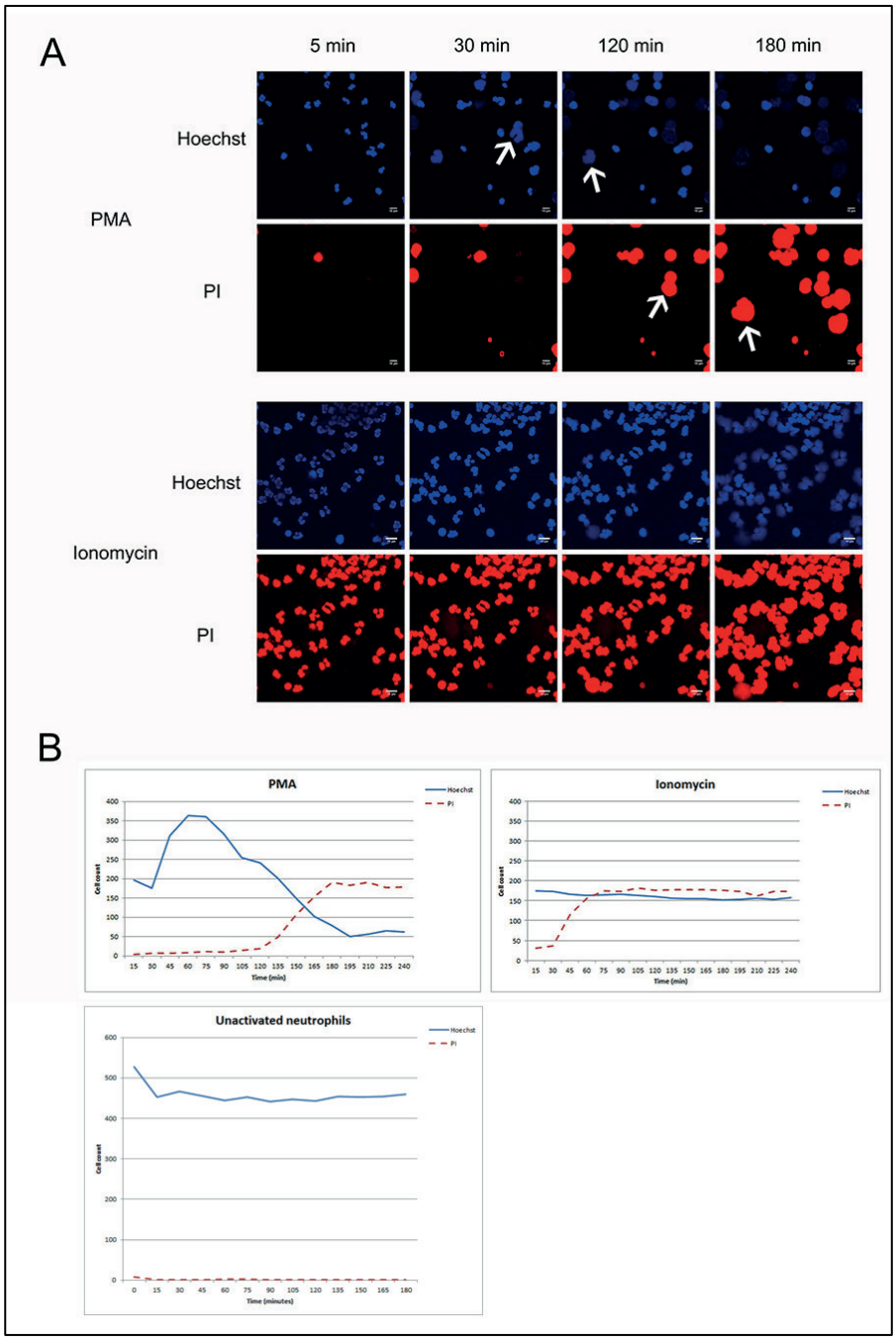

Figure 5: The effect of Ionomycin compared to PMA. (A) Time lapse images of PMA and Ionomycin at different time frames. The arrows indicate the decondensation of the nuclei before (Hoechst, 405) and after (PI, 561) DNA extrusion. (B) The amount of Hoechst and PI positive cells in PMA and Ionomycin stimulated cells and unstimulated cells, show the

difference in the process of NETosis. In the PMA stimulated cells, the number of Hoechst positive cells go down as the PI positive cells (NETosis) go up. In the Ionomycin stimulated cells, the number of PI positive cells go up very rapidly, but the Hoechst positive cells remain similar. In unstimulated cells, the intensity of Hoechst staining remains high and no PI staining was detected.

Activated platelets

We did not observe NETosis after incubating the neutrophils with thrombin activated platelets, activated platelets supernatant, platelets and LPS and activated platelets plus LPS (all n.s. n=7). However, in two experiments, NETosis was observed after incubating the neutrophils with LPS and activated platelets supernatant, while in the other experiments (n=7) no NETosis was induced. A possible explanation for this variation could be the variance between donors, though blood samples were taken from healthy donors, and none of the observed results could be linked to either sex or age.

Discussion

Our *in vitro* study, performed in a well-defined and well-controlled time-lapse setting, revealed that PMA, bacteria and Ionomycin were robust inducers of NETosis. The other reported NETosis inducers were less potent.

First, we performed a systematic literature review of NETosis inducers. This is the first systematic review to address NETosis inducers. NETosis is currently intensively investigated and therefore a systematic review on this topic is very needed. Our literature search revealed that PMA and bacteria were consistent inducers of NETosis. Both are being used in a more routine way in research now. PMA is used to mainly investigate the effect of other inducers and the ROS-pathway.

Studies on other inducers presented conflicting results. The difference in experimental setting, timing and dosing might contribute to the variation in results. Therefore, we performed *in vitro* experiments in a standardized laboratory setting. In these experiments, we used concentrations based on literature and a time frame of 3 hours, PMA was also used as a positive control

in our experiments, as it was a consistent inducer throughout the literature. In the imaging of NETosis by Ionomycin, we observed a different sequence of events, but according to the definition of NETosis that we use in this paper, Ionomycin is also a qualified inducer. NETosis was not observed with other tested inducers.

Bacteria and bacterial products

In our standardized experiments, living gram negative as well as living gram positive bacteria were strong and consistent NETosis inducers. Several studies, using a variation of experimental conditions, support our findings [12-15,214].

Our study also showed that different species gave a different time of onset of NETosis and a different percentage of the neutrophils that underwent NETosis. This is in line with Pilsczek *et al* [14]. We saw more NETosis after the induction with *S. aureus* compared to *E. coli*.

Dead bacteria did not induce NETosis in our experiments. Isolated LTA (derived from gram-positive bacteria) and LPS (derived from gram-negative bacteria), both bacterial-wall proteins, have been described to induce NETosis. We hypothesized that dead bacteria also expose these proteins and, therefore, were expected to be potent NETosis inducers. We killed the bacteria with two methods (heat and UV), however, did not observe NETosis in either situation.

When we added LPS, no NETosis was induced. In literature, contradictory reports are found regarding LPS as a NETosis inducer. To test whether the type of LPS explains this contradiction, we used three different types of LPS (derived from *E. coli* O55:B5, *E. coli* O111:B4 and *P.* aeruginosa), which also were used in literature. Data are shown in Figure 2. Post Hoc testing showed no difference between LPS and unstimulated neutrophils. These results are in line with our experiment where dead gram-negative bacteria, with LPS on the surface, also failed to induce NETosis. Therefore, the ability of LPS to induce NETosis should be studied further.

Glucose

In literature, glucose is described as NETosis inducer[58,183]. In our experiments, glucose did not induce NETosis. One study suggested that high levels of glucose make neutrophils more sensitive to NETosis inducers such as cytokines or LPS[58]. In contrast, other literature that claims that neutrophils

become insensitive to stimuli when maintained in high glucose concentrations [19].

Calcium ionophore

Incubation of the neutrophils with the Ionomycin resulted in an extrusion of DNA, however, this process differed from the PMA induced NETosis. Ionomycin opens the calcium channels of cells, thus causing high intracellular Ca^{2+} levels. This resulted in pore formation in the cellular membranes and positive staining for PI in the cells, followed by leakage of PI-positive material out of the cells. In the NETosis induced by PMA, nuclear swelling is seen as the first step. Still, we considered the process after Ionomycin induction NETosis, since in other forms of cell death, i.e. necrosis and apoptosis, the nuclear envelope remains intact, which prevents DNA excretion from the dead cell [12].

We emphasize that it is important to visualize the whole NETosis process and not only rely on end stage measurements. We have shown that there is variation in the process of DNA extrusion with Ionomycin. Since other studies on Ionomycin only measured at the end of the NETosis process, they may have missed these variations.

Platelets

In our experiments, resting platelets did not induce NETosis. This results is in contrast with literature [10,44]. We also did not see NETosis induction when we incubated neutrophils with activated platelets or activated platelets plus LPS, as described by one other study [175]. The majority of the studies, however, described that the excretion of growth factors by activated platelets (stimulated by for example LPS or PAF) will activate neutrophils and stimulate NETosis [10,44,173,174].

Difference in neutrophil function

Interestingly, we observed that NETosis induction with activated platelets was variable amongst healthy individuals, since strong NETosis was observed in two samples while absent in five other samples. Our donors were healthy individuals. Individual variation in neutrophil response might be an explanation for variable results. Therefore, all experiments with neutrophils should include blood samples from multiple healthy donors and should be repeated multiple times to obviate as much variation as possible.

Study limitations and recommendations

To our knowledge this is the first *in vitro* study that compares a comprehensive panel of NETosis inducers under standardized experimental conditions using time-lapse imaging, allowing a direct quantification of the NETosis strength using image analysis to quantify the data. We consider it a strong point of our study that time-lapse images allow visualization of the actual NETosis process. Therefore, NETosis can be identified with higher certainty than when using single images. Our approach was, for example, very helpful in interpreting the experiments with Ionomycin. In our study our medium contained 10% FCS. While this is widely used for cell culture purposes, in NETosis experiments this could affect NETosis, since FCS contains nucleases, which have been described to break down NETs *in vitro* [215]. However, nucleases break down the NETs after they have formed, and we did not observe this in our time-lapse analysis. Also, it is unlikely that either the use of DMEM or FCS would have an effect on any of the tested inducers. For example, the most used medium for NETosis experiments is RPMI-1640, but studies have also reported LPS, one of the most contradictive inducers in our panel, not to have much effect in their studies [16,18,21]. We are aware that Ca^{2+} in buffers can have an effect on NETosis. Therefore, we used PBS free from Ca^{2+}, and our HEPES buffer only contained a minimal concentration of 12 µM Ca^{2+}.

A drawback of any *in vitro* setting obviously is that an *in vitro* setup cannot completely reflect the *in vivo* situation. Inducers like LPS also are expected to trigger an immune response *in vivo*, which could trigger alternate pathways that induce NETosis.

NETosis can be found in many pathological conditions such as thrombosis and sepsis, which leads to a rising interest in exploration of its pathways. These pathways could be further explored *in vitro* in a setup similar to ours.

Conclusion

Our literature research showed that living gram positive and negative bacteria, PMA and Ionomycin are strong NETs inducers. Other inducers are less potent. Our additional experiments, which were performed under one experimental condition confirmed these our results found during our literature research.

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Chapter 3

Staphylococcal Protein A (SpA) is a key factor in Neutrophil Extracellular Traps (NETs) formation

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Abstract

Staphylococcus aureus (*S. aureus*) are strong inducers of Neutrophil Extracellular Traps (NETs), a defense mechanism of neutrophils against pathogens.

Our aim was to explore the role of Protein A in *S. aureus*-induced NETosis. We determined the Protein A production of four different *S. aureus* strains and found a direct relationship between the strength of NETosis induction and Protein A production: strains producing higher concentrations of Protein A evoke significantly more NETs. A *S. aureus* strain in which Protein A as well as a second binding protein for immunoglobulins (*Sbi*) have been knocked-out (*ΔSpA ΔSbi*) induced significantly less NETosis than the wild type strain. NETosis induction by this knockout strain can be rescued by the addition of purified Protein A. Dead *S. aureus* did not induce NETosis.

In conclusion, Protein A is a determinant for NETosis induction by *S. aureus*.

Introduction

Upon encountering bacteria, neutrophils can form neutrophil extracellular traps (NET) as part of their antimicrobial defense mechanism. During NETosis, neutrophils excrete their DNA into the extracellular space, along with histones and other antimicrobial factors. These NETs trap bacteria and thereby limit bacterial spreading [1,2]. NETs have been shown to play an important role in the seriousness of several pathological conditions, such as chronic wounds [3], thrombosis [4-6] and sepsis [7-9].

The bacterial potency of NETosis is different between bacterial species [10]. A very potent inducer of NETosis is *Staphylococcus aureus* (*S. aureus*) [11,12]. *S. aureus* is a gram positive bacterium that can cause many different infections and, particularly when dealing with Methicillin Resistant *S. aureus* (MRSA), can cause critical problems in hospitals. *S. aureus* possesses multiple evasion strategies against the human immune system, such as the production of immune-modulators [13-17] and the secretion of nucleases, which enables them to escape NETs [16].

S. aureus also can evade phagocytosing neutrophils by blocking neutrophil rolling on activated endothelial cells and by targeting both antibodies and opsonins, necessary for pathogen recognition by neutrophils [18]. One of the main bacterial proteins involved in phagocytosis evasion is Protein A. Staphylococcal Protein A (*SpA*) is a 42 kD large protein which is covalently linked to the staphylococcal surface and can be secreted into the extra-bacterial environment [17,19,20].

Staphylococcal Protein A is known to be able to manipulate or to avoid early host adaptive immune responses. It can bind to the Fcγ domain of IgG and, therefore inhibit opsonization that precedes phagocytosis [17,19-22]. Furthermore, it can induce apoptosis in B-cells by binding to the Fab regions of the B-cell receptor and act as a B-cell superartigen [23]. However, little is known about its direct effect on innate immune cells, particularly neutrophils. Because neutrophils are one of the earliest effector host immune cell against *S. aureus* invasion and because of their ability to form NETs, we were interested to study whether Protein A is also involved in NETosis. To achieve this, we determined the Protein A production in different *S. aureus* strains and its relationship with NETosis inducing capacity. Next, we obtained more insight in the role of Protein A in NETosis by studying the rescue of NETosis with Protein A in a *S. aureus* Protein A knockout strain.

Material and methods

Bacterial strains

Bacterial strains used in this study are listed in Table 1. Strains were obtained from the bacterial collection of Department of Medical Microbiology and Infectious Diseases, Erasmus MC Rotterdam.

Table 1: Overview of the *S. aureus* strains used in this study.

Strain	Genetic background	Description	Source
Newman	ST8	Wild type, Laboratory strain	Hansenova *et al*
USA300	ST8	Clinical Strain	Sultan *et al*
M116	ST238, ST8	Clinical Strain	Sultan *et al*
RN6390	ST8	Laboratory strain, derivative of 8325-4	Hansenova *et al*
Newman *ΔSpA ΔSbi*	ST8	Laboratory Strain, derivate from Newman strain	van den Berg *et al*

Bacterial growth condition

All strains were cultured on Trypticase™ Soy Agar (TSA)(Becton Dickinson, Breda, The Netherlands) with 5% sheep blood overnight at 37 °C. Protein A and the second binding protein for immunoglobulins (*Sbi*) double knockout Newman strain (Δ*SpA* Δ*Sbi*) was cultured on TSA containing 5 µg/ml gentamycin and 5 µg/ml tetracycline to maintain its knockout status. After an overnight incubation at *37 °C* bacteria were suspended in NaCl 0.9% solution (OD 0.5 at OD_{600nm}) and 200 µL was added to a sterile Erlenmeyer flask containing 100 ml Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, Bleiswijk, The Netherlands).

The flask was then incubated for 24 hours at 37 °C at 150 rpm. The next day, based on OD_{600nm} measurements, the individual strains were concentrated to reach a final concentration of $2x10^{10}$ bacteria/ml. Heat killed bacteria were generated by incubating the bacteria at 96 °C for 10 minutes. Bacteria were then harvested and transferred to new IMDM medium. To control for the effectiveness of the heat treatment, the heat-killed bacteria were stained with Propidium Iodide (PI, diluted 1:400, Sigma Aldrich, Zwijndrecht, The Netherlands) and a sample was cultured to check for growth by plating.

Secreted Protein A measurement

The concentrations of released Protein A by *S. aureus* strains were measured using a sandwich ELISA type assay specific for Protein A (Enzo, Bruxelles, Belgium) according to the manufacturer's protocol. The detection range of the kit was 15.6-1000 pg Protein A/ml. *S. aureus* strains Newman, USA300, RN6390, M116 and Newman *ΔSpA ΔSbi* were cultured as described above and after overnight culturing, 20 µL of the supernatant was collected, centrifuged at 4000 rpm and then filtered. Supernatant from Newman *ΔSpA ΔSbi* bacteria was included as a negative control. The optical density at 450nm was measured using a Biotek plate reader (Biotek) with Gen5 software and used to calculate the protein A concentration.

FACS analysis of surface associated Protein A

Four milliliter of IMDM was inoculated with an overnight culture of *S. aureus* (Newman, USA300, RN6390 and M116) to obtain OD_{600nm} of 0.05. The individual cultures were incubated for 24 hours at 37 °C with continuous shaking at 230 rpm. The OD_{600nm} of the bacteria culture was normalized to

0.300 and the bacteria were washed 3 times with PBS, followed by centrifugation for 5 minutes at 4000g. The individual bacterial pellets were suspended in 100 µl PBS. Ten microliters of each bacterial suspension was mixed with either 10 µl of 1:50 dilution of anti-protein A IgY-FITC (FITC-labeled Chicken anti-Protein A, (Gallus Immunotech Inc., Fergus, Canada) or with 10 µl PBS, used as negative control, in a U shape 96-well microplate (Greiner Bio-One, Oberösterreich, Germany). The plate was then incubated at 10 °C for 45 minutes at 800 rpm in the dark. After washing 3 times with 200 µl PBS, bacteria were centrifuged for 5 minutes at 3500g. Bacteria were suspended in 50 µL of PBS and their fluorescence (emission 488-522 nm) was quantified with Accuri C6 Flow Cytometer and analyzed using Accuri C6 Software (version 1.0.264.21) (both BD Bioscience, Breda, The Netherlands). Values are expressed in Mean Fluorescence Intensity (MFI).

Neutrophil isolation

Neutrophils were isolated as described previously [24]. Briefly, medium Lymphoprep™ (Stemcell Technologies) was used to isolate neutrophils from donor blood. Red blood cells were lysed using Erythrolysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA, pH 7.4) and then neutrophils were washed two times with HEPES buffer. A final neutrophil concentration of $2x10^7$ cells/ml was used. All experiments were approved by the Medical Ethics Committee of the Erasmus MC.

NETosis induction and imaging

Neutrophils were stained for DNA with Hoechst 34580 (diluted 1:10 000, Life Technologies) and for extracellular DNA with Propidium Iodide (PI, diluted 1:400, Sigma Aldrich) in 500 µl DMEM culture medium (Biowhittaker, Lonza). The cells were allowed to attach to gelatin-coated coverslips at 37 °C for at least 1 hour.

To induce NETosis, 500 µl $2x10^{10}$ bacteria/ml were added to 500µl $2x10^7$ neutrophils/ml in a Attofluor Cell Chamber (Thermo Fisher Scientific, Bleiswijk, The Netherlands). The chamber was sealed and the neutrophils were continuously imaged with a confocal microscope (Leica SP5 AOBS). Hoechst and PI were excited by 405 nm (emission BP 450-550 nm) and 561 nm (emission 570-620 nm) lasers, respectively. NETs were visible as PI positive elongated structures and were quantified (see quantification).

In order to study the effect of Protein A on NETosis induction by *S. aureus* Newman *ΔSpA ΔSbi* strain, 100 µl of either 0.01, 0.1 or 1 mg/ml of purified Protein A (Sigma Aldrich, Zwijndrecht, The Netherlands) was added to the Newman *ΔSpA ΔSbi* strain prior to co-incubation with neutrophils (final concentration range 0.9-90 µg/ml Protein A).

NETs quantification

Z-stack images were taken from randomly distributed fields of view within the cell chamber every 3-5 minutes, in a time frame of 5-40 minutes. Every image had the same X, Y and Z dimensions. In each image, NETs were manually traced in every z-stack by using ImageJ (Version 1.49, National Institutes of Health, USA). The total volume (μm^3) of NETs was calculated from 10-15 images per strain. The average percentage of NETs coverage was then calculated: total volume of NETs/total volume.

Statistical analyses

Data are presented as mean ± standard error of the mean (SEM). A statistical t-test with Statistical Package for the Social Sciences (SPSS, IBM, version 21) was used to analyze differences between groups. Results were considered significant if $p<0.05$.

Results

The secretion of Protein A in the supernatant of the overnight grown *S. aureus* strains Newman and USA300 (ST8) (0.44 µg/ml and 0.42 µg/ml, respectively) was almost 8 times higher than that of M116 and RN6390 (0.08 µg/ml and 0.06 µg/ml, respectively)(Figure 1A). No Protein A was measured in the Protein A knockout Newman *ΔSpA ΔSbi*.

The amount of Protein A associated to the bacterial surface was higher in Newman and USA300 (3703 ± 404 MFI and 3767 ± 163 Mean Fluorescence Intensity (MFI), respectively) compared to M116 and RN6390 (2336 ± 288 MFI and 1649 ± 254 MFI, respectively) (Figure 1B). In literature it was described that Protein A is in particular expressed in classical bacterial growth media during the exponential growth phase. To determine if this is the case in IMDM, we as well made a growth curve and determined at 3 time points the amount of surface associated Protein A. Interestingly, the level of the surface associated Protein A during stationary phase (2.5 and 3.5 hours) is higher

compared to its exponential phase (24 hours, Figure 1C), except for strain RN6390.

