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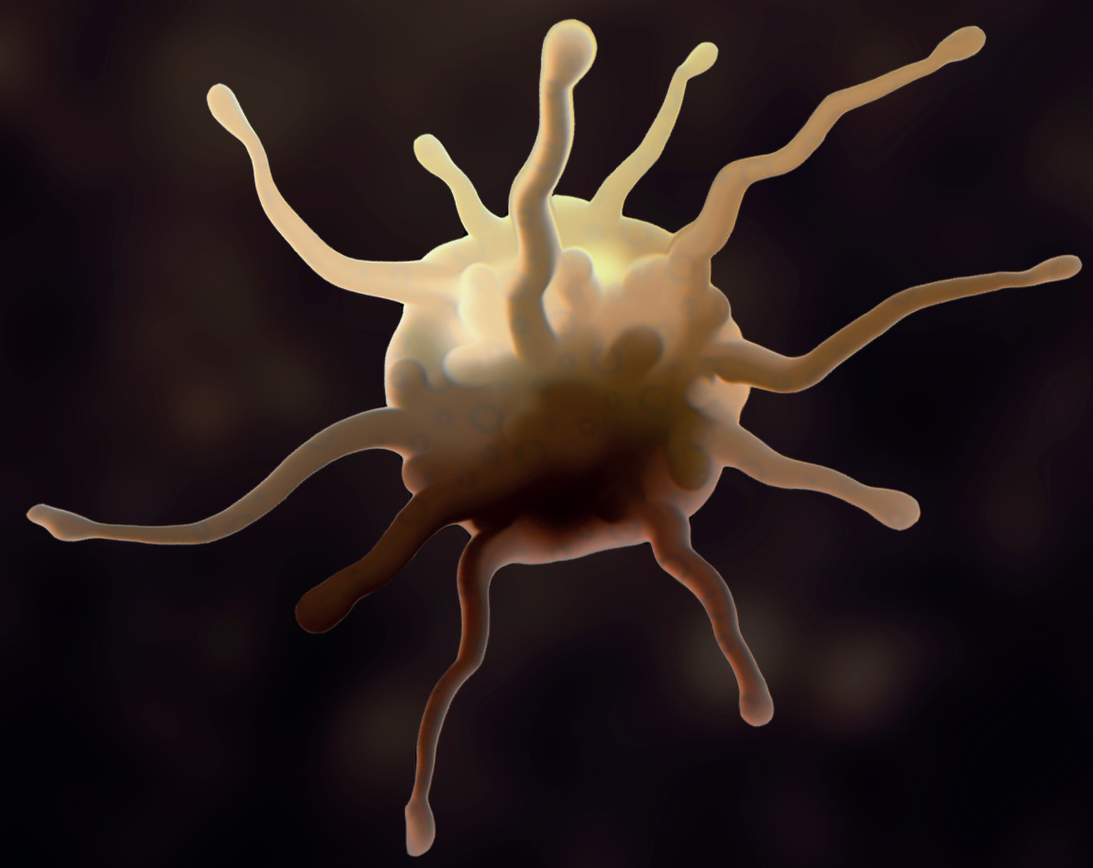

The role of complement and neutrophils in infection, inflammation and immunity.

Yasmin Elisah Sophia de Wit

HOLD YOUR PLATELETS

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Yasmin de Wit

Hold your platelets:
The role of complement and neutrophils in infection, inflammation and immunity