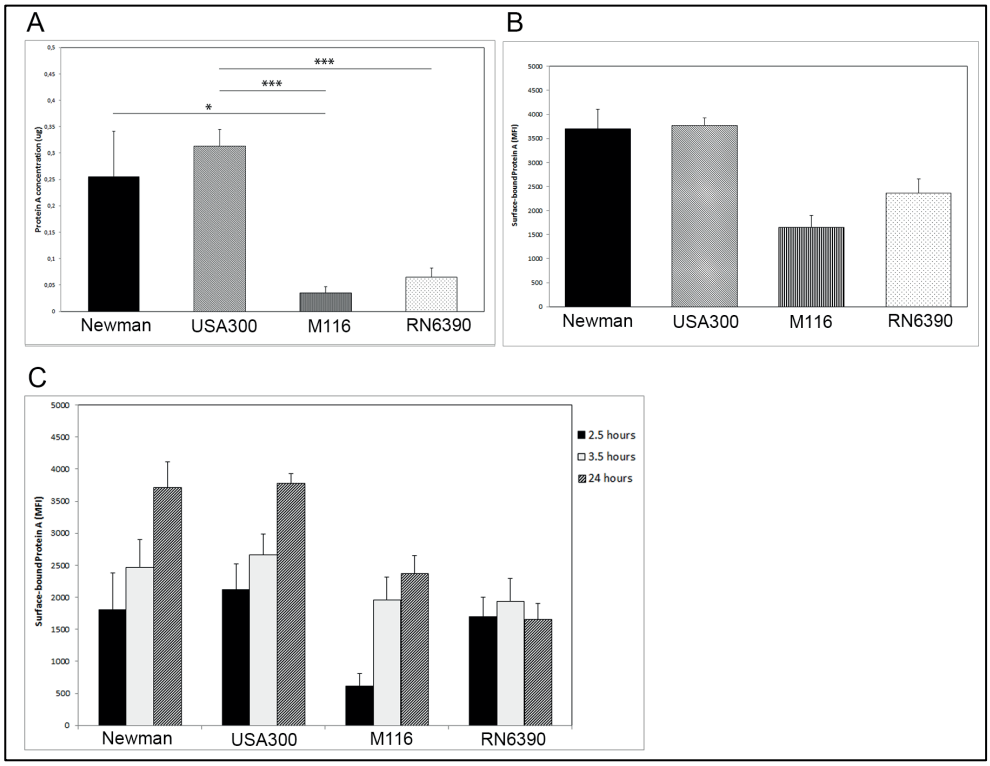

Figure 1: Protein A produced by *S. aureus*. A: Protein A secretion is higher in *S. aureus* strains Newman and USA300 compared to *S. aureus* strains M116 and RN6390, as determined by an ELISA assay (n=1). B: A higher amount of Protein A is bound to the surface of *S. aureus* strains Newman and USA300 compared to *S. aureus* strains M116 and RN6390, as determined by FACS (n=3). C: Amount of Protein A bound to the surface of *S. aureus* measured on 2.5, 3.5 and 24 hours as determined by FACS. Except for strain RN6390, the amount of surface bound Protein A is increasing over time (n=3).

When we tested these *S. aureus* strains to see whether the amount of Protein A plays a role in NETosis, we observed a positive correlation between Protein A levels and NETosis. High Protein A producing strains Newman and USA300 induced significantly more NETosis (Newman 10.7 ± 1.9 % and USA300 13.5 ± 3.8 % of the total volume) compared to the low Protein A producing strains M116 (0.7 ± 0.2 %, $p<0.001$ and $p=0.003$, respectively) and RN6390 (3.4 ± 1.4 %, $p=0.005$ and $p=0.02$, respectively) (Figure 2). Corresponding NETs volumes are 3750 ± 675 μm^3 (Newman), 4738 ± 1347 μm^3 (USA300), 245 ± 72

µm³ (M116) and 1208 ± 482 µm³ (RN6390). NETosis in all strains started within 5 minutes and within 40 minutes all neutrophils had formed NETs.

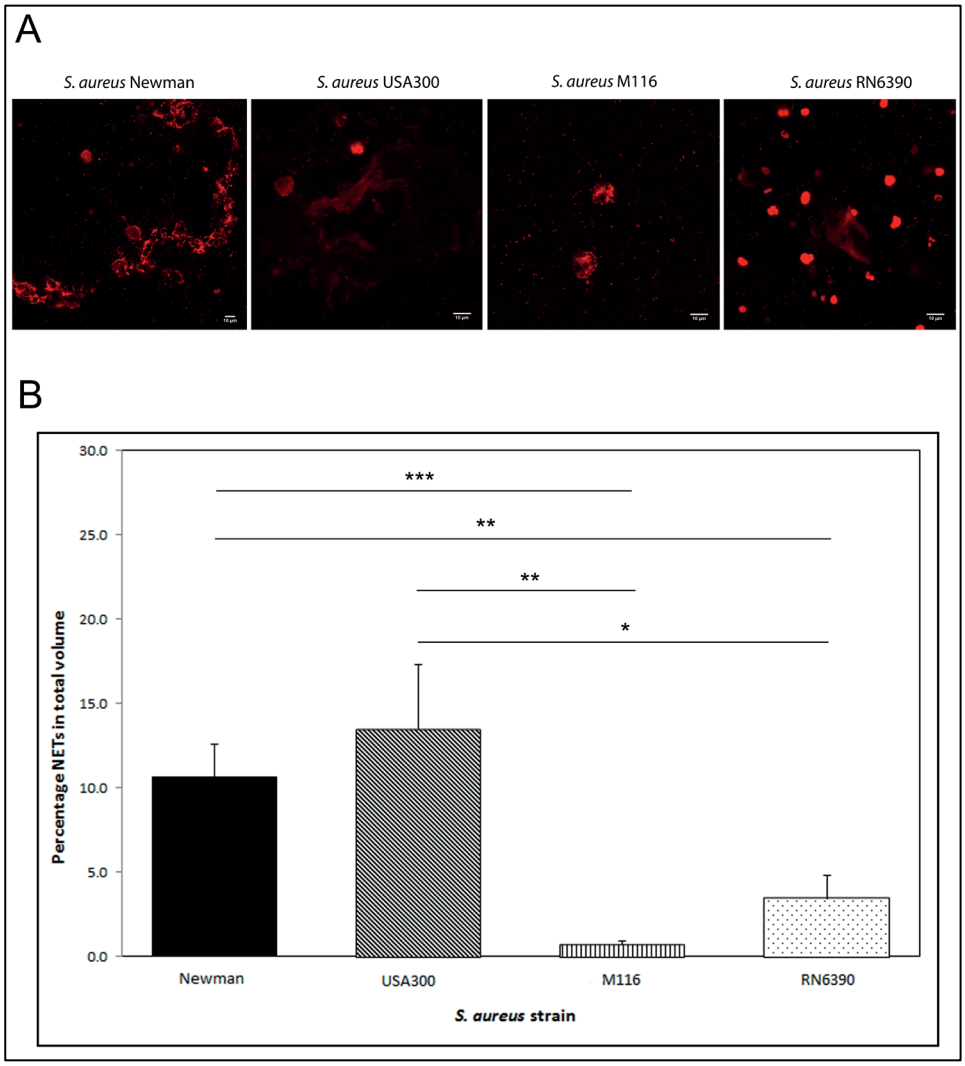

Figure 2: NETs induction by different *S. aureus* strains Newman, USA300, M116 and RN6390. A: in vitro NETs formation as indicated by PI (red). B: Strains Newman and USA300 induce significantly more NETs than M116 and RN6390.

*** p< 0.05**
**** p< 0.01**
***** p<0.001**

To further determine the contribution of Protein A to NETosis induction, we used a *SpA Sbi* double knockout strain of *S. aureus* Newman (*ΔSpA ΔSbi*) to

induce NETosis. Only very modest NETosis was observed (2.3 ± 0.9 %), which was significantly lower than of the WT Newman strain ($p<0.001$). We could recover NETosis by the knockout strain by adding purified Protein A. Addition of 0.9, 9 or 90 µg/ml of purified Protein A to the knockout strain, prior to NETosis induction, rescued NETosis induction (8.1 ± 1.9 %, $p=0.01$; 7.4 ± 1.2 %, $p=0.005$; 7.2 ± 1.7 %, $p=0.02$, respectively)(Figure 3) to comparable levels that were observed for WT Newman strain ($p=0.36$, $p=0.26$ and $p=0.19$, respectively).

Additionally, we also were able to rescue NETosis formation to the level induced by WT Newman when Protein A was added to the modestly NETosis inducing strain M116 (0.7 ± 0.2 % in M116 vs 9.1 ± 2.0 % in M116 plus Protein A, $p<0.001$), giving it the same NETosis induction rate compared to WT Newman ($p=0.63$, Figure 4).

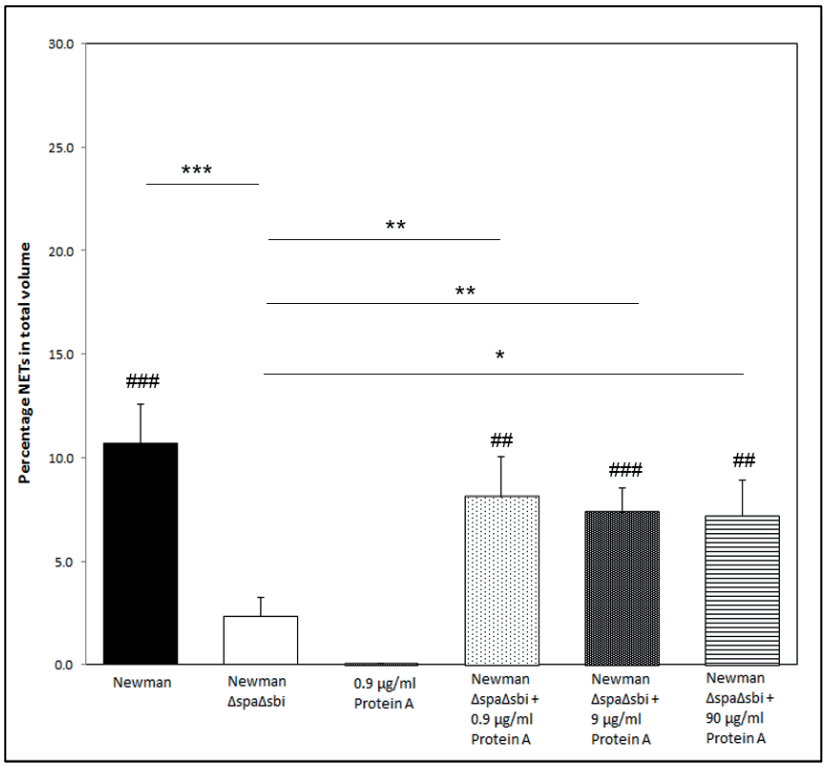

Figure 3: Reversed effect of significantly reduced NETosis by *S. aureus* knockout strain after the addition of purified Protein A. Except for *S. aureus* Newman *ΔSpAΔSbi*, all conditions induce more NETosis than Protein A only. * indicates significant difference from *S. aureus* Newman *ΔSpAΔSbi*. # indicates significant difference when compared to neutrophils stimulated with 0.9 µg/ml Protein A.

*** / # p< 0.05**

**** / ## p< 0.01**
***** / ### p<0.001**

Additionally, we also were able to rescue NETosis formation to the level induced by WT Newman when Protein A was added to the modestly NETosis inducing strain M116 (0.7 ± 0.2 % in M116 vs 9.1 ± 2.0 % in M116 plus Protein A, p<0.001), giving it the same NETosis induction rate compared to Newman (p=0.63, Figure 4).

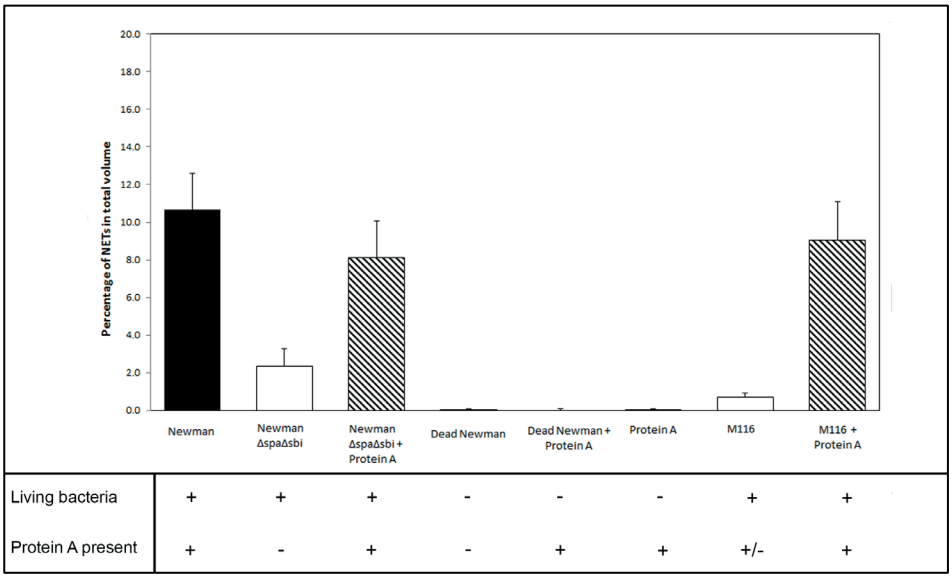

Figure 4: The effect of living and dead *S. aureus* and the presence of Protein A (both produced by *S. aureus* and added) on NETosis in different rescue experiments.

No NETosis was observed when 0.9, 9 or 90 µg/ml of purified Protein A was added to the neutrophils without bacteria (0.0 ± 0.0, Figure 3). To explore the effect of bacterial viability on NETosis induction, 0.9 and 9 µg/ml Protein A were added to dead bacteria (WT Newman). No NETosis was observed (Figure 4) which indicates that living bacteria are needed in order to induce NETosis. We observed that dead bacteria were phagocytosed by neutrophils, however, when 0.9-90 µg/ml Protein A was added, bacteria were not cleared by the neutrophils (Figure 5).

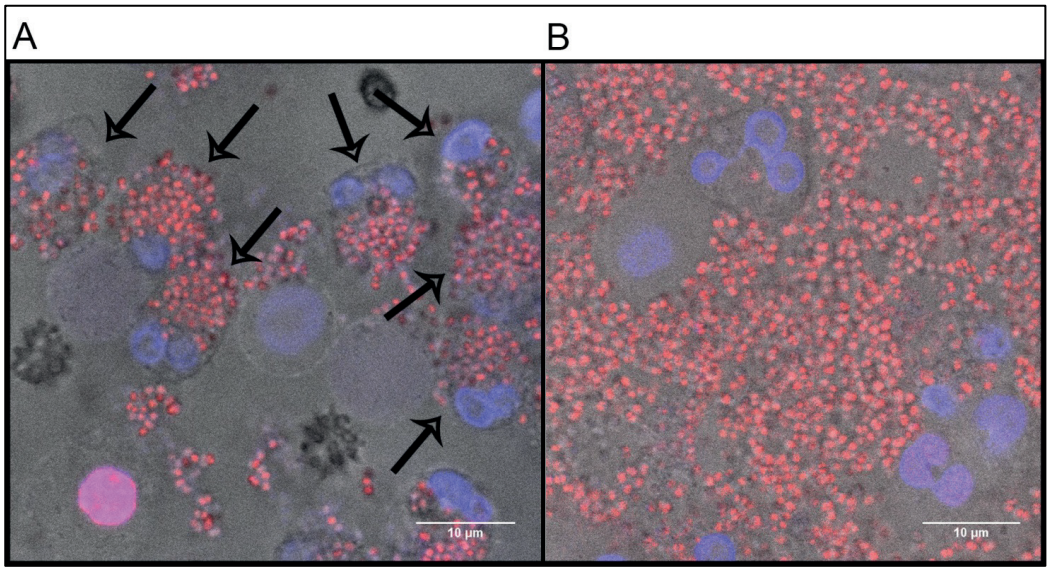

Figure 5: The effect of Protein A on dead bacteria in the presence of neutrophils after 40 minutes of incubation. A: Dead *S. aureus* Newman incubated with neutrophils. Arrows indicate phagocytosis. B: Dead *S. aureus* Newman incubated with 0.9 µg/ml Protein A and neutrophils. Blue: DNA. Red: Dead bacteria.

Discussion

In this study we demonstrated that Protein A secretion is positively correlated to NETosis induction by *S. aureus*. Previously, it has been described that different bacterial species induce different NETosis responses [10,12,24]. We showed that between different *S. aureus* strains, differences in NETosis response are seen, which is correlated to the amount of Protein A present. Furthermore, NETosis induction by *S. aureus* with little to no Protein A present can be rescued by adding Protein A.

In our study, the bacterial cell wall-associated protein A level is higher during the stationary phase than during the exponential phase. In a previous study by Gao et al., using trypticase soy broth (TSB) medium, the *spa* gene, that encodes Protein A in *S. aureus*, is upregulated in the exponential growth phase of the bacteria and the expression could be regulated via various factors [25]. In our experiments, the use of IMDM over TSB is preferred, since we work with neutrophils, and, most importantly, because the composition of culture media for mammalian cells, such as IMDM, is more closely resembling the

composition of human conditions. *S. aureus* behaves differently In IMDM than when cultured in TSB medium [26], which is an interesting and important observation.

Protein A is one of the important virulence factors that *S. aureus* uses to evade the immune system. By Protein A binding to the Fcγ region of immunoglobulin IgG, bacteria can avoid being opsonized and phagocytosed by neutrophils and other immune cells, as it inhibits binding to the neutrophils Fc receptor [27]. In previous experiments we already reported that dead bacteria are phagocytosed by neutrophils and do not induce NETosis [24]. We now observed that phagocytosis was inhibited when Protein A was added to dead bacteria, although in our experiment, to our knowledge, no IgG was present. Protein A has also been described to bind to the TNFR1 of immune cells, causing production of pro-inflammatory cytokines such as IL-6 and TNF-α [28]. Both of these cytokines have been described as inducers of NETosis [29,30]. Additionally, Protein A can activate NADHP oxidase [31], on which NETosis depends [32]. Collectively, this would indicate that Protein A alone could activate NETosis by binding to TNFR1. However, in our experiments no NETosis was observed when Protein A was added to the neutrophils without bacteria, indicating that another stimulus, for example a different cofactor secreted by living *S. aureus*, is needed in addition to Protein A.

The observations in our study that Protein A is both a determinant of NETs formation and can inhibit phagocytosis without IgG are interesting additions to previous studies, where living *S. aureus* bacteria were reported to degrade the NETs [16,33]. These studies speculated that the ability of NETosis induction by bacteria might be a function of bacteria to kill human neutrophils, due to the high survival of bacteria that were able to escape the NETs. Our findings contribute to this hypothesis, and suggests that *S. aureus* is not only able to evade phagocytosis by secreting Protein A, but also to destroy the neutrophils by evoking NETosis, from which they can escape by producing nucleases.

In this study, we used the double knockout strain *S. aureus* Newman for SpA and Sbi. The NETosis inducing capacity of this strain also might have been affected by Sbi. Sbi has a similar function and structure as Protein A, and it can interact with complement protein C3, thereby inhibiting opsonisation and therefore phagocytosis [33,34]. However, in our experiments we were able to fully rescue NETosis induction by only adding purified Protein A, indicating

that Protein A is the important determinant of NETosis in this experimental setting.

In chronic wound infections such as osteomyelitis and ulcers, *S. aureus* is one of the most frequently isolated bacteria[3,35]. By understanding more about direct interaction between the human immune system, in this case neutrophils, and *S. aureus*, we may find more ways to efficiently target the bacteria by interfering with the bacterial products that influence and inhibit our immune system. In conclusion, the amount of Protein A present is an important determinant of NETosis induction by *S. aureus*, and NETosis inducing capacities of strains with little to no Protein A present can be enhanced by addition of commercial Protein A.

Acknowledgements

None.

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Chapter 4

The Role of Nuclease during the Early stages of Biofilm formation

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* Contributed equally

In preparation

Abstract

The chronic character of *Staphylococcus aureus* infections, like osteomyelitis and foot ulcers, is often associated with biofilm formation. eDNA is thought to play a crucial role in the structural stability of biofilms but it also induces host immune responses. Studying the bacterial products made during biofilm formation, we observed that *S. aureus* already during the early stages produces nuclease 1 when grown in IMDM. As eDNA is supposed to play an important role during biofilm formation, we studied the implications of Nuclase1 production on biofilm accumulation in both TSB and IMDM. In TSB, nuclease production is delayed compared to IMDM grown bacteria. Furthermore, biofilms grown in TSB showed accumulation of dead cells eDNA while IMDM grown biofilms produced vast amounts of PIA. Illustrating that biofilms developing in IMDM do not need eDNA for structural stability. Based on these results, we investigated the effect of nuclease Nuc1 production on NETosis and ROS production by PMNs using IMDM medium and could show that nuclease can degraded the biofilm-induced NETs. Interestingly, ROS production by PMNs was decreased when biofilm was present but was not affected by nuclease production. Our findings suggest that *S. aureus* produces nuclease in both early and late biofilm, and that nuclease contributes to immune evasion of biofilms.

Introduction

Staphylococcus aureus (*S. aureus*) is a well-known human pathogen that can cause infections from mild subcutaneous to life threatening invasive infections. When infections caused by *S. aureus* become chronic, biofilm formation can often be observed. The non-cellular component of the bacterial extracellular matrix (ECM), that encloses a biofilm, is a complex matrix that consists of polysaccharides, glycolipids, proteins and extracellular DNA (eDNA) [1]. eDNA formation in *S. aureus* biofilms is facilitated by an autolysis process that mimics apoptosis of eukaryotic cells [1] and is thought to play a crucial role in the structural stability of biofilms [2].

In a previous study from our group (Sultan, *et al.*, unpublished data) we observed that, during the early stages of biofilm formation, *S. aureus* produces immune modulators like SCIN, CHIPS and FLIPR, facilitating the defense of the developing biofilms against host early immune responses. Furthermore, we

observed the production of nuclease during the early stages of biofilm formation when *S. aureus* was grown in Iscove's Modified Dulbecco's Medium (IMDM). *S. aureus* can produce two types of nucleases: Nuc1 and Nuc2 [3]. The major type of nuclease produced by *S. aureus* is Nuc1[4]. Nuc1 production is particularly conserved across all strains of *S. aureus* and it has been used as one of the specific markers to identify *S. aureus* [5,6]. It is known that *in vitro* transcription and production of Nuc1 by *S. aureus* can be regulated by the 2-component system *SaeRS* [7]. Nuclease production is associated with prevention of bacterial cell attachment and biofilm formation [1,2,8].

Whilst eDNA stabilizes the biofilm structure, it also induces host immune responses. For example, unmethylated CpG motifs of bacterial eDNA can be recognized by TLR9 [9,10], causing further host immune activation via sequential recruitment of sequential recruitment of MyD88 and TRAF-6 [9,10]. In biofilm, nuclease Nuc1 is an enzyme that can dissolve and degrades eDNA that interconnects between bacterial cells within biofilm [8]. Furthermore, in a study with planktonic *S. aureus*, nuclease was described to be associated with evasion of neutrophil extracellular traps (NETs), a mechanism of cell death of the polymorphonuclear cells (PMN), that trap and kill bacteria [11,12]. Additionally, Berends *et al* [11] demonstrated in a murine respiratory tract infection model that *S. aureus* nuclease also plays a role in immune avoidance against NETs *in vivo*. Taken together, nuclease production seems to be an important strategy for *S. aureus* to modulate the innate host response, allowing a biofilm to develop. On the other hand, nuclease formation is thought to destabilize biofilms as well.

To approach this controversy, we aimed to study, in the context of a developing biofilm, the consequences of nuclease production on a) biofilm accumulation and b) defense against the innate host immune response. Therefore, we first determined biofilm accumulation, composition and nuclease production in the mammalian cell culture medium IMDM and as comparison in the classical medium, tryptic soy broth (TSB). Furthermore, we studied the NETosis inducing abilities of early biofilms and the effect of nuclease on NETs degradation and ROS production.

Materials and Methods

Bacterial strains and growth condition

The *S. aureus* strains used in this study are listed in Table 1. The strains were plated on Trypticase™ Soy Agar (TSA) with 5% sheep blood overnight at 37 °C (Becton Dickinson, Breda, The Netherlands).

Table 1: Strains of *S. aureus* that are used on this study.

Strain	Genetic Background	Description	Source
Newman	ST8	Laboratory strain	
8325-4	ST8	Laboratory strain	A. Cheung
M82	ST20	Clinical isolate from a patient with an osteomyelitis infection in Indonesia	Sultan, *et al*.
M116	ST239, ST8	Clinical isolate from a patient with an osteomyelitis infection in Indonesia	Sultan, *et al*.
RWW50	ST8, USA300	Clinical isolate from the collection of MMIZ Erasmus MC, The Netherlands	W. van Leeuwen
MW2	ST1, USA400	Clinical isolate	
Mu50	ST5	Clinical isolate	

Bacterial growth and biofilm assessment

Biofilm formation was assessed *in vitro* using a dynamic, microtiter plate, biofilm formation assay as previously described [13-16] with slight modifications. *S. aureus* bacteria cultured overnight on TSA were suspended in 4 ml of 0.9% NaCl to reach OD_{660nm} of 2.0 (± 0.2). One microliter of this suspension was added to 199 µl Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, Bleiswijk, the Netherlands) in separate wells of a sterile flat-bottom 96-well polystyrene tissue culture plate (CELLSTAR® Greiner Bio-One, Alphen aan de Rijn, the Netherlands) [16]. Plates were incubated for 1, 2, 4 and 24 hours under 150 rpm orbital shaking at 37 °C. At these times points, the formed biofilms were washed once with 200 µl sterile water, air dried for 30 minutes at room temperature and stained for 2 minutes with 50 µL of 1% crystal violet (Sigma

Aldrich, Zwijndrecht, the Netherlands). Excess of crystal violet was removed by washing the plates five times with 200 µL of sterile water. The biofilms were dissolved in 200 µL extraction solution consisting of 50% dH_2O 40% ethanol (Sigma Aldrich) and 10% acetic acid (Sigma Aldrich). Lastly, to determine the biofilm mass, the absorbance of crystal violet was measured at OD_{490nm} in a microplate reader (Epoch 2 Microplate reader, BioTek Instruments, Inc., Winooski, VT, USA).

Determination of nuc presence

Presence of *nuc* gene was performed according to a PCR protocol as described previously [17].

Production of immune modulators during S. aureus biofilm formation

S. aureus Newman, clinical strains M82 (ST20), M116 (ST239) and also RWW50 (USA300) were cultured as described above. We cultured the biofilm for either 1, 3 or 23 hours. At these time points we discarded the supernatant to remove all non-attached or planktonic bacteria, washed the biofilm with 200 µL IMDM once and added 200 µl of fresh IMDM medium and proceeded incubation for 1 hour at the conditions described above. Subsequently, we collected the supernatants, and labelled them as the 2nd, 4th, and the 24th hour biofilm supernatants. All individual supernatants from all time points and all different strains were filtered through a 0.2 µm sterile filter (Whatman® GE Healthcare Life Sciences, Little Chalfont, UK) and stored at -20 ˚C until further usage. The supernatant collected after 1 hour of biofilm formation served as negative control.

Quantification of nuclease production with competitive Luminex assay

Nuclease production during biofilm formation was quantified using a multiplex competitive luminex assay [13,18]. Nuclease production was measured in the biofilm supernatant of the 1st, 2nd, 4th and the 24th hour of biofilm formation. Briefly, the diluted biofilm-derived supernatants were incubated separately with 1/200 diluted human pooled serum (HPS) at 22 ˚C for 35 minutes. This mixture was used for the simultaneous semi-quantification of the non-captured IgG's specific for *S. aureus* biofilm derived immune modulators using Luminex bead based flow cytometry (Luminex Corporation, Austin, Tx, USA). Nuclease antigen was coupled to MagPlex beads (Luminex Corporation) according to the manufacturer's protocol The Luminex assay,

analyzed using a Luminex BioPlex 200 System using Bio-Plex® Manager software version 6.1 (Bio-Rad Laboratories, Inc., USA) was performed as described previously [19] with one modification; that is, we measured 50 beads per region [20]. Negative control beads (beads to which no antigen was added during coupling) were included in all assays. All data are based on three separate experiments. The mean fluorescence intensity (MFI) values of these triplicates were averaged if the CV value was lower than 25%; and the standard error of the mean (SEM) was calculated. For each sample, and each individual antigen we calculated the proportion (in %) of uncaptured IgG specific for nuclease. This was achieved using a semi-quantitative measurement of the antigen specific antibody absorption from HPS by biofilm supernatant, which indirectly reflects the presence of the immune modulators in the biofilm supernatant [21] and subtracting this percentage from 100%. Statistical differences of nuclease production between the time points of measurements were calculated by comparing the percentage of uncaptured anti-nuclease IgG from the 2nd, 4th and the 24th hour time points with the 1st hour as reference control.

Identification of the immune modulators by mass spectrometry

One hundred microliter of supernatants derived from the 4th hour during biofilm formation of the RWW50 (USA300) and Newman strains were precipitated at 1:10 v/v with ice-cold acetone and centrifuged for 10 minutes at 18407x g at 4 °C. Subsequently, pellets were washed twice with 50 µl of cold acetone. Then, pellets were air dried for 30 minutes and dissolved in 10 µl of 50 mM NH_4HCO_3. Solution digestion was performed according to an adjusted protocol as described by Dekker, *et al* [22]. Ten microliters of 0.2% Rapigest (Waters Corporation, Milford, MA, USA), which was previously diluted in 50 mM NH4HCO3, was added into 10 µL sample. This mixture then was reduced with 5 mM dithiothreitol (DTT) (Thermo Fisher Scientific, Waltham, MA, USA) at 60 °C, 450 rpm for 30 minutes. After cooling down to room temperature, this mixture was alkylated in the dark with 15 mM iodoacetamide (Thermo Fisher Scientific) at ambient temperature for 30 min, and digested overnight with 1:100 (w/w) trypsin (Promega Corporation, Fitchburg, WI, USA) at 37 °C and 450 rpm. Five percent trifluoroacetic acid was added to obtain a final concentration of 0.5% trifluoroacetic acid (pH<2). After 45 minutes of incubation at 37 °C and 450 rpm, the samples were centrifuged at 13,000 x g for 10 minutes and the supernatant was used for liquid chromatography–mass spectrometry (LC-MS). Mass spectrometry and data analyses were performed

as described elsewhere [22]. Database search was performed against the uniprot_sprot_v151112 database (selected for Bacteria, 332280 entries).

Quantification of nuclease activity with FRET-based assay

We quantified the nuclease activity using a previously described fluorescence resonance energy transfer (FRET)-based assay [23]. The FRET substrate was purchased from Eurogentec (Maastricht, the Netherlands) [24]. The FRET substrate was diluted to 2 µM in tris Buffer (20 mM Tris, pH 8.0, and 10 mM $CaCl_2$) [23,24]. To measure the activity of biofilm associated nuclease of each strain, biofilms were grown in a flat-bottom 96-well black polystyrene tissue culture plate (Costar, Corning incorporated, NY, USA) at 37 ˚C with continuous 150 rpm agitation. After incubation for 3 hours, biofilms were washed once with IMDM or TSB. Twenty-five microliter of new IMDM or TSB (with or without tested compounds) and 25 µL diluted FRET substrate was added into the biofilms. The biofilms were then incubated in a FLUOstar Optima microplate fluorescence reader (BMG lab technologies) at 30 ˚C with 150 rpm periodic rotational shaking for 2 hours. The accumulation of fluorescence during these 2 hours, used as a measurement for nuclease activity, was determined automatically every 5 minutes (excitation 552 nm/emission 580 nm/Gain 800). Results are reported as fluorescence units. Mean Fluorescence Intensity (MFI) of three separate experiments were calculated.

Live/Dead - WGA staining for biofilm

Biofilms were stained with LIVE/DEAD® BacLight Bacterial Viability Kit (Thermo Fisher Scientific, Bleiswijk, The Netherlands) and Wheat Germ Agglutinin (WGA) - Alexa Fluor® 350 conjugate (Invitrogen BV, Breda (The Netherlands), according to the manufacturer's protocol with slight modification. Four hours old biofilms were washed once with 200 µL of PBS. The biofilms were then incubated with 50 µL of PBS, 50 µl of 15 µM propidium iodide (PI), 50 µl of 2.5 µM SYTO9 and 0.5 µl of 1 mg/mL WGA-Alexa fluor® 350 conjugate on each well. The plate was incubated at 22 ˚C on an orbital shaker (300 rpm) in the dark for 35 minutes. The Biofilms were imaged using a Olympus IX51 fluorescence microscope (Olympus Nederland B.V., Zoeterwoude, The Netherlands) with 40x magnification.

NETosis assay

Human neutrophils were isolated as described previously 25 and stained with Hoechst 34580 (1:10 000, Life Technologies, Landsmeer, The Netherlands)

and Propidium iodide (PI, 1:400, Sigma Aldrich, Zwijndrecht, The Netherlands). One hundred microliters of 107/mL neutrophils were added to a three hours old biofilm formed by Newman or RWW50 (in presence or absence of 5 µg/mL nuclease), in a flat-bottom 96-well polystyrene tissue culture plate (CELLSTAR® Greiner Bio-One, Alphen aan den Rijn, The Netherlands). Biofilm – PMN interactions were imaged with a confocal microscope (Leica SP5 AOBS) after 20 minutes of incubation at 37 °C. Hoechst and PI were excited by 405 nm and 561 nm lasers. NETs were visible as PI positive elongated structures and were quantified.

Cellular ROS activation assay

Three hours old biofilms prepared in IMDM were washed and refreshed with 100 µL new IMDM (in presence or absence of 5 µg/mL nuclease). One hundred microliters of 107/mL neutrophils then added to the biofilms. The biofilms were then prepared for the Cellular ROS activation assay (GeneCopoeiaTM, Nivelles, Belgium) according to the manufacturers protocol. The kinetics of the first 145 minutes of ROS activation was measured in FLUOstar Optima microplate fluorescence reader (excitation 552 nm/emission 580 nm/Gain 800). Results were reported as fluorescence units. Mean Fluorescence Intensity (MFI) of three independent experiments were calculated.

Statistical analysis

Statistical analysis was performed by using the Prism 5.0 package (Graph Pad Software, San Diego, CA, USA) and Microsoft Excel 2010.We used unpaired t-test or one-way ANOVA for data analysis, where $P \leq 0.05$ was considered as statistically significant. All experiments were independently repeated for three times and the mean with Standard Error of the Mean (SEM).

Results

Nuclease expression by early biofilms

To confirm our initial observation that during early biofilm development in IMDM, *S. aureus* generates Nuc1, Nuc1 production was studied after 1, 2, 4 and 24 hours by competitive Luminex assay. Nuc1 was found in the supernatant of biofilms from all isolates (Fig. 1B-F). During the 4th hour of biofilm formation, Nuc1 was significantly expressed by M82 ($P \leq 0.01$), M116 ($P \leq 0.001$) and RWW50 ($P \leq 0.001$) (Fig. 1). Additionally, a positive correlation between nuclease production and biofilm mass was found for M82 (r=0.903; p =0.053), M116 (r=0.813; p=0.187), 8325-4 (r=0.999; p=0.0002), RWW50 (r=0.847; p=0.152) and for Newman (r=0.947; p=0.053) (Fig. 1B-F). We further confirmed Nuc1 production during early stages of biofilm formation using mass-spectometry (Table 2).

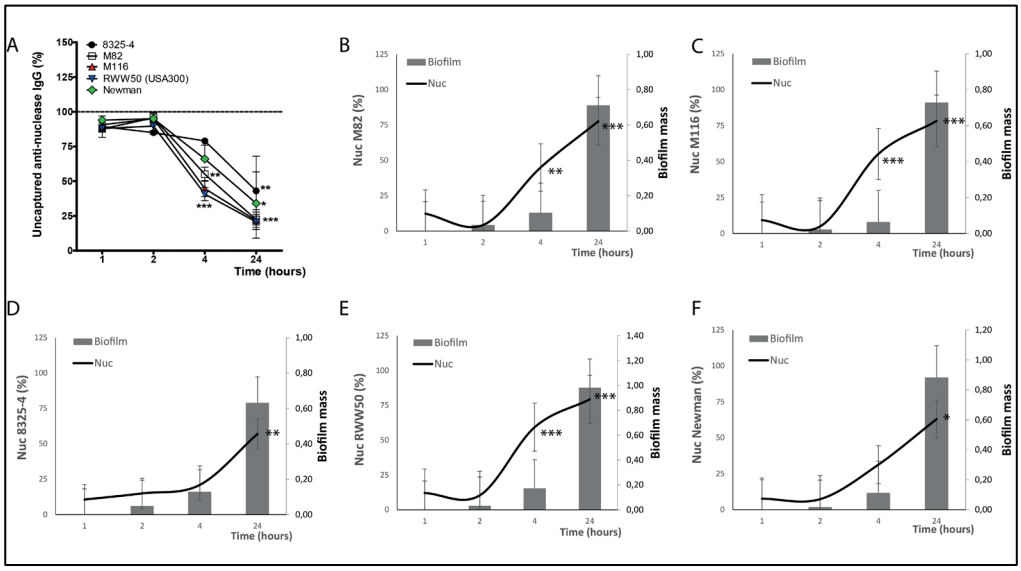

Figure 1: Nuclease expression during biofilm formation. The competitive Luminex assay monitored the presence of nuclease (A) indirectly in the supernatants of the 2nd, 4th and the 24th hour during biofilm formation of S. aureus M82 (B), M116 (C), M116 (D), RWW50 (E) and Newman strains (F). Presented are biofim (bar) en Nuclease (line). These figures have been generated from three separate experiments. Error bars presented are mean and SEM.

Table 2: Mass-spectrometry for nuclease from the 4th and the 24 hour of biofilm formation.

Strains	Biofilm supernatant	Unique peptides detected*			Sequence coverage (%)*			Protein identification probability (%)*		
		I	II	III	I	II	III	I	II	III
Newman	t=4h	5	-	-	25.6	-	-	100	-	-
	t=24h	11	3	1	40.8	16.7	6.58	100	100	92,9
RWW50 (USA300)	t=4h	5	5	11	21.1	24.6	46.9	100	100	100
	t=24h	16	10	-	47.8	36.8	-	100	100	-

***Three separate experiments I, II and III. All biofilms had Protein Nuc and Protein accession number Q5HHM4.**

As nuclease activity is mostly described during later stages biofilm formation in conventional media and *nuc*-GFP transcription was noticeable approximately after 10 hours of biofilm incubation in BHI media [24] or while 6 hours in TSB [26], we setup an experiment in which we compared nuclease activity in biofilms grown in both TSB and IMDM**.** Therefore, we quantified nuclease activity for two hours in 3 hours old biofilms using a FRET-based assay. Here, we saw that nuclease activity of strains 8325-4, M116, Newman and MW2, was higher when grown in IMDM, compared to the Nuclease activity of these strains in TSB ($P \leq 0.001$) (Fig. 2). Interestingly, strain RWW50, which belongs to the USA300 family, and strain Mu50 show no significant different in nuclease activity between these two growth media ($p=0.05$).

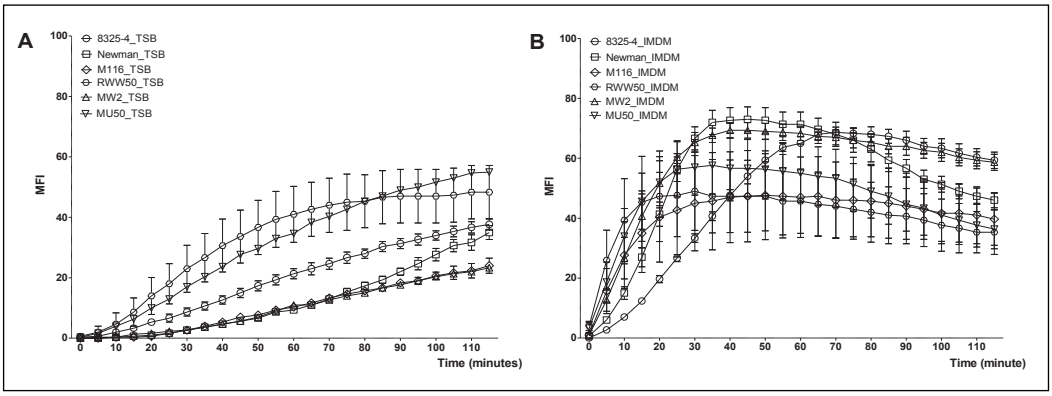

Figure 2: Nuclease production by biofilm of different *S. aureus* strains in TSB and IMDM. FRET-based measurement of Nuclease production during the 4th and the 5th hour of biofilm formation of *S. aureus* 8325-4, Newman, M116, RWW50, MW2 and Mu50 in TSB

(A) and IMDM (B). Figures have been generated from three separate experiments. Error bars represent SEM.

Contribution of eDNA to biofilm mass in the early stages of biofilm formation

If eDNA is an important factor in the accumulation of bacterial cells during biofilm formation, how is it possible that biofilm formation in IMDM does not seem to be hampered during the early stages? We therefore studied the biofilm content of 4 and 24 hour biofilms grown in TSB and IMDM. The composition of biofilm after 4 hours of biofilm formation showed that dead cells or eDNA were minimally present in both TSB and IMDM, but after 24 hours of biofilm formation, eDNA was observed, particularly in biofilm formed in TSB medium (Fig. 3). Interestingly, in biofilms grown in IMDM, the polysaccharide intercellular adhesin (PIA) was visible after 4 hours of biofilm formation and abundantly present after 24 hours (Fig. 3).

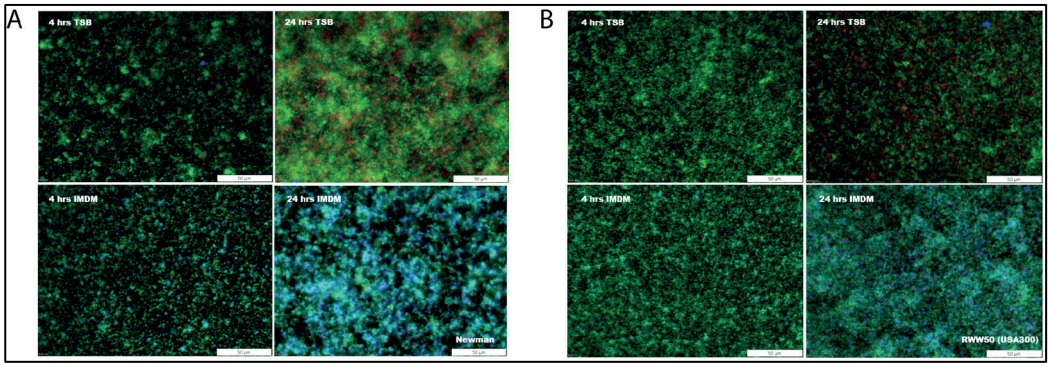

Figure 3: Extracellular DNA formation in biofilm. Four and twenty-four hours biofilms of *S. aureus* Newman (A) and RWW50 (B) in TSB and IMDM were stained with Syto9 (DNA, Green) and PI (extracellular DNA, Red) staining in combination with WGA (Blue) staining and visualized by fluorescence microscopy. Figures are a representative result of 3 separate experiments.

Biofilm activates ROS-independent NETs formation

In the experiments described above, we observed that biofilm formation, when grown in IMDM, is facilitated in particular by PIA, and we did not find reasonable amounts of eDNA. It could be that Nuc1 is playing a role in the defense against the immune system by prevention of eDNA accumulation during biofilm formation in IMDM. Furthermore, previously, it was described in a planktonic setting that nuclease can degrade NETs to ensure the survival of *S. aureus*[11,12]. In order to investigate if this is also happening in a biofilm

setting, we added freshly isolated PMNs to 3 hours old washed biofilm. Here, we saw massive NETosis induction by both *S. aureus* Newman and by RWW50 biofilms. When 5 µg/mL of nuclease was added to the biofilm before incubation with PMN, almost no NETs were observed (Fig. 4). Furthermore, during the co-incubation of PMN with 3 hours old biofilm of Newman or RWW50, we saw that reactive oxygen species (ROS) levels of PMN were not significantly different with spontaneous ROS production by PMN itself (Fig. 5). This findings indicate that *S. aureus* biofilm-induce NETs formation is ROS-independent. Meanwhile, the addition of nuclease alone had no effect on ROS activation (Fig. 5).

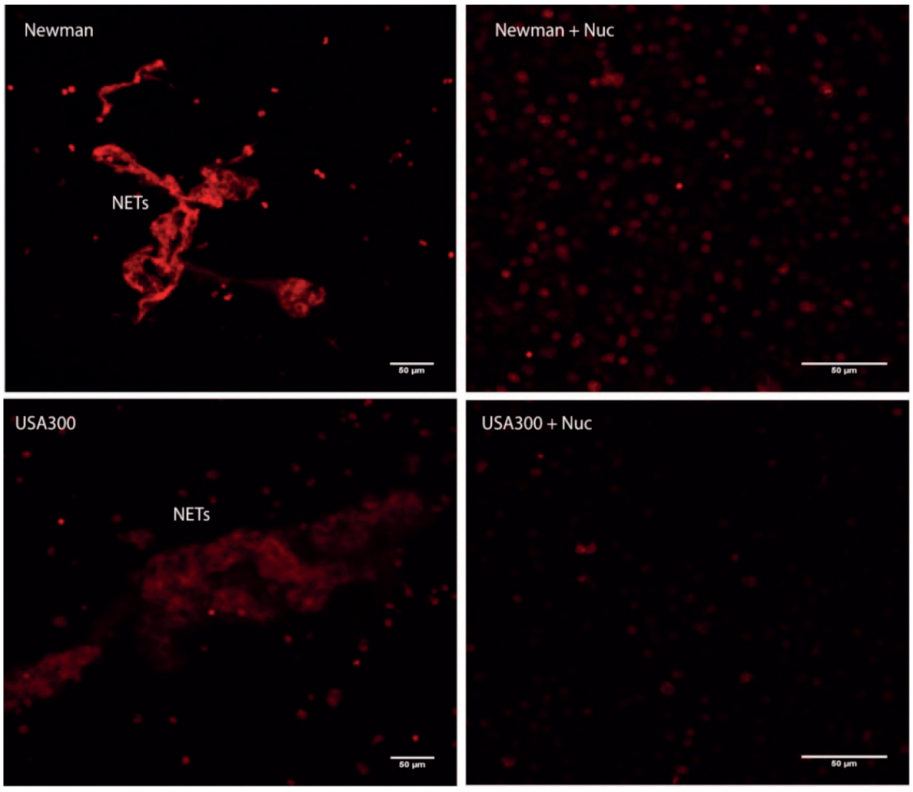

Figure 4: NETs activation by early biofilm. Exogenous nuclease inhibited the formation of NETs (red) during the 4th hour of biofilm formation of Newman (A-B) and RWW50 (C-D) in presence of human PMN cells. Figures are a representative result of 3 separate experiments.

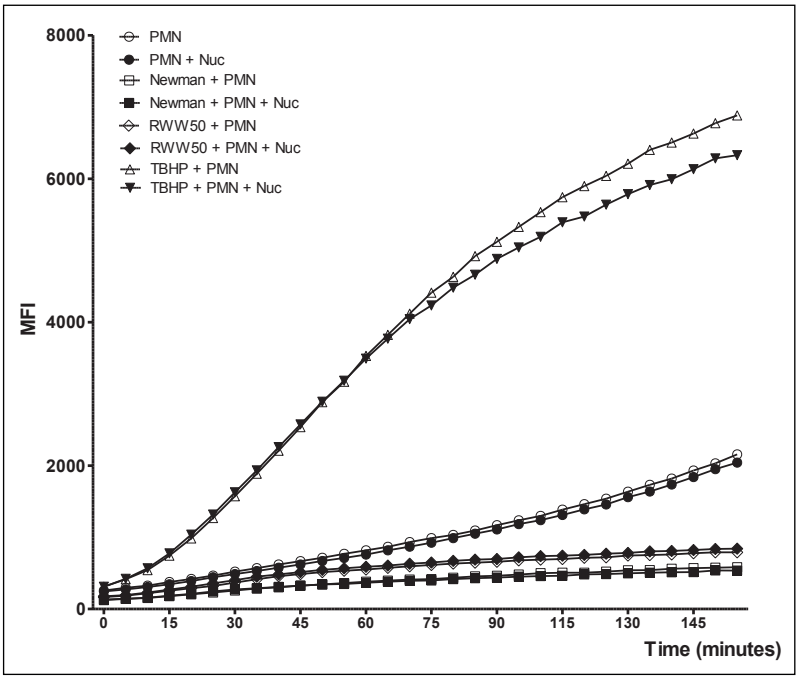

Figure 5: Biofilm inhibits PMN ROS activation. When PMN and 3 hours old biofilm were co-incubated for 2 hours, ROS activation by PMN was decreased compared to PMN without biofilm or to the positive control tert-butyl hydroperoxide (TBHP), which is a common ROS inducer. Nuclease had no effect on this ROS inhibition. MFI: mean fluorescence intensity.

Discussion

During the early stages of biofilm formation of *S. aureus* in the eukaryotic growth medium IMDM, we observed the production of nuclease Nuc1 using three different methods. Furthermore, this nuclease production was higher in IMDM compared to TSB in most of the tested strains.

Currently, it is expected that one of the roles of nuclease is to release the bacteria from the eDNA containing biofilm matrix and destabilization of biofilm [23]. However, most of the experiments leading to this assumption were performed in TSB. When we grew biofilms in IMDM, we observed biofilm accumulation even though the concentration of nuclease activity increased as well. This indicates that in IMDM, biofilm formation is independent of eDNA. Analysis of biofilm composition formed in IMDM and TSB revealed that indeed after 4 hours of growth, all biofilms contained almost no eDNA, but biofilms

formed in IMDM did contain PIA. After 24 hours of growth in TSB, dead cell/eDNA was observed, but was minimally present in IMDM grown biofilms. In addition after 24 hours, PIA was abundantly present in the IMDM cultured biofilms, but was not observed in the TSB cultured biofilms. This might be due to the activity of the *SaeRS* system [7]. *SaeRS* systems, which regulate *nuc* transcription, may be activated differently between these two growth media. In mammalian cell culture medium that contains plasma, the *Sae* system is required for biofilm formation, while in TSB, the *Sae* system is dispensable [27]. The *SaeRS* gene can be activated by limited or absence of Fe in medium, such as in IMDM [27], and may stimulate the production of both PIA and Nuclease [28-30]. This could, therefore, also explain why biofilms grown in IMDM produce more nuclease. Because nucleases break down DNA, it has been described to be produced by biofilm to regulate biofilm mass [8,24]. This would indeed be the case in biofilm grown in TSB, as it consists of eDNA that and can be regulated in this manner. However, when grown in IMDM, the early and robust production of nuclease might have broken down most eDNA in these biofilms, leaving only PIA. Since deacetylated PIA is positively charged [31,32], PIA can act as an extra protection for bacteria within the biofilm against many antimicrobial factors produced by, for example, innate immune cells [33].

In our study, we found that early biofilms are inducers of NETosis, and that nuclease Nuc1 can degrade these NETs. PMN are the first immune cells to respond to an injury, and one of their host defense responses is the formation of NETs [34]. When we incubated 3 hours old biofilm with PMN, we observed the formation of NETs, whereas no NETs were found when the biofilm was incubated with nuclease beforehand. Previously, nucleases, secreted by planktonic *S. aureus*, have been described to break down NETs [12]. NETs have been described as an immune response against pathogens [34] and can be induced by bacteria [25,35], fungi [36] and bacterial products such as LPS [37]. *S. aureus* is a very strong inducer of NETosis [35,38], the process of NETs formation in which neutrophils excrete their DNA, histones and antimicrobial proteins into the ECM, which is activated in a ROS independent manner [11]. When present, bacteria are trapped in the NETs, thus bacterial spreading is limited. Recently, NETs have also been detected in biofilm [39]. However, other studies observe phagocytosis by PMN that invade biofilm formed by *S. aureus* [40]. Our results support that biofilms are also inducers of NETosis, but these NETs can be degraded by nuclease. Furthermore, it has been described that *S. aureus* biofilm can produce SasH (AdsA), which can convert the degraded DNA

products into deoxyadenosine (dAdo), an important immune modulator [41,42]. In this study, we present the first step in understanding the interaction between *S. aureus* biofilm and NETosis in the immune system. Future studies need to further elucidate how the balance between the parallel induction and inhibition of NETosis by biofilms is regulated and how it affects the role of NETosis in the pathophysiology of biofilm.

Finally, we did not observe ROS production of PMN in our biofilm experiments. PMN typically have three different antimicrobial mechanisms: (1) Phagocytosis: pathogens are engulfed into a phagosome and destroyed by NADPH-dependent mechanisms (ROS production) or antimicrobial proteins. (2) Degranulation: pathogens are killed by ROS and antimicrobial proteins, that are released into the ECM by PMN and (3) NETosis [43]. NETosis can be induced by both ROS-dependent and ROS-independent pathways [44]. ROS production, therefore, is essential in all PMN host defense mechanisms. In our biofilm experiments, we saw a ROS-independent formation of NETs, indicating as NETs are formed while at the same time ROS was inhibited by biofilm. This is in line with previous literature in planktonic *S. aureus* [35] and *Leishmania* [45].

Since there are indications that nuclease does not act alone to modulate early host innate responses [41,42], other immune modulators that are present during the early stage of biofilm formation should be explored. Investigation of the role of other *SaeRS*-related immune modulators such as Eap (map) [46] and sasH (AdsA) in immune modulation should be prioritized, since the role of these immune modulators in host immune evasion related to nuclease has already been described elegantly in planktonic *S. aureus* studies [41,42,47-49], but not yet in biofilm.

Many studies on biofilm are currently using classic growth medium such as TSB for biofilm experiments. On the contrary, IMDM seems to be a more reasonable choice as it better mimics the human environment. We have now demonstrated that biofilms grow and respond differently in IMDM compared to TSB. Iron-limited or free medium like IMDM or RPMI would be more suitable for *ex vivo* studies with biofilm, since these media could support both S. *aureus* biofilm and human cells, enabling co-incubation experiments. Therefore, the use of IMDM in biofilm related studies might be a better way to support *ex vivo* investigation of biofilm-related infections.

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Chapter 5

Neutrophil extracellular traps in children with meningococcal sepsis

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Abstract

Background: Children with meningococcal sepsis are highly at risk for fulminant disease, multi-organ failure and death. Recently, neutrophil extracellular traps (NETs) levels have been indicated as a marker for severity in different kinds of sepsis. Our aim was to study the role of NETosis in meninogococcal sepsis in children.

Methods: We measured MPO-DNA, a marker for NETs, in serum of meningococcal sepsis patients upon admission to PICU, at 24 hours, and at 1 month and studied the association with clinical outcome. Subsequently, we tested whether *N. meningitidis*, isolated from children with meningococcal sepsis, were able to induce NETosis, using confocal microscopy live imaging.

Results: MPO-DNA levels at admission (n=35, median 0.21 AU/mL, IQR 0.12-0.27) and at 24 hours (n=39, median 0.14 U/mL, IQR 0.09-0.25) were significantly higher than the MPO-DNA levels after 1 month (controls, n=36, median 0.07 AU/mL, IQR 0.05-0.09, $p<0.001$). We did not observe a correlation between MPO-DNA levels and mortality, cell-free DNA or other inflammatory markers. In addition, *N. meningitidis* are fast and strong inducers of NETosis.

Conclusion: Children admitted to PICU for meningococcal sepsis have higher NETs levels at admission and after 24 hours than controls. NETs levels were not associated with outcome, cell-free DNA or other inflammatory markers. These NETs may be induced by *N. meningitidis*, since these are strong NETosis inducers.

Introduction

Meningococcal sepsis is notorious for its rapid progression to fulminant disease, multi-organ failure and death [1,2]. Complex interplays between host, pathogen, and environmental factors, including immune evasion mechanisms, determine the severity of *Neisseria meningitidis* infections, ranging from harmless colonization to lethal disease [3-5].

A recently identified mechanism that may play a role in the pathology of meningococcal sepsis is NETosis. The release of neutrophil extracellular traps (NETs) is an important part of innate immune defense [6,7]. NETs are an

extracellular DNA matrix, containing also granule proteins and histones, released by neutrophils to degrade virulence factors and to kill bacteria [8]. NETs are primarily considered as a protective mechanism against a broad range of microorganisms, including gram-negative and gram-positive bacteria, because they prevent bacteria from spreading and contain toxic histones [8,9]. Several inducers of NETosis are known, including bacterial species such as *Staphylococcus aureus* [10]. Multiple studies describe a negative influence on outcome in mice and humans [11,12], since the tissue damage caused by NETs may contribute to disease severity [13,14]. Also cell free DNA (cfDNA), the backbone of a NET, has been described to contribute to disease via the formation of microthrombi and to inhibit fibrinolysis [15].

Studies on the relationship between NETs and *Neisseria meningitidis* in sepsis are lacking. Our objective was to study the role of NETs in children with meningococcal sepsis. We measured levels of NETs, cell-free DNA and other inflammatory markers in serum from children with meningococcal sepsis and studied the association with severity of disease. We also investigated whether *N. meningitidis* isolates from patients are able to induce NETosis.

Material and methods

Patients and samples

From 1988 to 2005, children with meningococcal sepsis presenting to the pediatric intensive care unit (PICU) of Erasmus MC-Sophia Children's Hospital (Rotterdam, The Netherlands) were prospectively enrolled in meningococcal studies [16-19]. These studies were conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. All individual meningococcal studies as well as the current laboratory study (MEC-2015-497) were approved by the ethical committee of Erasmus MC, and written informed consent was obtained from parents or legal guardians. All patients fulfilled internationally agreed criteria for sepsis [20]. Blood samples were taken on admission to PICU, at 24 hours after PICU admission, and at 1 month after PICU admission. Since all children were healthy at 1 month, the 1 month convalescence samples were used as controls. Samples were processed on ice and serum was stored at -80°C until analysis. This is a retrospective laboratory study in remaining samples of prospectively collected samples.

Clinical data collection

Clinical data were collected prospectively. Disease severity was indicated by Pediatric Risk of Mortality (PRISM) [21], predicted death based on the Rotterdam score [17], disseminated intravascular coagulation (DIC) score [22], and the base excess and platelet count at presentation (BEP) score [23]. Patients were classified as *death* if death occurred during PICU-stay. PICU-free days in patients who died were considered zero.

NETosis measurements

Our MPO-DNA ELISA assay was performed as reported earlier [24]. For detection of NETs in serum, we adjusted the commercial human ELISA kit that measures cell death (Cell death detection ELISA^{PLUS}, Cat. No 11920685001, Roche Diagnostics Nederland B.V., Almere, the Netherlands) [25]. Briefly, ELISA plates were coated with a mouse-anti-human myeloperoxidase (MPO) monoclonal antibody (AbD Serotec, Oxford, UK), a NETs marker, overnight at 4 °C. The plates were then washed with Phosphate Buffered Saline (PBS) containing 0,05 % v/v Tween®-20 and incubated with blocking solution (1% BSA/PBS) overnight at 4 °C. Next, samples were added to the plate and incubated with MPO-DNA immunoreagens for 2 hours at 300 rpm at RT, washed and incubated with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) reagens for 30 min at 250 rpm at RT. The reaction was stopped with stop solution from the kit and plates were measured using a Biotek reader (FLX 800, Austria) at 405 nm with a 490 nm reference filter. The NETs reference curve was created from neutrophils that were transferred to FBS serum and stimulated with phorbol-myristate-acetate (PMA) for 4 hours. Samples were serially diluted and stored at -80°C. Values are expressed as arbitrary units (AU/mL).

Inflammatory markers CRP, fibrinogen, soluble TNFr, IL-1B, IL-6, and IL-8 and cell-free DNA (nucleosomes) were measured in previous studies from our research group [16-19,26].

Neutrophil isolation

Neutrophils were isolated as previously described [10]. Briefly, red blood cells and granulocytes were isolated from blood from adult healthy donors, as approved by the Medical Ethics Committee of the Erasmus MC, using Lymphoprep™ (Stem cell Technologies, Grenoble, France). Granulocytes were further purified by lysing the erythrocytes with erythrolysis buffer (3.1M

NH_4Cl, 0.2M $KHCO_3$, 0.02M EDTA, pH 7.4) and washing the neutrophil rich pellet twice with HEPES (0.115M NaCl, 0.012mM $CaCl_2$, 1.51mM $MgCl_2$, 4mM KCl, 0.01M HEPES, pH 7.4) buffer.

In vitro NETosis

For experiments with meningococcal bacteria, neutrophils were transferred to Dulbecco's Modified Eagle Medium (DMEM) culture medium without any additions (Biowhittaker, Lonza, Walkersville, USA) in a confocal ring. Propidium Iodide (PI, 1:400, Sigma Aldrich, Zwijndrecht, The Netherlands) was added to visualize extracellular DNA during live imaging.

To visualize extracellular DNA (NETs), neutrophils were stained for DNA with Hoechst 34580 (1: 10000, Life Technologies, Landsmeer, The Netherlands) and PI (1:400, Sigma Aldrich, Zwijndrecht, The Netherlands) prior to contact with *N. meningitidis* as described previously by our group [27]. Clinical isolates of *N. meningitidis* bacteria were cultured as described previously [28]. After 4 hours of culturing, 100 µl $1x10^8$/ml bacteria were added to 500 µl $2x10^6$/ml neutrophils. NETs were visualized using confocal microscopy (Leica SP5 AOBS). Excitation of Hoechst with a 405 laser and a BP 420-500 emission filer, and excitation of PI with a 561 141 and BP 580-620 emission filter. Elongated PI positive structures larger than 10 µm (the average diameter of a neutrophil) were defined as NETs. Round PI positive structures equal or smaller than 10 µm were defined as necrotic neutrophils.

Statistical analysis

Patient characteristics and MPO levels are presented as numbers and proportions (means and percentages) or medians and interquartile ranges. To compare MPO-DNA levels between the three time points, we used the Kruskal-Wallis test, including the post-hoc Mann-Whitney rank-sum test. To compare MPO-DNA level between survivors and non-survivors, we used the Mann-Whitney rank-sum test. Correlations were assessed using Spearman's rank correlation. Linear regression analyses were performed on logarithmically transformed data. Graphs were created with GraphPad Prism 7.00. All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS, IBM, version 21). Two-sided p-values <0.05 were considered to indicate statistical significance.

Results

NETs levels in meningococcal sepsis

In this study we included 60 children with meningococcal sepsis (58% male, median age 2 years and 10 months [IQR 21 months-9 years]), of whom 35, 39, and 36 serum samples were available originating from admission to PICU, after 24 hours, and at 1 month, respectively. Patient characteristics are presented in Table 1.

Table 1: Patient characteristics at admission to PICU (n=60). Characteristics for 2 patients are unknown.

	Total group (n=60)	Non-survivors (n=11)	Survivors (n=49)	P-value
Male gender (%)	36 (58%)	7 (64%)	29 (59%)	ns
Age (years)	2.9 (1.8-9.6)	2.2 (0.8-2.8)	3.5 (1.9-10)	0.08
PRISM score	17 (10-25)	25 (21-34)	17 (12-24)	0.003
P (death Rotterdam)*	12 (2– 72)	98 (83–99)	5 (1–25)	<0.001
Presence of DIC$ (%)	26 (52%)	10 (100%)	16 (40%)	<0.001
DIC score#	6 (4-7)	7 (6-7)	5 (4-6)	ns
P (death BEP)~	6 (3-19)	30 (20-59)	5 (3-12)	<0.001

*** Data are available for 49 patients; 11-non-survivors and 38 survivors. $ Data are available for 50 patients; 10 non-survivors and 40 survivors. These data represent the values collected at t=0. # Data are available for 36 patients; 6 non-survivors and 30 survivors. ~ Data are available for 53 patients; 11 non-survivors and 42 survivors. Categorical variables are presented as percentages, continuous variables are presented as median (IQR). Abbreviations: PRISM = pediatric risk of mortality[21], P (death Rotterdam) = predicted death rate based on the Rotterdam score[17], DIC=Disseminated intravascular coagulation[22], P (death BEP) = predicted death rate based on the BEP score [23].**

MPO-DNA levels at admission (n=35, median 0.21 AU/mL, IQR 0.12-0.27) and at 24 hours (n=39, median 0.14 AU/mL, IQR 0.09-0.25) were significantly higher than the convalescent MPO-DNA levels in the control group of survivors after 1 month (n=36, median 0.07 AU/mL, IQR 0.05-0.09, p<0.001) (Figure 1A). The MPO-DNA level in children who eventually died did not differ significantly from survivors at admission to PICU (non-survivors: n=11, median 0.23 AU/mL, IQR 0.12-0.47; survivors: n=23, median 0.16 AU/mL, IQR 0.12-0.25, p=0.14) and after 24 hours (non-survivors: n=3, median 0.14 AU/mL; survivors: n=34, median 0.15 AU/mL, IQR 0.09-0.26, p=0.48) (Figure

1B).

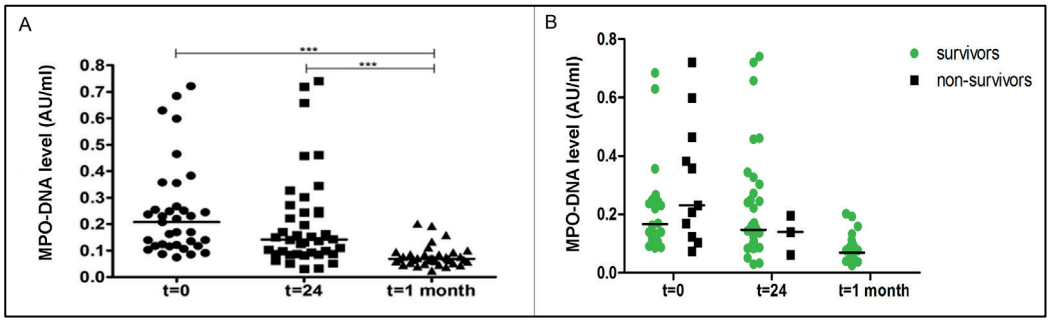

Figure 1: MPO-DNA levels measured at different time points (A) and in relation to survival (B).

NETs and inflammatory markers in sepsis

Since NETs can be induced by inflammatory markers, and NETs in turn can lead to an increased inflammatory response, we explored the correlations between inflammatory markers (neutrophil count, and serum C-reactive protein (CRP), fibrinogen, soluble Tumor Necrosis Factor (TNFr), Interleukine (IL)-1B, IL-6 and IL-8 levels) and MPO-DNA levels in our samples. We did not observe any significant correlation of NETs levels with any of these markers (Table 2).

Table 2: Correlation of MPO-DNA levels with pro-inflammatory factors.

Laboratory parameter	R-Spearman T=0	P-value	R-Spearman T=24	P-value
Neutrophils	0.043 (23)	0.85	0.211 (27)	0.29
CRP	0.105 (28)	0.60	-0.114 (29)	0.86
Fibrinogen	0.035 (29)	0.86	0.037 (36)	0.82
Soluble TNFr	0.238 (17)	0.36	-	-
IL-1B	0.391 (17)	0.12	-	-
IL-6	0.326 (17)	0.20	-0.068 (20)	0.78
IL-8	0.35 (17)	0.17	-0.291 (20)	0.21

Number of patients are indicated between brackets.

NETs and cell-free DNA

Because nucleosome levels in serum reflect cell-free DNA, we tested how serum levels of nucleosomes were correlated to serum levels of NETs. We did not find a significant correlation between nucleosomes and MPO-DNA at any

of the time points (t=0: r=0.162, p= 0.54; t=24: r=0.044, p=0.86; t=1m: r=0.025, p=0.93).

In vitro formation of NETs in the presence of *Neisseria meningitidis*

In our pilot experiment, we saw that clinical isolates from *N. meningitidis* induce NETs *in vitro* in adult neutrophils. NETs were visible after 15 minutes of incubation of neutrophils with the bacteria (Fig. 2).
In the control experiment with unstimulated neutrophils without bacteria, no NETs were seen.

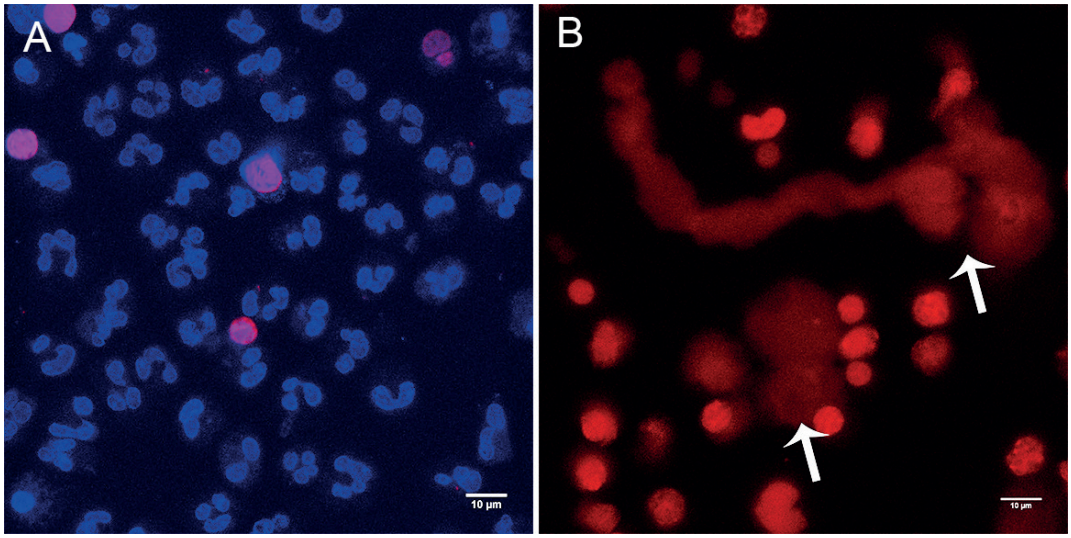

Figure 2: Unstimulated neutrophils (A) vs NETs (see arrows) formed by *N. meningitidis* (B). Blue: Hoechst staining for DNA. Red: PI staining for extracellular DNA (NETs) and dead cells.

Since neutrophils started dying approximately 30-40 minutes after contact with *N. meningitidis,* we only observed NETs within this time frame.

Discussion

This is the first study to describe NETs in children with meningococcal sepsis, describing both *in vitro* and *in vivo* data. The main findings of our study are that NETs levels in these children are higher at admission to PICU and after 24 hours compared to levels after one month. We also found that NETs levels were not associated with clinical outcome in our cohort. Lastly, in a pilot experiment we found that *N. meningitidis,* isolated from children with meningococcal sepsis, are strong NETs inducers in adult neutrophils.

In our study the NETs levels, measured by MPO-DNA levels, in children with meningococcal sepsis are higher in the acute phase of disease, i.e. at admission to PICU and at 24 hours after admission, compared to 1 month. Similar results were previously observed in adult studies, reporting increased NETs levels during the acute phase of sepsis [11,13,29].

Multiple animal and patient studies associated NETs levels with sepsis severity and outcome [13,14]. This effect of NETs in sepsis likely results from the interplay between NETs, platelets and thrombin, which activates coagulation and inhibits fibrinolysis, leading to more severe DIC [13,14,30]. In our study, we did not find an association between NETs, severity parameters, and outcome. Our study group was relatively small, and may have been underpowered to detect these effect. Alternatively, the positive and negative effects of NETosis, respectively containing meningococcal infection and inducing tissue damage and deregulate coagulation might be balanced in meningococcal sepsis in children.

Previously, Zeerleder et al (2013) [31] measured nucleosomes in this cohort and reported that nucleosomes are correlated with several factors, such as organ dysfunction, several cytokines and patient outcome. In some studies, nucleosomes are used as marker for NETosis [32]. We did not observe a correlation between MPO-DNA and nucleosome levels in this study, indicating that the nucleosomes in these patients are probably cell-free DNA originating of other cell death mechanisms than neutrophils in NETosis. In sepsis patients, severe tissue and organ damage are the main cause of death. Cell-free DNA is therefore very likely originating from these damaged cells.

We have shown in a pilot experiment that *N. meningitidis* isolated from sepsis patients, are able to induce NETosis in neutrophils *in vitro*. This is in line with a previous study on NETs formation by *N. meningitidis* [28]. This article describes that *N. meningitidis* is also able to evade NETs different from *S. aureus*, which excretes nucleases to break down the NETs [33]. *N. meningitidis* secretes small outer membrane vesicles (SOMVs) that bind to the NETs, blocking the binding of the bacteria themselves. In our experiments, we observed that after 30-40 minutes, neutrophils underwent necrosis next to NETosis, as expected since the bacteria are known to be toxic. Altogether, our results indicate that *N. meningitidis* has developed multiple mechanisms to

avoid and destroy neutrophils, which might contribute to the severe pathogenesis of these bacteria in sepsis.

This is the largest prospective cohort of children with severe meningococcal disease of which detailed clinical and extensive laboratory data are available. Although a total number of 60 patients might be relatively small compared to adult sepsis cohorts, the advantage from our study is that these children are all affected by the same bacterium, *N. meningitidis*. The detailed assays performed and measurements of several factors over time allowed us to study correlations with markers assessed in both the current and previous studies [26,31]. Also, we were able to follow these patients over time and collect data on many variables, including NETs and inflammatory markers.

We have shown that *N. meningitidis* is capable of inducing NETosis in neutrophils isolated from healthy adult donors in vitro. Unfortunately age matched donors were not available, as volumes obtained for diagnostic tests are limited in children. Therefore we cannot exclude that neutrophils of children might react differently [34]. However, as NETs were present in our patient serum samples, such a difference *in vitro* is not expected.

Conclusions

N. meningitidis are strong NETs inducers. Children admitted to PICU for meningococcal sepsis have higher NETs levels at admission and after 24 hours than controls. NETs levels were not associated with positive or negative outcome or other inflammatory markers, indicating that the beneficial and detrimental effects of NETosis in meningococcal sepsis might be balanced. We also did not find a correlation between MPO-DNA and nucleosomes, indicating that nucleosomes are not only NETs but all cell free DNA.

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Chapter 6

Complement factor H and von Willebrand factor size in young patients with arterial thrombosis

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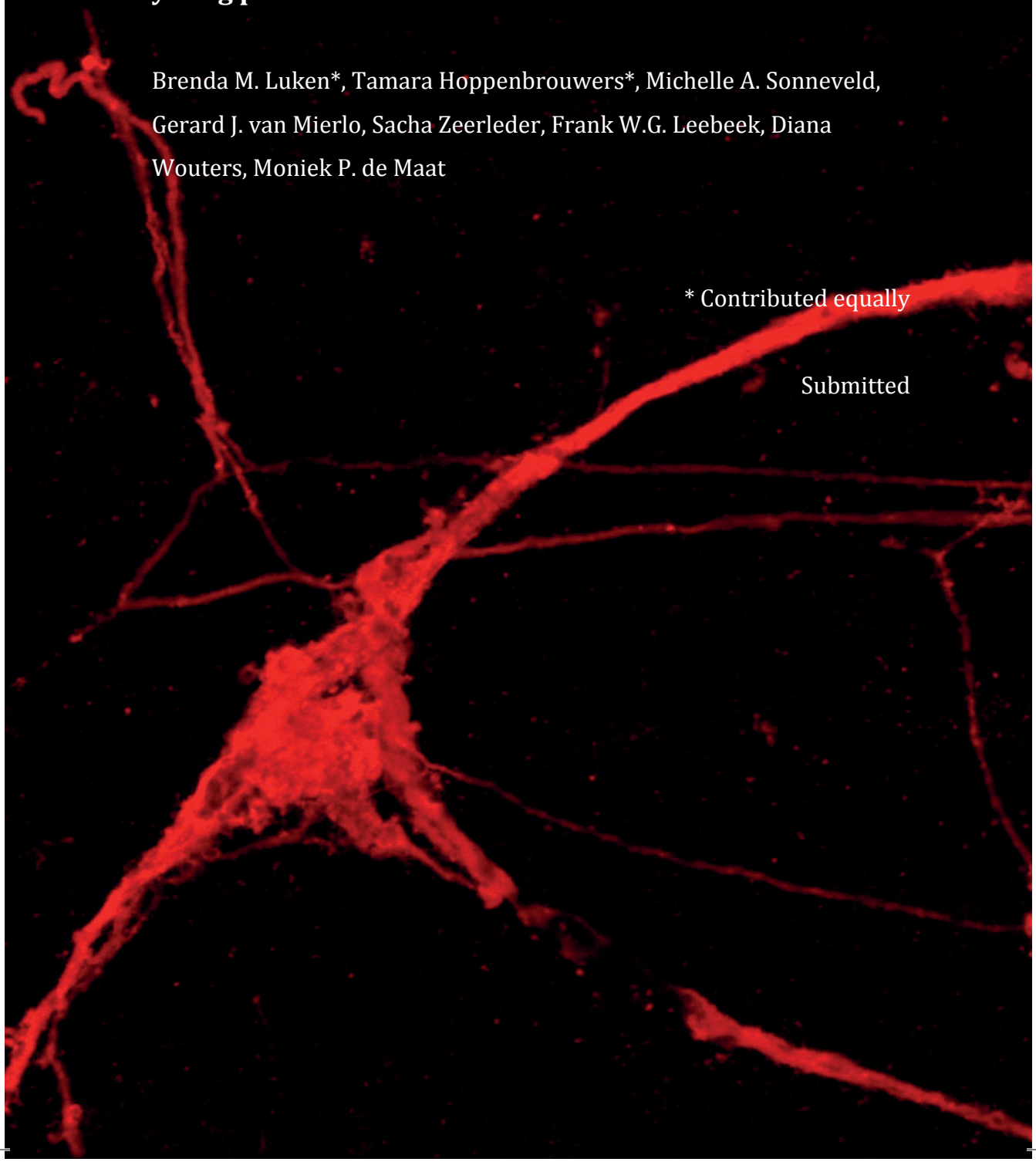
* Contributed equally

Submitted



Abstract

Complement factor H (FH) has been shown to bind to VWF and to influence its cleavage by ADAMTS13 *in vitro*. No data on this interaction in humans is available.

We aimed to study whether FH influences the relationship between VWF and ADAMTS13 and the risk of arterial thrombosis.

We determined plasma FH antigen levels in 350 young patients with a first event of arterial thrombosis and 293 healthy controls from the ATTAC study cohort, and studied the effect of FH on the known relationship of VWF antigen (VWF:Ag) and ADAMTS13 activity (ADAMTS13:Act) with the risk of arterial thrombosis. Moreover, we specifically determined the relationship between FH and the VWF collagen binding activity (VWF:CB) over VWF:Ag ratio as a surrogate marker for VWF multimer length. Finally, we determined correlations of FH with classic risk factors for arterial thrombosis.

FH levels were higher in cases compared to controls, and negatively correlated with VWF:CB/VWF:Ag ratio. In our population, we found no effect of high FH on the association of VWF:Ag and ADAMTS13:Act with arterial thrombosis risk. FH levels were linked to markers of the metabolic syndrome including body mass index (BMI), HbA1c, age and cholesterol and also C-reactive protein, which suggests a possible role of FH in the turnover of oxidized lipids.

In conclusion, FH does not seem to affect the interaction between VWF, ADAMTS13 and arterial thrombosis risk.

Introduction

Inflammation plays a central role in the pathophysiology of arterial thrombosis. Endothelial cells respond to inflammatory stimuli by expressing a range of different proteins, such as adhesion receptors for interactions with platelets and leukocytes and secreted proteins, including the large glycoprotein von Willebrand factor (VWF). VWF forms large multimer strands and may form the largest protein in the bloodstream in terms of its length. These long VWF strands are highly reactive towards platelets and mediate the initial adhesion of platelets to the vessel wall under high shear stresses such as encountered in the arteries. VWF multimer size is regulated by the plasma protease ADAMTS13, which cleaves VWF into smaller, less platelet-reactive, multimers of average length. The involvement of both VWF and ADAMTS13 in

arterial thrombosis has previously been found, as reviewed by us and others [1-3]. We have previously observed that VWF levels were increased in patients with arterial thrombosis compared to healthy controls, whilst low ADAMTS13 level formed an independent risk factor [4-6].

During inflammation, the complement pathways also become activated and further contribute to thrombosis [7]. Recent *in vitro* studies have suggested that complement factor H (FH), an important regulator of the alternative complement pathway, interacts with VWF and may affect its proteolysis by ADAMTS13. Factor H is best known for its role in atypical hemolytic uremic syndrome (aHUS), a disease characterized by thrombotic microangiopathy, although the underlying mechanism between FH and thrombotic microangiopathy is still largely unclear [8]. Turner and Moake were the first to describe that FH binds to ultra-large VWF strings anchored to endothelial cells *in vitro* [9]. In further *in vitro* studies it was shown that FH may act as a reductase of VWF, thus reducing VWF multimer length [10].Thereafter, FH has been shown to co-precipitate with VWF from serum and that FH enhanced the proteolysis of the VWF A2 domain by making VWF more susceptible for cleavage by ADAMTS13 and the cleavage of VWF-platelet strings on cultured endothelial cells under flow [11]. However, in a different study it was found that FH inhibited the proteolysis of VWF by ADAMTS13 in a static assay and that FH led to an increased VWF mediated platelet aggregation in a ristocetin cofactor assay [12].

Together, these *in vitro* studies suggest that the interaction of FH with VWF, either positively or negatively, impacts on ADAMTS13 mediated regulation of VWF multimer length and thus its platelet binding properties.

In this study we determined levels of the complement regulator FH in young patients with arterial thrombosis to evaluate whether FH influences the risk of thrombosis development. In addition, we assessed the relation of FH level to VWF antigen (VWF:Ag), ADAMTS13 activity and the collagen binding activity (CB) of VWF as ratio of VWF antigen (VWF:CB/VWF:Ag) as surrogate marker for VWF multimer size, as it is directly related to the number of collagen binding domains. Moreover, we determined possible associations of FH with inflammatory markers, including the acute phase C-reactive protein (CRP) and the levels of extracellular nucleosomes as marker for cell death. Finally, we assessed the relation of FH antigen level to classic markers of thrombosis,

including markers of the metabolic syndrome and hypertension.

Material and methods

Patients

We performed a case control study of 350 young patients with a first event of arterial thrombosis and 293 healthy controls, part of the previously reported ATTAC study cohort [13,14]. Measurements were performed on currently available plasma from patients included before April 2005, in which ADAMTS13 activity was previously determined. Males were younger than 45 and females younger than 55 years. The cohort consisted of three subgroups: a coronary heart disease (CHD) group containing patients with acute myocardial infarction and unstable angina pectoris, an ischemic stroke (IS) group also containing patients with a transient ischemic attack, and a peripheral arterial disease (PAD) group. Definitions have previously been described [13,14]. Population based controls were neighbours or friends of the patient without a history of cardiovascular disease and fulfilling the same age criteria. Medical ethical committee approval was obtained from Erasmus MC, Rotterdam, the Netherlands. All patients and controls gave informed consent for inclusion in the study. Citrate plasma was collected 1-3 months after the thrombotic event, to prevent influence of the acute phase reaction.

ELISA and bioassays

The antigen levels of complement factor H were determined in citrate plasma using a previously established ELISA [15]. In brief, 1 μg/mL monoclonal anti-FH.16 directed against complementary repeats 16-17 was coated on Maxisorp plates (Nunc), samples were diluted in high performance ELISA buffer (HPE, Sanquin), and after washing detected using 0.25 μg/mL horse-radish peroxidise labelled polyclonal goat anti-human FH antibody (Quidel) and TMB as a substrate. A pool of sera from 400 donors containing 288 μg/mL FH was used as a standard. VWF:Ag, VWF:CB, and ADAMTS13:Act levels were previously determined [13].

Levels of extracellular nucleosomes were determined as previously described [16]. In summary, Maxisorp plates (Nunc) were coated with 5 μg/mL anti-histone H3 monoclonal antibody, samples diluted in HPE and after washing detection was performed using 0.5 μg/mL biotin labelled $F(ab')_2$ fragment of a monoclonal antibody against the histone H2A/H2B/DNA complex, followed by

a streptavidin-HRP step and detection using TMB substrate. One arbitrary unit is the amount of nucleosomes released by ≈100 Jurkat cells, which corresponds roughly to ~1 ng histone based on total protein quantitation. Other values (CRP, cholesterol, HbA1c) were determined using commercial assays as part of earlier studies of the ATTAC cohort.

Statistical analyses

The medians with 95% confidence intervals are presented as not all values were normally distributed. Mann-Whitney U test was performed to compare cases and controls. Correlations of FH with VWF and ADAMTS13 parameters, markers of inflammation and other markers associated with thrombosis were determined using Spearman rank correlation analysis. Comparison between subgroups of arterial thrombosis was performed using Kruskall Wallis statistics. For logistic regression analysis, levels were divided into quartiles based on the distribution in the control group. The lowest quartile was used as the reference group, except for ADAMTS13 activity where the highest quartile was used as reference. For extracellular nucleosomes 2 groups were formed based on the lower detection limit of the assay of 5 U/ml, since most levels were below this detection limit. Using logistic regression analysis, odds ratio (OR) and 95% confidence interval (95% CI) were calculated. We adjusted for age and gender, or performed extensive adjustments for cardiovascular risk factors (age, gender, smoking, diabetes mellitus, and hyperlipidemia). A *p*-value of <0.05 was considered statistically significant to reject the null hypothesis. All statistical analyses were performed using the SPSS software package version 21 (IBM SPSS Statistics, release 21.0.0.1)

Results

FH in patients with arterial thrombosis

The levels of FH were determined in 350 patients with arterial thrombosis and 293 healthy controls. As expected in thrombotic patients, Body Mass Index (BMI), and the incidence of smoking and diabetes were higher in cases (Table 1). Median FH levels were higher in cases compared to controls, 343 (interquartile range, 288-410) µg/mL versus 318 (95% CI 268-378) µg/mL ($p=0.006$). From previously determined VWF:Ag and VWF:CB values we calculated the VWF:CB/VWF:Ag ratio, as a surrogate marker for VWF multimer size. In contrast to our expectations, VWF:CB/VWF:Ag was slightly

lower with a median ratio of 1.10 (IQR 0.92-1.29) in cases and a ratio of 1.12 (IQR 0.99-1.30) in controls (p=0.07), whilst ADAMTS13 activity levels were lower in cases at 144% (IQR 103-167) compared to 163% (IQR 138-184) in controls (p<0.001). To determine the contribution of inflammation to FH levels we analysed the correlation with CRP, the general marker of inflammation, and extracellular nucleosomes. CRP levels were higher in cases, median 0.81 mg/L (IQR 0.30-2.43) compared to 0.61 mg/L (IQR 0.26-1.67) in controls (p=0.02). It is unclear whether nucleosome levels were different, as these were below the 5 U/mL detection limit in ~70% of cases and controls.

Table 1. Baseline characteristics

	Controls *n*=293	**Cases** *n*=350
Age in y, median (IQR)	39 (28-50)	43 (35-51)
Male (%)	36%	43%
BMI in kg/m², median (IQR)	24.4 (19.4-29.4)	26.3 (20-32.6)
Smoking (%)	52%	81%
Diabetes (%)	1%	9%
Use of statins (%)	1%	89%

Medians with interquartile range (IQR) are given for age and BMI, other data are counts (yes or no) with percentage calculated. Smoking includes both past and present smoking. Diabetes and hyperlipidemia, which was defined as cholesterol level >5 mmol/l or receiving lipid lowering treatment, were based on clinical diagnosis.

The correlation of FH antigen level with VWF, ADAMTS13, and other markers

The relationship between FH on the one hand and VWF:Ag, VWF:CB, VWF:CB/VWF:Ag, ADAMTS13:Act, CRP and nucleosomes on the other hand was performed using Spearman Rank correlation analysis as several parameters were not normally distributed (Table 2). FH levels were significantly correlated with CRP in both controls and cases, R_S=0.29 (p<0.001) and R_S=0.15 (p=0.006), respectively, whilst FH levels correlated to levels of VWF:Ag, VWF:CB/VWF:Ag, and also nucleosomes in the cases only, with R_S=0.12 (p=0.02),

R_S=-0.15 (p=0.004) and R_S=0.13 (p=0.018) respectively. No association of FH antigen level with VWF:CB and ADAMTS13:Act was found in the cases and controls of this study population.

Table 2. Correlation of FH with VWF and ADAMTS13 parameters and markers of inflammation and known risk factors of thrombosis.

	Controls ***n*=293**		**Cases** ***n*=350**	
	Rs	***P***	***Rs***	***P***
VWF:Ag	0.05	0.38	0.12*	0.02
VWF:CB	0.05	0.38	0.03	0.55
VWF:CB/VWF:Ag	-0.03	0.65	-0.15**	**0.004**
ADAMTS13 activity#	0.01	0.89	-0.06	0.36
CRP	0.29**	**<0.001**	0.15**	**0.006**
Nucleosomes	0.11	0.07	0.13*	**0.02**
Age	0.09	0.12	0.14**	**0.008**
BMI	0.25**	**<0.001**	0.36**	**<0.001**
SBP	0.06	0.32	0.03	0.56
DBP	0.04	0.55	0.02	0.78
Cholesterol†	0.20**	**0.001**	N.A.#	N.A.#
HbA1c†	0.10	0.12	0.24**	**<0.001**

Spearman rank correlation test. *Rs:* Spearman rank correlation coefficient, CRP: C-reactive protein, BMI: body-mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, HbA1c: hemoglobin A1c.
#ADAMTS13 activity was determined in 222 controls and 261 cases. †Cholesterol levels were determined in 270 controls and 350 cases, ‡HbA1c levels were determined in 268 controls and 344 cases. # Not determined since 89% of cases use statins.

Next, we determined the association of FH antigen levels with other known risk factors of arterial thrombosis, including age, BMI, systolic and diastolic blood pressure, cholesterol level and the level of glycated hemoglobin (HbA1c), a marker used in diabetes control (Table 2). FH antigen levels strongly correlated with BMI in both controls and cases, R_S=0.25 (p<0.001) and R_S=0.36 (p<0.001) respectively. Moreover, FH antigen level correlated to

age and HbA1c in patients, R_S=0.14 (p=0.008) and R_S=0.24 (p<0.001), and with cholesterol level in controls, R_S=0.20 (p=0.001).

Logistic regression and subgroup analysis

We then performed logistic regression analysis to determine the risk of arterial thrombosis (Table 3). No relation was found between FH levels and risk of thrombosis between the highest quartile (odds ratio (OR) 1.37 with 95%CI 0.86-2.19) compared to the lowest quartile, after adjustment for age and gender. The non-significant increase of 40% was largely lost upon extended adjustment for smoking, diabetes and hyperlipidemia, (OR 0.90, 95%CI 0.53-1.54). Upon performing a similar analysis for the VWF:CB/VWF:Ag ratio, we also found no differences in thrombosis risk with OR 0.81 (95%CI 0.51-1.26) in the highest quartile (Q4) when compared to the lowest quartile (Q1), after adjustment for age and gender, which remained the same after extended adjustment OR 0.77 (95%CI 0.47-1.26). The influence of ADAMTS13 activity on arterial thrombosis risk was most pronounced; with an OR of thrombosis in quartile Q1 of 4.47 (95%CI 2.49-8.01) or 3.73 (95%CI 1.96-7.09) after extended adjustment, when compared to quartile Q4. Although increased, no significant differences were found in risk of thrombosis correlated to CRP levels: OR 1.28 (95%CI 0.80-2.04). After adjustment, the OR was only 1.01 (95%CI 0.61-1.68).

Table 3. FH, VWF:CB/VWF:Ag ratio, ADAMTS13, CRP, and nucleosomes and the risk of arterial thrombosis

Complement factor H (ug/ml)				**adjustment age/gender**		**extended adjustments**	
	boundaries	n, controls	n, cases	**OR**	95%CI	**OR**	95%CI
Q1	<268	73	65	**1 (ref)**		**1 (ref)**	
Q2	296-318	74	65	**0.88**	0.53-1.44	**0.67**	0.38-1.17
Q3	319-378	73	100	**1.24**	0.77-1.99	**0.87**	0.51-1.49
Q4	>379	73	116	**1.37**	0.86-2.19	**0.90**	0.53-1.54

VWF:CB/VWF:Ag ratio							
Q1	<0.99	73	121	**1 (ref)**		**1 (ref)**	
Q2	1.00-1.24	73	66	**0.56**	0.36-0.89	**0.58**	0.34-0.96
Q3	1.25-1.30	73	78	**0.68**	0.43-1.07	**0.76**	0.46-1.27
Q4	>1.31	73	85	**0.81**	0.51-1.26	**0.77**	0.47-1.26
ADAMTS13 activity (%)							
Q1	<137	55	117	**4.47**	2.49-8.01	**3.73**	1.96-7.09
Q2	138-163	56	64	**2.14**	1.17-3.91	**1.80**	0.93-3.48
Q3	164-184	56	51	**1.84**	0.99-3.43	**1.83**	0.91-3.64
Q4	>185	55	29	**1 (ref)**		**1 (ref)**	
CRP (mg/ml)							
Q1	<0.26	73	76	**1 (ref)**		**1 (ref)**	
Q2	0.27-0.61	77	70	**0.78**	0.48-1.26	**0.95**	0.56-1.60
Q3	0.62-1.67	71	87	**1.06**	0.66-1.70	**1.31**	0.78-2.22
Q4	>1.68	69	112	**1.28**	0.80-2.04	**1.01**	0.61-1.68
Nucleosomes (U/ml)							
D1	<5	207	240	**1 (ref)**		**1 (ref)**	
D2	>5.01	86	110	**0.95**	0.66-1.35	**1.05**	0.70-1.56

Levels were divided in quartiles based on the distribution in the control group. The lowest quartile was used as the reference, except for ADAMTS13 activity where the highest quartile was used as reference. For extracellular nucleosomes 2 groups were formed based on the lower detection limit of the assay of 5 U/ml. Odds ratio (OR) and 95% confidence interval (95% CI) were calculated using logistic regression. We adjusted for age and gender, or performed extensive adjustments for cardiovascular risk factors (age, gender, smoking, diabetes, and hyperlipidemia).

Addition of the VWF ratio to the model with FH, age and gender did not change the risk estimates of arterial thrombosis (-2 log likelihood of the model are 613 and 605, respectively, P[$\chi^2(4)$]=n.s.). Further addition of ADAMTS13 to the model strongly improved the risk prediction (-2 log likelihood of the model becomes 569, P [$\chi^2(4)$]<0.001). In our analyses, ADAMTS13 activity forms the main parameter that determines the risk of arterial thrombosis and no significant contribution of FH or VWF to the model is seen in this young arterial thrombosis patient group.

Finally, we performed a subgroup analysis of FH levels in CHD, IS and PAD (*n* of 206, 98 and 46 cases respectively). The FH levels were statistically significant different between the different patient subgroups with the Kruskal-Wallis test ($\chi^2(2) = 12.41$, $p = 0.002$), with a median (range) of 356 (IQR 299-418) µg/mL for CHD, 317 (IQR 262-371) µg/mL for IS and 343 (IQR 307-413) µg/mL for PAD (p=0.002 Kruskal Wallis, p<0.001 between CHD and IS).

Discussion

In patients with arterial thrombosis, FH levels were associated with the VWF:CB/VWF:Ag ratio. No association was found between FH levels and ADAMTS13 activity. In the cases, FH levels were higher than in healthy controls, but no association with risk of thrombosis was observed.

We found a negative correlation between FH levels and the VWF:CB/VWF:Ag ratio. *In vitro* studies have shown that FH binds to ultra large VWF multimeric strings and makes VWF more susceptible to cleavage by ADAMTS13 [10,11]. FH was found to bind to the A2 domain of VWF [11], which also contains the ADAMTS13 cleavage site. Addition of FH enhanced the proteolysis of platelet decorated VWF strings at the surface of cultured endothelial cells [10]. We used the VWF:CB/VWF:Ag ratio as a surrogate marker for VWF multimer length to determine a possible effect of FH on the cleavage of VWF by ADAMTS13. The collagen binding activity of VWF is directly related to the number of collagen binding VWF A3 (and A1) domains of VWF. The observed negative correlation between FH level and VWF:CB/VWF:Ag ratio is compatible with a mechanism where FH binding to VWF catalyzes its proteolysis by ADAMTS13.

Upon analysis of the relationship of the VWF:CB/VWF:Ag ratio with the risk of thrombosis, we found that this ratio was lower in patients with arterial

thrombosis compared to controls in Q2 and Q3 compared to Q1, which was in contrast to our expectations. No differences were found between Q1 and Q4. However, both VWF:CB and VWF:Ag were significantly higher in patients with arterial thrombosis, as expected. Hence, these levels are likely to have risen as a consequence of endothelial activation that results in the release of large VWF multimers. It is possible that some of the largest VWF multimers remain sequestered to the endothelial cell surface and would therefore not show up in the circulation.

It has been suggested that FH and VWF are both inflammatory factors [17,18]. Indeed, we found a positive association between FH and VWF:Ag levels, which also forms a marker of endothelial activation and inflammation. We also found that FH levels correlated with CRP levels, both in patients and controls. It has been suggested that both FH and CRP interact with oxidized phospholipids that arise during inflammation, which form damage-associated molecular patterns (DAMP) that activate the innate immune system [19]. The authors of that study speculate that this recognition may enable the innate immune system to mediate important physiological housekeeping functions, including the removal of dying cells. Indeed, FH was also correlated with extracellular nucleosome levels, a marker of cell death, in our patients. Extracellular nucleosomes in the blood may potentially also be derived from neutrophil extracellular traps (NETs) that are released in inflammation and which are known contributors to both venous and arterial thrombosis[20]. Of interest, FH has been implicated in the regulation of NETs formation, although no such effect was observed with soluble rather than immobilized FH [21]. However, the extracellular nucleosome levels in our study population were low and often even below the detection limit, probably because plasma samples were obtained 1-3 months after the acute thrombotic event, this in contrast to a previous study where extracellular nucleosomes associated with the presence of deep vein thrombosis at the time of occurrence [22].

We found correlations between FH with classic risk factors for thrombosis, e.g. BMI, HbA1c, age and cholesterol levels. These factors are all part of the metabolic syndrome, which is linked to obesity and the development of diabetes. Our results are consistent with previously reported associations between FH and circulating lipids and indices of body fat, including BMI, body fat, triglycerides, cholesterol and also HbA1c and insulin in a population of healthy individuals [23]. As virtually no information on the regulation of FH

antigen levels and its clearance exists, further research into the mechanisms that contribute to the observed association with the metabolic syndrome are needed.
Since our data render it plausible that FH modulates the effect of ADAMTS13, we studied the association between FH and the risk of arterial thrombosis. Indeed, FH levels were higher in cases than in controls. In our subgroup analysis on the type of arterial thrombosis, we saw that FH levels were higher in CVD patients than in IS patients. However, in our logistic regression models we did not identify FH as a predictor of thrombosis risk, neither on its own nor by interaction with VWF and ADAMTS13 in a combined model. In this population, ADAMTS13 was the major determinant of the risk of thrombosis. We previously published this relationship between ADAMTS13 and risk of CVD in the ATTAC population [13] and also in prospective studies for both ischemic stroke and coronary heart disease [4-6]. Our data suggest that the effect of FH on ADAMTS13 cleavage of VWF is present, but that other mechanisms are more important in determining the risk of arterial thrombosis.

In conclusion, we found high FH, high VWF:Ag and low ADAMTS13:Act in patients with arterial thrombosis. FH antigen levels in arterial thrombosis are linked to markers of the metabolic syndrome and suggest a possible role in the turnover of oxidized lipids.

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Chapter 7

Fibrin improves skin wound perfusion in a diabetic rat model

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Abstract

The fibrin matrix of the thrombus that is formed directly after wounding, is an important determinant of the success of the early phase of wound healing. This phase is often impaired in patients with diabetes. A promising approach to improve skin wound healing is the application of a pro-angiogenic fibrin matrix onto the wound. We studied this in 59 female WAG/RijCrl diabetic rats, in which we created two dorsal full-thickness wounds of which one was treated with a human physiological fibrin matrix (2 mg/ml) and one with PBS as control. Wound healing parameters were determined at different time points. The wound closure was significantly improved in fibrin-treated wounds on day 3 and 7. Also, fibrin-treated wounds showed a significantly higher perfusion on day 28 and 35 compared to control wounds ($p<0.05$). CD68 staining revealed that human fibrin did not induce an immune response. In conclusion: the application of a fibrin matrix on a diabetic wound showed improved perfusion and an increased early closure rate of the wound area.

Introduction

An important first step in wound healing is the formation of a fibrin clot. Fibrin is formed from the soluble plasma protein fibrinogen, a molecule of 340 kDa that is generated in the liver and circulates in blood with a concentration of approximately 2 mg/ml. It consists of six polypeptide chains connected by disulfide bonds; two Aα, two Bβ and γ chains (1-3). Fibrin is the end product of the coagulation cascade, formed by the cleavage of fibrinopeptides A and B from fibrinogen by thrombin (3-5). The primary function of the fibrin matrix is to stop bleeding. In addition to its role in hemostasis, the fibrin matrix also stimulates the attraction, migration, adhesion and proliferation of a variety of cells that are important in wound healing, such as inflammatory cells, fibroblasts (6) and endothelial cells (1, 7).

Patients with Diabetes mellitus (DM) often have disturbed wound healing, which is a growing clinical problem since the prevalence of DM is increasing in Western societies. Worldwide, about 9% of all adults suffer from DM and every year about 1.5 million people die from the effects of DM (8). These numbers show a dramatic increase over the last 30 years (9). One of the major limitations in the healing of wounds in DM patients is circulatory insufficiency due to, among others, stiffening of the microvessels. When blood circulation is

improved, the delivery of nutrients and oxygen to the wound area will be enhanced. Since oxygen is one of the most important molecules in angiogenesis, increased circulation and oxygenation will improve wound healing (10).
Fibrin has been used in studies on wound healing, However, these studies used fibrin glue consisting of fibrinogen with a high concentration of 20-40 mg/ml with a high concentration of 4 U/mL thrombin is used as a delivery tool of growth factors in wound healing (11-13). Fibrin matrices from this high-concentration fibrinogen have a very tight matrix structure that allows none to only very limited angiogenesis and fibrinolysis. In our previous research we have already shown that using an optimized fibrin matrix in a wound, based on a concentration of fibrinogen of 2 mg/ml, creates a fibrin matrix with the highest capacity of endothelial cell ingrowth *in vitro* and *in vivo* in a normal rat wound healing model (14). As patients with DM often have circulatory insufficiency, we now hypothesize that a fibrin matrix can also improve angiogenesis in delayed diabetic wound healing.
We studied the effect of a physiological fibrin matrix on delayed wound healing in a diabetic delayed wound healing rat model.

Material and methods

Animals

Sixty five female WAG/RijCrl rats (6-8 weeks old), purchased from Charles River (l'Arbresle, France), were housed in pairs under standard 12 hour light/dark cycles. Food and water were available ad libitum. The rats were allowed to acclimatize to their environment for one week prior to diabetes induction. The experimental protocol was approved by the Animal Experiments Committee under the Dutch national Experiments on Animals Act and adhered to the rules laid down in this national law that serves the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe (1986), Directive 86/609/EC. All surgery was performed under isoflurane anesthesia and the animals received Temgesic two days after surgery to minimize suffering.

Diabetes induction

Animals (weight >120 grams, 8-10 weeks of age) were fasted overnight and received an intraperitoneal injection of 60 mg/kg Streptozotocin (STZ, Sigma-

Aldrich, St. Louis, MO) in 0.05 mol/L sodium citrate buffer (pH=4.5). The diabetic condition was carefully monitored for at least 5 weeks before the start of the experiment. The diabetic state of the animals was monitored via physiological changes (e.g. elevated blood glucose levels and urine production, thinner skin and weight loss).

Full-thickness ulcer wound model and treatment

After a stable diabetic period of at least 5 weeks, the rats received two dorsal full-thickness wounds. The left wound was covered with 300 µl PBS pH 7.4 and served as a control. The right wound was covered with 300 µl fibrin matrix, consisting of human fibrinogen (2 mg/ml in PBS pH 7.4, Enzyme Research Laboratories, South Bend IN, USA), containing 0.43 U/ml FXIII (determined photometrically with Berichrom Factor XIII, Siemens Healthcare diagnostics, Deerland, IL), depleted of plasminogen, von Willebrand Factor and fibronectin mixed with human thrombin (1U/ml, Siemens Healthcare Diagnostics, Breda, the Netherlands) immediately before application to the wound. Before surgery, animals were anesthetized with a mixture of isoflurane and oxygen. After removal of the dorsal hair, two full-thickness wounds with a diameter of 15 mm (3 cm apart, 4 cm caudal from the scapulae) were created with surgical scissors as previously described by Tong et al (15). Initial wound sizes in control and fibrin-treated wounds were similar. After application of the fibrin matrix, the animals were left in a dorsolateral position for 15 minutes to allow the fibrin matrix to form. All animals received Temgesic (0,05 mg/kg, Rekitt Benckiser Healthcare, Ltd., Hull, East Yorkshire, UK) i.m. before surgery and twice a day for two days after surgery. Animals were followed for either 3 (n=6), 7 (n=20), 21 (n=20) or 42 (n=19) days, randomly assigned, and sacrificed for wound histology and gene/protein expression assays.

Perfusion measurements

Perfusion in the wounded area and the normal (unwounded) skin was measured weekly from 14 days after surgery onwards using O2C (LEA Medizintechnik, Giessen, Germany). Measurements were performed according to manufacturer's recommendations. Four perfusion parameters were measured: oxygen saturation (SO2), relative hemoglobin (rHb), blood flow in the microcapillary network and blood flow velocity. SO2 and rHb levels were

measured using a white light probe. Blood flow and flow velocity were measured by using changes in laser light frequency within vessels (16).

Vessel capacity measurements

Vessel capacity in the wounded area was measured weekly from 14 days after wound induction onwards with a Laser Doppler monitor (LDPM, Perimed Periflux System 5000, Perimed AB). A pre-heated 33 ^{0}C probe was placed onto the wound for approximately 3 minutes for baseline measurements. Subsequently, the probe was heated to 44 ^{0}C and measurements were continued for another 3 minutes. Vessel capacity was calculated relative to baseline measurements at 33 ^{0}C as the % increase and expressed in perfusion units (PU) (15). Only wounds without crust were measured.

Macroscopic wound area measurements

Each week, the wounds were photographed and area measurements were performed (3, 7, 14 and 21 days) using ImageJ 1.49q (Wayne Rasband, National Institutes of Health, USA). Each wound area was measured after selecting the wounded area with a tracing tool. On t=0, the wound surface was considered 100% and calculated wound areas revealed the % of wound closure per time point.

Immunohistochemistry

Collected wound tissue specimens were fixed in 4% formalin for 24 hours. Immunohistochemistry was performed on paraffin sections (5 μm). Slides were rehydrated and treated with 0.05% trypsin with 0.1% calcium chloride in distilled water for antigen retrieval. To inhibit endogenous peroxidase, slides were washed with 3% H_2O_2 in PBS pH 7.4. After blocking with 5% skim milk, slides were incubated overnight at 4 ^{0}C with primary antibodies polyclonal goat anti-rat CD34 (dilution 1:200, R&D systems, cat. nr. AF4117, lot ZDP0107081) or monoclonal mouse anti-rat CD68 (dilution 1:100, Serotec, cat. nr. NLA59-MCA3 41R, batch 060509) diluted in 1% BSA/PBS pH 7.4. Biotinylated secondary antibodies rabbit anti goat (1:200, DAKO) or goat anti mouse (dilution 1:200, DAKO) were applied after washing, respectively. All antibodies were validated by using a positive and negative control according to the manufacturer's recommendations in each staining. For visualization, slides were incubated with streptavidin-HRP (dilution 1:300, Biotech) and DAB (3,3' Diaminobenzidine, Sigma Aldrich) and counterstained

with haematoxylin. In CD68 slides, the positive-signal density was classified by three independent, blinded observers as absent (1), low (2), medium (3), high (4) and very high (5). In CD34 slides, vessels per mm^2 were counted. Here, only day 7 was included for analysis due to aspecific binding of the antibody to mature collagen on later time points. The thickness of the epidermis was evaluated on haematoxylin and eosin (H&E) stained slides.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Group differences were analyzed by univariate ANOVA with Statistical Package for the Social Sciences (SPSS, IBM, version 21). Results were considered significant if $p<0.05$.

Results

Diabetic state of the animals

After diabetes induction, 65 rats had an average glucose level of 29.1 ± 0.5 mmol/L. Weights in the different groups were similar. 5 animals recovered from their diabetic state and were, therefore, not included in this study. No animals died during the experimental procedure.

Fibrin matrix improves wound healing

Fibrin-treatment of the diabetic wound significantly increased the wound closure rate in the first days after wounding, both on day 3 (14.9 ± 5.4 % closure in controls vs 34.6 ± 3.2 % closure in fibrin-treated wounds, $p=0.02$) and on day 7 (64.0 ± 1.8 % closure in controls vs 71.5 ± 1.9 % closure in fibrin-treated wounds, $p=0.006$) (Fig. 1 and Table 1). On day 21 the wound closure was nearly complete in both groups (93.9 ± 0.4 % in controls vs 94.6 ± 0.6 % in fibrin-treated wounds, $p=0.29$). No difference was seen in epidermal thickness. Similar numbers of CD68 positive cells were seen in the wound area of fibrin-treated and control wounds, indicating that the human fibrin did not trigger an enhanced immune response in the rats.

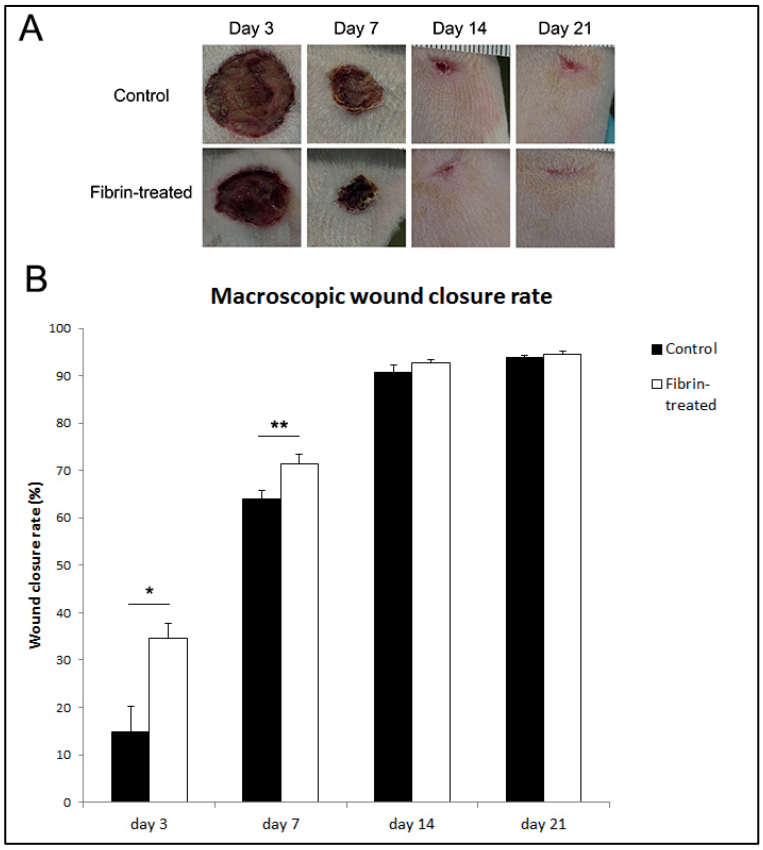

Figure 1: Improved wound closure rate (A and B) on day 3 and 7 of fibrin-treated wounds compared to control wounds. Scale bars are 5 mm. *p<0.05, **p<0.01.

Table 1: Wound healing in the diabetic rat model.

	Control	Fibrin-treated	P-value
SO2 (%)			
○ Day 14	87.9 ± 1.6	92.2 ± 3.3	0.27
○ Day 21	90.8 ± 1.2	90.0 ± 1.8	0.73
○ Day 28	80.3 ± 2.6***	94.7 ± 1.3***	*0.00002*
○ Day 35	85.9 ± 2.7**	94.6 ± 0.8**	*0.004*
○ Day 42	69.7 ± 3.9	76.7 ± 2.2	0.13
rHb (AU)			
○ Day 14	61.8 ± 3.4	63.5 ± 3.2	0.71
○ Day 21	30.0 ± 3.1	35.7 ± 4.5	0.30
○ Day 28	31.4 ± 2.6**	44.1 ± 3.3**	*0.005*
○ Day 35	32.4 ± 1.7***	43.4 ± 1.8***	*0.00009*
○ Day 42	32.3 ± 2.5	38.0 ± 2.0	0.08

Blood flow (AU)			
○ Day 14	308.8 ± 33.5	330.0 ± 42.0	0.70
○ Day 21	136.3 ± 14.1	187.7 ± 24.7	0.08
○ Day 28	175.1 ± 17.9*	242.1 ± 22.2*	*0.03*
○ Day 35	234.1 ± 15.7*	319.8 ± 31.4*	*0.02*
○ Day 42	228.1 ± 12.7	199.4 ± 26.0	0.33
Blood flow velocity (AU)			
○ Day 14	40.8 ± 2.2	41.3 ± 2.6	0.86
○ Day 21	26.7 ± 1.4*	32.2 ± 2.0*	*0.03*
○ Day 28	29.8 ± 1.3**	36.3 ± 2.0**	*0.009*
○ Day 35	33.9 ± 1.3**	40.8 ± 2.2**	*0.01*
○ Day 42	33.7 ± 1.1	31.2 ± 1.9	0.28
Vessel capacity			
○ Day 14	25.8 ± 6.2	19.4 ± 4.2	0.39
○ Day 21	20.0 ± 3.7	25.2 ± 5.3	0.43
○ Day 28	33.9 ± 5.3	34.2 ± 3.6	0.96
○ Day 35	26.8 ± 4.1	32.9 ± 4.9	0.35
○ Day 42	29.8 ± 5.2	35.4 ± 8.6	0.58

Data are given as mean ± SEM. Oxygen saturation (SO2) are percentages, relative hemoglobin levels (rHb), blood flow and blood flow velocity are artificial units. Vessel capacity is expressed relative to baseline measurements at 33 ^{0}C as the % of increase.

*p < 0.05.

**p < 0.01.

***p < 0.001.

The fibrin matrix promotes perfusion

In the early phase of wound healing, the wound closure was faster in the fibrin-treated wounds. On day 21 and day 28, wound perfusion was significantly higher in the fibrin-treated wounds compared to the control wounds, as indicated on day 21 by blood flow velocity (32.2 ± 2.0 AU fibrin-treated vs 26.7 ± 1.4 AU control, p=0.03) (Fig. 2) and on day 28 by oxygen saturation (94.7 ± 1.3 % fibrin-treated vs 80.3 ± 2.6 % control, p<0.0001), relative hemoglobin (44.1 ± 3.3 AU fibrin-treated vs 31.4 ± 2.6 AU control, p=0.005), blood flow (242.1 AU ± 22.2 fibrin-treated vs 175.1 ± 17.9 AU control, p=0.025) and blood flow velocity (36.3 ± 2.0 AU fibrin-treated vs 29.8 ± 1.3 AU control, p=0.009). Similar effects were observed on day 35 (Table 1). Both SO2 and rHb showed significant higher values in fibrin-treated wounds (p<0.0001) and blood flow velocity showed a positive trend (p=0.069) over time. Vessel capacity measurements by Laser Doppler (Table 1) and CD34 counts were similar in both groups.

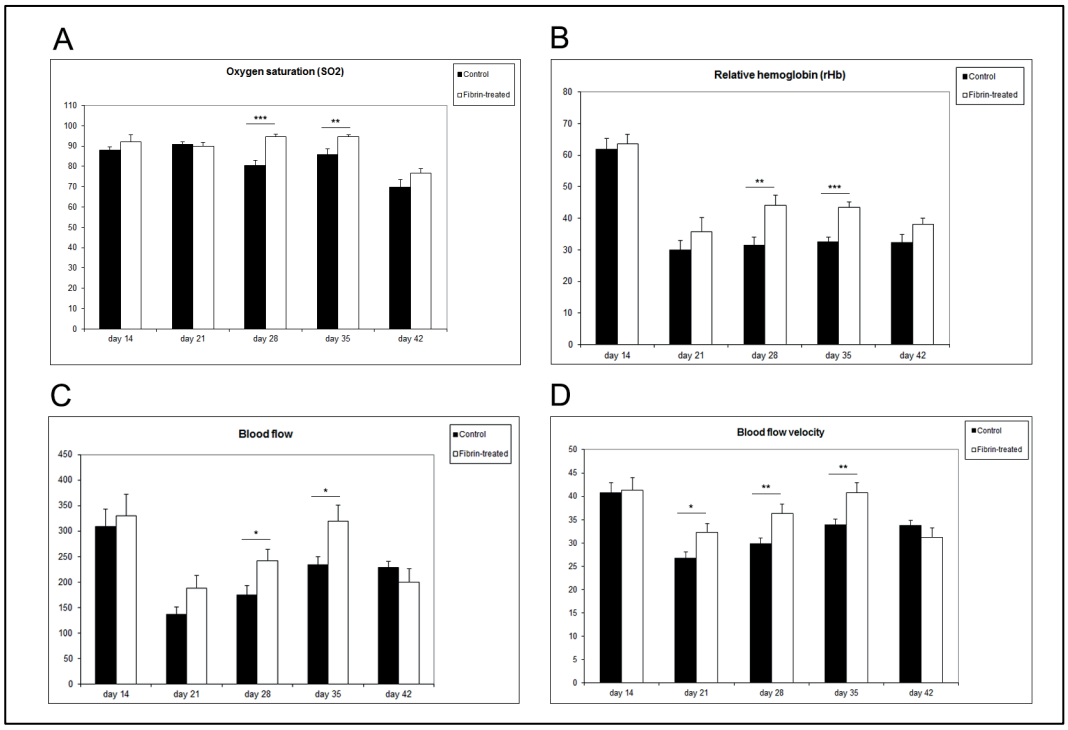

Figure 2: Improved O2C values on time points 21d, 28d and 35d after wounding. A: % of the oxygen saturation. B: Relative values of the relative hemoglobin levels. C: Relative values of the blood flow. D: Relative values of the blood flow velocity. *p<0.05. **p<0.01. ***p<0.001.

Discussion

The most important finding from our study was that fibrin improves wound perfusion and wound closure in delayed-healing skin wounds in diabetic rats. This study is the first to apply a fibrin matrix with a physiological concentration (2 mg/ml) in a diabetic delayed wound healing model. Our results show an improved wound healing rate and an increased blood flow, representing the extensiveness of the microcapillary network. In our previous studies, both our *in vitro* data and our healthy rat model also showed improved angiogenesis with a fibrin matrix (1, 14). Previous studies have been performed with fibrin in wound healing, but these used fibrin as a growth factor delivery tool, and were made from fibrinogen with very high concentrations of 20-40 mg/ml. These concentrations form a dense fibrin sealant or film rather than a matrix in which endothelial cells can invade for angiogenesis, in which the structure of the fibrin matrix is crucial (17). Thus

there is an essential difference in the physiological fibrin matrix that we used in this study.

In this study, control wounds were treated with PBS, in which the fibrinogen was also diluted. With this model we aimed to simulate the clinical situation in which wounds are not directly covered by a matrix. The observed effect of fibrin on wound healing could be due to different mechanisms, including the effect of a protective film onto the wound or, as previously suggested, angiogenesis. Based on previous literature describing fibrin as an angiogenesis promoting protein, we suggest that the angiogenic properties of the fibrin matrix has the strongest effect on wound healing.

In addition to the importance of the physiological fibrinogen concentration we used for our fibrin matrix, also FXIII may affect the results. FXIII stabilized the fibrin matrix by cross-linking the fibrin fibers, and the importance of this process is illustrated by the bleeding complications that are seen in patients suffering from FXIII deficiency (18). Additionally, low FXIIII levels increases pore size of the fibrin fibers, fiber thickness and increases the fibrinolysis rate of the matrix (19). In our fibrinogen preparation, FXIII is present at a concentration of 0.43 U/ml, which is well above the level of FXIII that gives clinical complications (1-3%). Furthermore, we used fibrinogen that is depleted from plasminogen, which has an important role in fibrinolysis and angiogenesis. In this study and in our previous experiments (14), we have shown that the plasminogen that is needed for the wound healing in our model is provided by the animals themselves.

Despite the improved perfusion, vessel capacity did not differ significantly between treated and non-treated wounds. From day 21, the mean values of the fibrin-treated wounds were slightly higher than the mean values of the non-treated wounds, however did not reach significance. This pattern is consistent with the positive trend found in O2C measurements.

The number of monocytes and macrophages in the wound area did not differ between treated and non-treated wounds, indicating that the addition of a human fibrin matrix does not increase the immune response in the early phase of wound healing. This suggests that treatment of human diabetic ulcers with fibrin will be well tolerated.

Our diabetic rat model shows the characteristics of diabetes (e.g. weight loss/hampered weight increase, thinner skin, high glucose levels and elevated urine production) and delayed wound healing (20), as previously described in other diabetic rat models (16, 21, 22). The difference between rats and humans is that rats have a muscle layer under their skin, causing wound contraction. By using an internal control, it is expected that the difference observed in wound size between fibrin-treated and control wounds are due to treatment and not wound contraction.

Disturbed wound healing is a problem in patients with DM. Of all patients, approximately 15% will develop delayed healing wounds, of which 24% ultimately result in lower limb amputation (23). After amputation, morbidity and mortality is reported 50-60% on a 2-year survival rate (24). Given the high number of diabetic delayed healing wounds, it is of clinical importance to provide a treatment that is well accepted, affordable and time efficient. Fibrin is already a known substance in the clinic, as high concentration fibrinogen is applied as fibrin glue. However, these fibrin matrices do not promote cell migration due to the high density of the fibrin network (25). With our model, we show that the addition of a healthy human fibrin matrix with a physiological concentration may be a promising and safe treatment for improving diabetic wound healing by improving perfusion. Furthermore, the addition of a fibrin matrix can be easily translated and applied in the clinic on diabetic delayed healing wounds.

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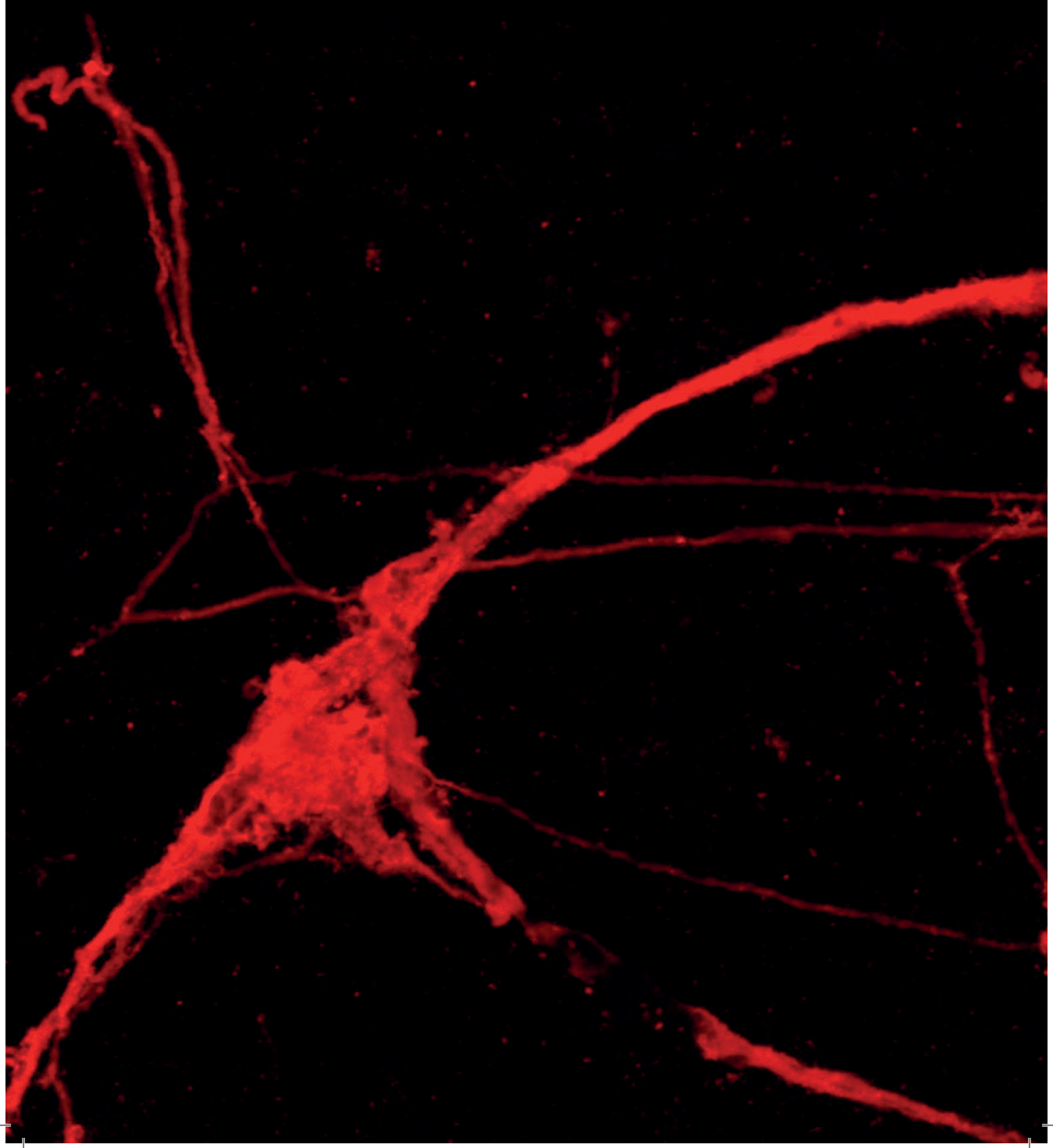
Chapter 8

General discussion



NETs as a common thread (or threat?) in several diseases

NETs play a key role in chronic wound healing, which is the scope of this thesis, but also in diseases such as sepsis and thrombosis. In the consecutive chapters of this thesis, we have investigated how NETs are formed (chapters 2,3 and 4) and what their role is in various diseases (chapters 5 and 6). As an intertwining thread, the importance of fibrin in chronic wounds was also explored (chapter 7). Whereas it is clear that a physiological concentration of fibrin improves wound healing, the effect of NETs in wound healing is less clear. NETs, like other immune cells, can act as a double-edged sword: favorable to wound healing is that they can trap and destroy pathogens, but detrimental is that they can also cause collateral damage to the surrounding tissue. In thrombi, NETs have been described to strengthen the clot, therefore the clots become more resistant to lysis, which may worsen patient outcome [1,2]. Furthermore, some bacteria like *S. aureus* have developed strategies to evade the immune system, for example by breaking down the NET[3] or blocking phagocytosis[4].

In sepsis, NETs have previously been described as a predictor of patient outcome[5]. We explored in chapter 5 the correlation between patient outcome and plasma levels of NETs, measured in the serum of children with severe meningococcal sepsis. In a previous study from our group in this population, a significant correlation between nucleosomes (a marker for extracellular DNA) and patient outcome was found[6]. Therefore, the obvious hypothesis was that NETs levels are also correlated to patient outcome. However, in contrast to our expectations and the literature, we did not see a clear effect of NETs on patient survival. Also, no correlation was found between nucleosomes and NETs, which we quantified using an MPO-DNA ELISA, indicating that the nucleosomes marker alone measures not the same as the NETs levels. Other studies found a correlation between nucleosomes and neutrophil elastase (NE) levels, another marker for NETs[7]. However, NE is a common marker for neutrophil levels, and like we used MPO-DNA complex levels in our study, also NE complexed to DNA should be investigated. Then, in future studies, the correlation between MPO-DNA and NE-DNA could be explored. It should be noted that our patient group was relatively small. Nevertheless, as we did not find any indication of an association between patient survival and NETs, we do not expect to find significant results in a larger cohort. In our studies, in chapters 2 and 3, we observed that the type of bacteria is important in the amount of NETs formed. This has also been reported in earlier studies[8]. This

could indicate that different types of bacterial species are responsible for inducing a different amount of NETs, and that these NETs only become a problem when extensively present. This might be the case in other forms of sepsis, but in our meningococcal sepsis patient group, this was not observed.

The hypothesis that different intensities of NETosis play a role in different types of sepsis is very obvious, considering the enormous variation in NETosis induction rate between and even within bacterial species[8]. We explored this potency to induce NETosis in chapter 2 with *S. aureus* and *E. coli*, and in chapter 3 with different strains of *S. aureus*. Especially in the studies presented in chapter 3, it became clear that every bacterial strain has its own unique defense mechanism against our immune system, causing variation in response by our neutrophils. This may also explain the difference in severity of NETs in sepsis caused by different bacterial species.

In chapter 6, the effect of FH and nucleosome levels on VWF cleavage by ADAMTS13 was explored in a case-control study of young patients with arterial thrombosis (ischemic stroke and myocardial infarction). Here, we observed that FH was correlated to nucleosome levels, indicating an interaction between FH and the removal of dead cells. However, the most important finding in this study was that FH levels were increased in patients, as compared to controls, and that these levels were negatively correlated with VWF:CB/VWF:Ag ratio, which indicates VWF multimer size, as it is directly related to the number of collagen binding domains. FH can bind to VWF, and previously this has been associated with higher amounts of high molecular weight VWF multimers and increased VWF mediated platelet aggregation[9]. Therefore, our hypothesis was that, in our patient group, this mechanism could play a role in arterial thrombosis. However, no association with FH levels or nucleosome levels and thrombosis was observed. It would be interesting to measure MPO-DNA complex levels in these patients, to see if there is a correlation between NETs levels and nucleosome levels. It also would be interesting to correlate the NETs levels from the plasma to NETs found in thrombi derived from these patients. It should be taken into account, however, that the plasma from these patients was derived when the acute inflammatory state was over, therefore, less NETs could be expected.

The process and appearance of NETosis

NETosis can be induced by different stimuli, which has been explored in chapter 2. One of our most interesting findings was that different stimuli had

different effects regarding the extrusion of the DNA complex by the neutrophils, including breaking of the nuclear membrane and mixture of the cellular components. In experiments with PMA, DNA extrusion could take up to 4 hours. With other inducers, like bacteria or Ionomycin, this process was much faster: 5 minutes and 1.5 hours, respectively. We observed that NETosis by these inducers clearly differed from each other. In the case of Ionomycin, the cellular membranes became porous within 15 minutes, allowing PI to bind to the DNA whilst still in the nucleus. Thereafter, the DNA mixed with the other cellular components and extruded from the neutrophil. When only looking at the final image after 4 hours, neutrophils stimulated by PMA and Ionomycin look similar, which underpins the importance of time-lapse imaging to follow the process of NETosis. However, in the case of bacterial experiments, time-lapse imaging was not possible due to the fast NETosis induction by bacteria. Not only was the process much faster, also the morphology of the NETs differed greatly from NETs resulting from neutrophils stimulation with PMA or Ionomycin. Whereas in PMA and Ionomycin-stimulated neutrophils the DNA often remained close to the remainder of the neutrophil, DNA, extruded after stimulation with bacteria, often appeared elongated and thread-like. The extension of these NETs depended largely on the type and number of bacteria added. Some bacterial strains, as mentioned before and as described in chapter 3, induced significantly less NETosis than others, for example, *S. aureus* Newman produced much more NETs than *S. aureus* ST239-M116. In the latter, NETs remained more closely to the neutrophil remains.

The morphology of our PMA-induced NETs very much resembles the NETs (and NETosis process) as described previously by other groups[10,11]. However, there also are reports in which PMA-induced NETs appear thread-like and resemble the NETs found after bacterial induction of NETosis[12-17]. Some of these papers show imaged NETs using SEM, which is a more detailed imaging technique than our confocal and SIM images. More often, NETs structures were stained with MPO and NE after fixation and immunohistochemistry (IHC). It is possible that during IHC, the morphology of the NETs change compared to NETs found in live imaging, like the ones we showed in chapter 2. In our experience, NETs were very fragile and were lost during the washing steps of our IHC protocols.
In summary, it is difficult to judge differences in morphology of NETs due to their fragileness and susceptibility to change. Therefore, it would be

interesting to observe the change in morphology when NETosis is induced by, for example, PMA, and movement is induced in the vicinity of moving bacteria. In this way, it could be explored if bacterial movement causes the difference in morphology, or if bacteria have a different NETosis inducing mechanism.

NETosis as a defense mechanism

One of the most interesting discussion points in this thesis is the functionality of the NETs, as discussed in chapter 3. The earliest research on NETosis states that NETs are a defense mechanism to trap and kill bacteria that is more efficient than phagocytosis[13]. Later, and in line with our findings, it was observed that multiple bacterial species can escape and survive the NETs[3,18,19]. *S. aureus* generally has developed multiple ways to evade the human immune system. For example, *S. aureus* is able to excrete Protein A, which has been described in literature as a regulator of phagocytosis and the complement system[4,20]. We have now shown another function of Protein A: the quantity of NETosis is dependent on the amount of Protein A secreted by *S. aureus*. This could indicate NETosis regulation, as bacteria can excrete Protein A in response to immune cells. From perspective of the bacteria, neutrophil destruction could be a positive act: this way phagocytosis is prevented and when NETs are released, *S. aureus* can dismantle these by secreting nuclease, as was observed in chapter 4. Additionally, *S. aureus* can form biofilms, which are protecting the bacteria by secreting nucleases at an early stage. We saw that these biofilms are, when grown in IMDM to simulate the human cell environment, largely consisting of PIA. It is known that deacetylated PIA can protect bacteria from innate immune cells because of its positive charge [21-23]. Also, when neutrophils are co-incubated with *S. aureus* biofilm, ROS production is inhibited. As mentioned previously, ROS production is a large part of the neutrophil's defense mechanism. Summed up, *S. aureus* has developed a spectrum of mechanisms to protect itself from human neutrophils, including Protein A production and biofilm formation.

The role of fibrin in wound healing

In chapter 7, we explored the potential of adding unfractionated human fibrin as a novel matrix to wounds in diabetic rats in order to improve the healing process. In previous studies by our group, fibrin has been shown to provide a good scaffold for angiogenesis *in vitro*, and it improved wound healing, angiogenesis and perfusion when applied onto the wounds of healthy rats [24]. Therefore, we hypothesized that in diabetic rat wounds, the effects would be

even more prominent. In our study, we saw improved wound closure on day 3 and 7 and improved wound perfusion after 21 days. We attempted to explore the underlying mechanism and found a 176 times upregulation of the eNOS gene in a qPCR, but our Western Blot data did not confirm this finding. Here, the eNOS protein was only 1.03x higher in the fibrin-treated wounds compared to the controls. Therefore, the mechanism should be further explored. However, fibrin effects on tissue perfusion warrant further investigation of its clinical use. We were able to conclude that fibrin on its own has potential to improve wound healing in a clinical setting. Previously, platelet rich plasma (PRP)[25-27] and platelet rich fibrin (PRF)[28] have been implemented in clinical trials. Even though these studies show promising results, they often include small patient groups, only report positive results for the beginning of the trial or do not include a proper control group.

Future perspectives

One of the most promising findings in this thesis is the ability of unfractionated fibrin to improve diabetic wound healing. Because of its angiogenic abilities, we hypothesized that fibrin would improve wound healing by boosting perfusion in rats, which we indeed found. The next step is to test human fibrin, in a physiological concentration (2 mg/ml), in human wounds. At the moment, fibrin is already used in the clinic as tissue glue, which has very high fibrinogen and thrombin concentrations (60-80 mg/ml and 800 U/ml, respectively). When applied to wounds, normal fibrin will be an optimal matrix for cellular ingrowth and new vessels and can be lysed normally, to be replaced by myofibroblasts and eventually collagen.

The relation between NETs and wound healing needs to be further explored. Are NETs advantageous in wound healing? The function of NETs seems the most efficient in wound healing, as many bacteria are present and NETs strengthen the fibrin matrix[29]. A paper by Wong and colleagues, however, suggests that in wound healing in diabetic mice, NETs may be disadvantageous as wound healing improved when the NETs are depleted with DNase[30]. They point out that NETs contain toxic components that destroy the ECM, therefore delaying wound healing. In addition, NETs, induced by PMA, have been described to hamper fibrinolysis[29] in normal plasma clots, which would also hamper wound healing. As diabetic wound healing is often hampered, it would be interesting to compare the effect of normal and diabetic NETs, induced by bacteria, on the fibrinolysis of normal and diabetic

human plasma clots. Furthermore, biopsies of non-healing diabetic wounds should be investigated. A biobank with chronic wound biopsies could be set up to explore NETs in these tissues, for example by immunofluorescent staining or ELISA. In both cases, levels of MPO-DNA complexes can be measured, similar to our methods in chapter 5, giving an indication of the amount of NETs compared to control tissue. Normal skin could be taken as a control.

S. aureus is a key player in chronic infection and thus inflammation in wounds. One of our main findings in this thesis was that *S. aureus* Protein A is a determinant of NETosis. We found that if more Protein A was present, more NETosis could be observed. Furthermore, when Protein A was added to a Protein A knockout strain of *S. aureus*, NETosis could be rescued. However, no NETosis was observed when Protein A alone was added to neutrophils, indicating that Protein A by itself is not enough to induce NETosis. We speculated that bacteria needed to be present to induce NETosis, however, when Protein A was added to dead *S. aureus*, no NETosis was observed. Therefore, we expect that a co-factor, secreted by living *S. aureus*, must be present in order to induce NETosis. To fully understand the mechanism behind NETosis induction by *S. aureus* via Protein A, this co-factor must be found.

In conclusion, NETs have been described in many different diseases, such as chronic wound healing, sepsis and thrombosis. However, in our studies, the effect of NETs was often much less clear than described previously. Fibrin, on the other hand, seems to have more potential in improving diabetic wound healing.

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Summary

The aim of this thesis was to investigate the effect of fibrin, NETs and the induction of NETosis, in wound healing. To achieve this, we created *in vitro* models to study the formation of NETs by several inducers, such as PMA, LPS, *S. aureus*, *E. coli* and *N. meningitidis*. Furthermore, we studied the role of NETs and extracellular DNA in sepsis and thrombosis. A diabetic rat model was used to study the effect of fibrin on wound healing.

In **chapter 2** we created a systematic review in order to obtain more insight in the different inducers of NETosis used in literature. Here, we found a wide variation in reported effectiveness of these inducers. Therefore, we investigated a subset of these inducers (PMA, *S. aureus* (alive and dead), *E. coli* (alive and dead), LPS, LPS + platelets, LPS + activated platelets, activated platelets and ionomycin), in our own standardized time-lapse model. We found that PMA, living bacteria and ionomycin were all robust inducers of NETosis. All other inducers were less potent. Furthermore, using time-lapse microscopy, we observed a difference in the NETosis induction process between PMA and ionomycin, indicating the importance of this technology.

In **chapter 3**, we investigated the role of Protein A, an important immune modulator produced by *S. aureus*, in NETosis induction. We used four different *S. aureus* strains to induce NETosis and determined their Protein A production. We saw a positive correlation between the amount of NETs and the amount of Protein A present. Significantly less NETosis was measured using a Protein A knockout strain to induce NETosis, which elevated to normal levels when commercial Protein A was added. When Protein A was added to dead *S. aureus*, no NETosis was observed. This indicates that Protein A plays an important role in the induction of NETosis by living *S. aureus*.

In **chapter 4**, the role of nuclease, as produced by *S. aureus* biofilm, was explored to obtain more insight in biofilm growth and defence against the innate immune system. Nuclease production by biofilm was studied using two different culture media: TSB, which is most commonly used in literature, and IMDM, a mammalian cell culture medium which more closely represents human cell environment. We observed that significantly more nuclease was produced when biofilm was grown in IMDM. Furthermore, mature biofilm grown in IMDM consisted mostly of PIA, whereas mature biofilm grown in TSB consisted mostly of eDNA. Additionally, biofilms were able to induce NETosis, and NETs could be degraded using nucleases. These nucleases, however, did

not have an effect on ROS production by neutrophils, which was lower when neutrophils were incubated with biofilm. Taken together, nuclease production by biofilms can regulate biofilm composition and protect the biofilm from the innate immune system by breaking down NETs.

In **chapter 5**, the contribution of NETosis on the severity of meningococcal sepsis in children was explored. Serum samples from these patients were used to measure MPO-DNA levels, a marker for NETosis. We aimed to study correlations between these levels and patient outcome and other inflammatory markers, such as CRP, IL6 and IL8. Whilst MPO-DNA levels were higher on admission and after 24 hours compared to controls, no associations were found between MPO-DNA levels and patient outcome or any other inflammatory markers. This indicates that in our study in children with meningitis, NETs might not be as important in severity of sepsis compared to previous sepsis studies.

In **chapter 6**, the role of Factor H and extracellular DNA (nucleosomes) on the cleavage of VWF by ADAMTS13 was studied in young patients with arterial thrombosis. FH levels in these patients were elevated, but had no direct association with thrombosis. A correlation between FH levels and nucleosome levels was found, which may indicate a role of FH on the activation of the immune system to clear dead cells. Furthermore, a negative correlation between FH and VWF:CBS/VWF:Ag ratio was found, which may indicate that the presence of FH in these patients gives increased amounts of high molecular weight VWF multimers and more VWF mediated platelet aggregation, leading to arterial thrombosis.

In **chapter 7**, the wound healing potential of human fibrin on diabetic wound healing was explored. Dorsal wounds were created in a diabetic rat model, of which one was treated with a placebo and one was treated with a fibrin matrix. Wound area and tissue perfusion were measured on several time points during wound healing. We found that fibrin significantly improved perfusion and early wound healing, indicating the potential of fibrin to boost wound healing of poorly-healing wounds.

Samenvatting

Het doel van dit proefschrift was het onderzoeken van het effect van fibrine, NETs en NETose inductie in wondgenezing. Wij hebben hiervoor *in vitro* modellen gecreëerd, om zo de formatie van NETs door verschillende inducers, zoals PMA, LPS, *S. aureus*, *E. coli* en *N. meningitidis* te bestuderen. Ook hebben we de rol van NETs en extracellulair DNA onderzocht in sepsis en trombose. Om het effect van fibrin in chronische wonden te bestuderen, hebben wij gebruik gemaakt van een diabeet ratmodel.

In **hoofdstuk 2** hebben we een systematic review verricht om zo meer inzicht te krijgen in verschillende inducers van NETose, die gebruikt waren in de literatuur. We vonden dat er veel discrepanties waren in de effectiviteit van deze inducers. Daarom hebben we een aantal van deze inducers (PMA, *S. aureus* (levend en dood), *E. coli* (levend en dood), LPS, LPS + plaatjes, LPS + geactiveerde plaatjes, geactiveerde plaatjes en ionomycin) getest in ons eigen gestandardiseerde time-lapse model. We vonden dat PMA, levende bacteriën en ionomycin sterke inducers van NETose waren. Andere induceres waren minder robuust. Ook zagen wij verschillen in het NETose inductie proces tussen PMA en ionomycin, wat benadrukt hoe belangrijk time-lapse technologie kan zijn.

In **hoofdstuk 3** keken we naar de rol van *S. aureus* Protein A, een belangrijke immuun modulator geproduceerd door *S. aureus*, in NETose inductie. We gebruikten vier verschillende *S. aureus* stammen om NETose te induceren en bepaalden van iedere stam de Protein A levels. We vonden een positieve correlatie tussen de hoeveelheid Protein A en de hoeveelheid NETs. Significant minder NETs werden gemeten als NETose was geïnduceerd met een *S. aureus* Protein A knockout stam. NETose kon worden hersteld tot normale levels door commerciële Protein A toe te voegen. Als Protein A werd toegevoegd aan dode bacteriën, zagen wij geen NETose. Dit impliceert dat Protein A een belangrijke rol spelt in de inductie van NETose door levende *S. aureus*.

In **hoofdstuk 4** bestudeerden we de rol van nuclease, geproduceerd door *S. aureus* biofilm, om meer inzicht te krijgen in biofilm groei en diens verdediging tegen het innate immuun systeem. Hiervoor hebben wij de nuclease productie door biofilms bekeken door gebruik te maken van twee verschillende cell culture media: TSB, wat in de literatuur het meest gebruikt wordt, en IMDM, een van zoogdieren afkomstig cultuur medium dat meer lijkt

op de humane omgeving van cellen. We zagen een verhoogde nuclease productie in biofilms die groeiden in IMDM. Ook bestonden volgroeide biofilms in IMDM vooral uit PIA, terwijl volgroeide biofilms in TSB vooral bestonden uit eDNA. Biofilms konden NETose induceren, en nucleases braken de NETs af. Deze nucleases hadden echter geen invloed op de ROS productie door neutrofielen, terwijl de ROS productie verlaagd was wanneer neutrofielen in de aanwezigheid waren van biofilm. Samengevat kan nuclease de compositie van biofilms reguleren en beschermt het de biofilm tegen het innate immuun systeem door NETs af te breken.

In **hoofdstuk 5** bekeken we het effect van NETose op de ernst van meningococcen sepsis in kinderen. MPO-DNA levels, een marker voor NETs, was gemeten in serum van deze patiënten. We onderzochten hiermee de correlatie tussen MPO-DNA levels en uitkomst en andere inflammatoire markers, zoals CRP, IL6 en IL8. MPO-DNA levels waren hoger in patiënten die net waren opgenomen en na 24 uur vergeleken met controles, maar geen associaties waren gevonden tussen MPO-DNA levels en uitkomst of de inflammatoire markers. Dit wijst erop dat in deze groep, NETs waarschijnlijk niet zo belangrijk zijn voor de ernst van sepsis vergeleken met voorgaande sepsis studies.

In **hoofdstuk 6** bestudeerden we de rol van Factor H en extracellulair DNA (nucleosomen) op het knippen van VWF door ADAMTS13 in jonge patiënten met arteriële trombose. FH levels in deze patiënten waren verhoogd, maar dit had geen directe associatie met trombose. We vonden een correlatie tussen FH levels en nucleosomen, wat mogelijk een rol impliceert van FH op de activatie van het immuunsysteem om dode cellen op te ruimen. Ook vonden we een negatieve correlatie tussen FH en VWF:CB/VWF:Ag ratio, wat erop kan wijzen dat de aanwezigheid van FH in deze patiënten zorgt voor meer high molecular weight VWF multimeren en meer door VWF gemedieerde plaatjes aggregatie, wat leidt tot trombose.

In **hoofdstuk 7** onderzochten wij de wondgenezende potentie van humaan fibrine in diabete wonden. Dorsale wonden in een ratmodel waren behandeld met een placebo of een fibrinematrix. Wondoppervlak en perfusie werden gemeten op verschillende tijdstippen. We vonden dat fibrine de perfusie en vroege wondgenezing significant verbeterde, wat de potentie van fibrine om wondgenezing in niet-genezende wonden te bevorderen, benadrukt.

Abbreviations

ADAMTS13	A Disintegrin-like And Metalloprotease with Thrombo Spondin motifs 13
aHUS	atypical hemolytic uremic syndrome
ANOVA	Analysis of Variances
ATTAC	Arterial Thrombosis TAFI and other Coagulation factors
AU	Arbitrary Units
BEP	Base Excess and Platelet count at presentation
Ca^{2+}	Calcium
CHD	Coronary Heart Disease
CRP	C-Reactive Protein
DIC	Disseminated Intravascular Coagulation
DM	Diabetes Mellitus
DMEM	Dulbecco's Modified Eagle Medium
ECs	Endothelial Cells
ECM	Extracellular Matrix
eDNA	extracellular DNA
ELISA	Enzyme-Linked Immunosobent Assay
F	Factor
FH	Factor H
FCS	Fetal Calf Serum
IFN-ɣ	Interferon ɣ
IL	Interleukine
IMDM	Iscove's Modified Dulbecco's Medium
IS	Ischemic Stroke
IQR	Inter Quartile Range
LPS	Lipopolysaccharide
LTA	Lipoteichoic Acid
MI	Myocardial Infarction
MPO	Myeloperoxidase
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSU	Monosodium Urate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NE	Neutrophil Elastase
NETs	Neutrophil Extracellular Traps
NETosis	The process of NETs formation

Nuc	Nuclease
OD	Optical Density
PAD	Peripheral Arterial Disease
PBS	Phosphate Buffered Saline
PDGF	Platelet-Derived Growth Factor
PI	Propidium Iodide
PIA	Polysaccharide Intercellular Adhesin
PICU	Pediatric Intensive Care Unit
PKC	Protein Kinase C
PMA	Phorbol-12-Myristate-13-Acetate
PMN	polymorphonuclear cells
PRISM	Pediatric Risk of Mortality
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RT	Room Temperature
saeRS	*staphylococcus aureus* exoprotein, 2-component system
Sbi	Staphylococcal Binding Immunoglobulin protein
SpA	*Staphylococcus aureus* Protein A
TAFI	Thrombin-Activatable Fibrinolysis Inhibitor
TF	Tissue Factor
TGF-β	Transforming Growth Factor β
TLR	Toll-like Receptor
TNF-α	Tumor Necrosis Factor α
tPA	tissue Plasminogen Activator
TSA	TrypticaseTM Soy Agar
TSB	Tryptic Soy Broth
VEGF	Vascular Endothelial Growth Factor
VWF	Von Willebrand Factor
VWF:Ag	Von Willebrand Factor antigen
VWF:CB	Von Willebrand Factor Collagen Binding activity

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Working on a thesis is an unique journey full of surprises, love, support, sometimes frustration, but luckily also a lot of laughter and crazy ideas. And being able to express creativity was one of the best things in this whole process. Writing one of the final chapters has made me realize how far I've come, helped me remember where I came from and more importantly, where I want to go from here. But all of this would never in a million years have been possible without everyone around me. So I want to say thank you, in a creative and joyful manner: with musical lyrics.

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- Klote – Want Want
- Ungrateful – Escape the Fate
- Haters Gonna Hate – Chunk! No, Captain Chunk!
- Last Resort – Papa Roach
- Growing Pains – Farewell Fighter

Tracklist:

1. The Press and The President – "Central Park."
2. Mercy Mercedes – "Lucky."
3. Like The Stars – "Better off Believing."
4. Nickelback – "Photograph."
5. Call it Off – "Do It All Again."
6. Paradise Fears – "Yours Truly."
7. Steel Panther – "Goin' In The Backdoor."
8. Indevotion – "Go Go Go Go Go!"
9. The Little Mermaid – "Part of Your World."
10. Beastie Boys –"Fight For Your Right."
11. Family Guy – "It's a Wonderful Day For Pie."
12. John Parr – "St. Elmo's Fire."
13. Alex Goot & Against The Current – "Find You."
14. Soul Control – "Chocolate."
15. Five Years Later – "Too Good To Let Go."
16. Owl City & Carly Rae Jepsen – "Good Time."
17. Destine – "Anywhere You Wanna Go."
18. Toto – "Stop Loving You."
19. The Young River – "Disappear."
20. Bon Jovi – "Always."
21. Theory of a Deadman – "Bad Girlfriend."
22. Steel Panther – "Community Property."
23. Paradise Fears – "Battle Scars."

Curriculum vitae

Tamara Hoppenbrouwers was born on the 19th of March 1990 in Spijkenisse. In 2013, she graduated from Leiden University, receiving her Master's degree in Biology (Evolution, Biodiversity and Conservation), after her final internship at the Leids Universitair Medisch Centrum (LUMC) on heart development. After one extra year of research at the LUMC on heart diseases, she started her PhD on NETs and fibrin in wound healing at the departments Hematology and Plastic and Reconstructive Surgery at the Erasmus MC in Rotterdam in 2014, supervised by Dr. J.W. van Neck and Dr. M.P.M. de Maat. As from September 2017, she will start working on a new research project as a Junior Postdoc on inherited eye diseases at the department of Genetics at the Radboud UMC in Nijmegen.

Publications

Poelmann, R. E., A. C. Gittenberger-de Groot, R. Vicente-Steijn, L. J. Wisse, M. M. Bartelings, S. Everts, **T. Hoppenbrouwers**, B. P. Kruithof, B. Jensen, P. W. de Bruin, T. Hirasawa, S. Kuratani, F. Vonk, J. M. van de Put, M. A. de Bakker, and M. K. Richardson. 2014. Evolution and development of ventricular septation in the amniote heart. PLoS One 9: e106569.

Gittenberger-de Groot, A. C., **T. Hoppenbrouwers**, L. Miquerol, Y. Kosaka, R. E. Poelmann, L. J. Wisse, H. J. Yost, M. R. Jongbloed, M. C. Deruiter, and L. Brunelli. 2016. 14-3-3epsilon controls multiple developmental processes in the mouse heart. Dev Dyn 245: 1107-1123.

Faniku, C., I. Castellano-Pellicena, **T. Hoppenbrouwers**, and H. I. Korkmaz. 2017. Report on the 1st European Tissue Repair Society Summer School, London 29th June - 1st July 2016. Wound Repair Regen.

Hoppenbrouwers, T., B. Tuk, E. M. Fijneman, M. P. de Maat, and J. W. van Neck. 2017. Fibrin improves skin wound perfusion in a diabetic rat model. Thromb Res 151: 36-40.

Hoppenbrouwers, T.*, A. S. A. Autar*, A. R. Sultan, T. E. Abraham, W. A. van Cappellen, A. B. Houtsmuller, W. J. B. van Wamel, H. M. M. van Beusekom, J. W. van Neck, and M. P. M. de Maat. 2017. In vitro induction of NETosis: Comprehensive live imaging comparison and systematic review. PLoS One 12: e0176472.

Hoppenbrouwers, T.*, A. R. Sultan*, T. E. Abraham, N. Lemmens-den Toom, S. Hansenova Manaskova, W. A. Cappellen, A. B. Houtsmuller, W. J. B. van Wamel, M. P. M. de Maat, and J. W. Van Neck. Staphylococcal Protein A (SpA) is a key factor in Neutrophil Extracellular Traps (NETs) formation. Accepted: Front. Immunol, 2018.

Sultan, A. R.*, **T. Hoppenbrouwers***, N. Lemmens-den Toom, M. P. M. de Maat, A. Verbon, J. W. Van Neck, and W. J. B. van Wamel. The role of nuclease during the early stages of biofilm formation. (In preparation)

Hoppenbrouwers, T.*, N. P. Boeddha*, E. Ekinci, M. Emonts, J. A. Hazelzet, G. J. Driessen**, and M. P. M. de Maat**. Neutrophil extracellular traps in children with meningococcal sepsis. Accepted: PCCM, 2018.

Luken, B. M.*, **T. Hoppenbrouwers***, M. A. Sonneveld, G. J. Van Mierlo, S. Zeerleder, F. W. G. Leebeek, D. Wouters, and M. P. M. de Maat. Complement factor H, and von Willebrand factor size in young patients with arterial thrombosis. (Submitted)

van Loon, P.S.E.L.M, P.L. Brinkhorst, **T. Hoppenbrouwers**. 2018. 'De verwaande groenteman' in De Sprookjessprokkelaar.

*/** These authors contributed equally.

PhD Portfolio

Summary of PhD training and teaching activities

Name PhD student: Tamara Hoppenbrouwers
Erasmus MC department: Hematology and Plastic and Reconstructive Surgery
PhD Period: July 2014 – July 2017
Promotor: Prof. Dr. F.W.G. Leebeek
Co-promotors: Dr. J.W. van Neck and Dr. M.P.M. de Maat

PhD Training	Year	Workload (ECTS)
General academic skills		
• Biomedical English Writing Molmed Erasmus MC Rotterdam	2015	2.0
• Biostatistical Methods I: Basic Principles NIHES Erasmus MC Rotterdam	2016	5.7
In-depth courses		
• Confocal microscopy introduction course Erasmus MC	2014	0.2
• The Microscopic Image Analysis: From Theory to Practice Molmed Erasmus MC Rotterdam	2015	0.8
• The Translational Imaging Workshop by AMIE: 'From mouse to man' Molmed Erasmus MC Rotterdam	2015	1.4
• Bacteria and Chronic Infections Coursera University of Copenhagen	2016	1.8
Seminars and workshops		
• Seminar COEUR Erasmus MC Rotterdam	2014	0.3
• Seminar Sanquin Sanquin Amsterdam	2016	0.2

• ETRS Summer School "Advancing Wound Research" London, UK	2016	1.8
• Workshop "Share Your Research with a Broad Audience" LUMC Leiden	2016	1.0
• Masterclass EC Marie Sklodowska-Curie Innovative Training Networks (ITN) Programme for Doctoral of Joint Doctorates Training Erasmus MC Rotterdam	2016	0.7
• Famelab Erasmus MC Rotterdam	2017	0.5
• Workshop Pitch jezelf (vena) Erasmus MC Rotterdam	2017	0.3
International conferences		
• 7th Joint Meeting of the European Tissue Repair Society (ETRS) with the Wound Healing Society Oral Presentation Copenhagen, Denmark	2015	2.4
Presentations		
• Department of Hematology (7x)	2014-2017	0.4
• Department of Plastic and Reconstructive Surgery (2x)	2014-2017	0.1
• Department of Pathology (1x)	2015	0.1
Supervising HLO intern • 1 student (20 weeks)	2016-2017	2.0
Grants		
• Trustfonds (€175,-)	2015	1.0
• Stichting Coolsingel (€25.000,-)	2016	1.0
• mRace Pilot (€40.000,-)	2016	1.0