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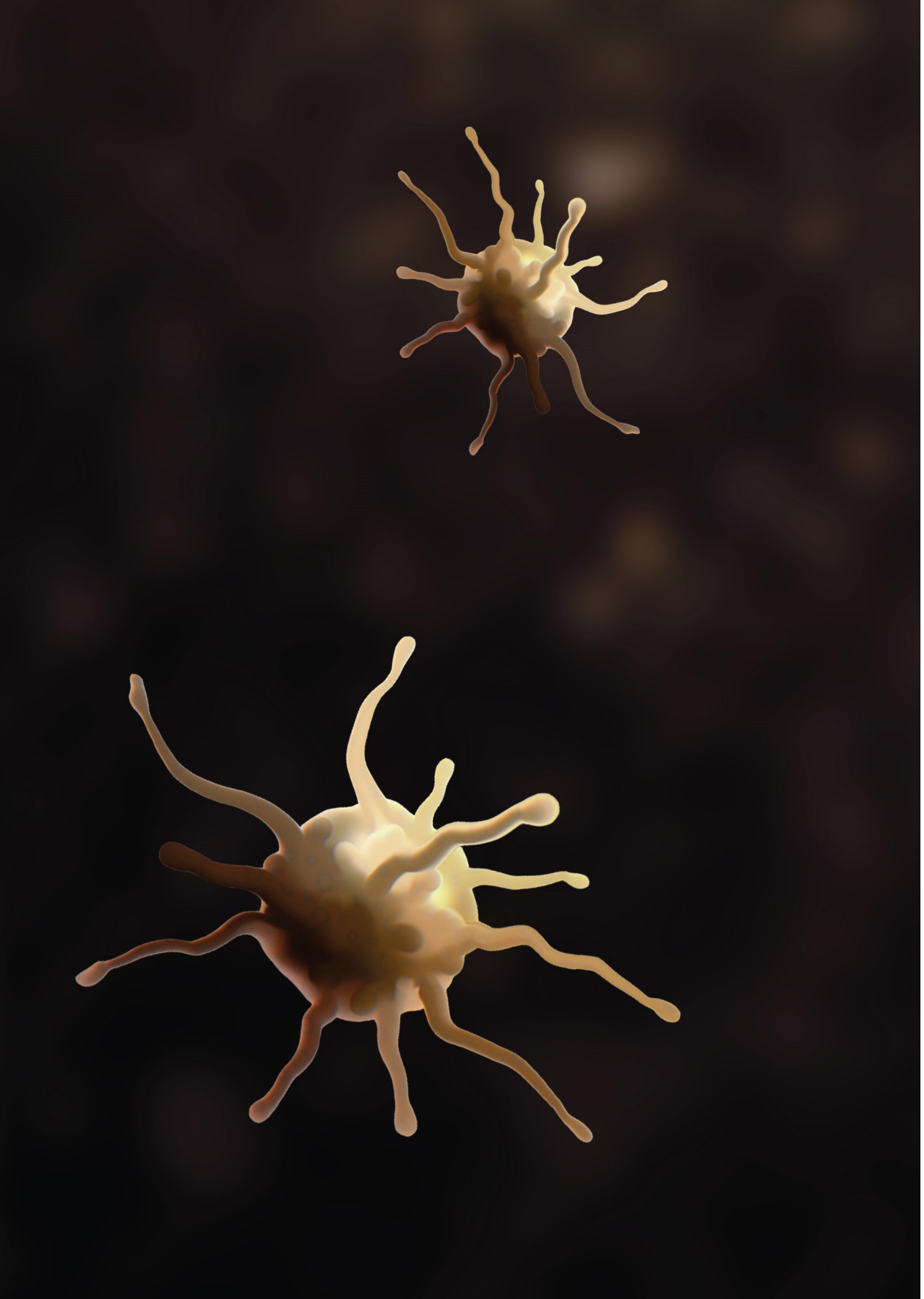

CHAPTER 1

General introduction and scope of the thesis

Platelets are the second most abundant blood cell type in the circulation. They are recognized as the primary cell type that regulates hemostasis and thrombosis. Upon activation by soluble ligands or subendothelial exposure of collagen, von Willebrand factor (vWF) or tissue factor (TF), platelets upregulate membrane-bound glycoproteins/integrins and excrete granules that contain soluble factors. Platelet α-granules contain a large spectrum of proteins, such as P-selectin (CD62p), thrombospondin, von Willebrand factor (vWF) and fibronectin[1,2]. These membrane-bound or soluble glycoproteins play a significant role in hemostasis, as they allow platelets to bind coagulation factors and agglutinate, but also to firmly adhere to the vessel wall. This leads to the formation of a hemostatic plug, also known as platelet thrombus **(Fig. 1)**. Secondly, vascular damage-induced exposure of subendothelial TF promotes thrombin generation. Via amplification loops in the coagulation system and in platelet activation, substantial amounts of thrombin are generated. Thrombin subsequently can, amongst other things, cleave fibrin, which stabilize the earlier formed hemostatic plug and activate additional platelets. At the same time, platelets secrete dense (δ) granules that are abundant in ADP, ATP, calcium and serotonin, which are important for constriction of injured blood vessels and reenforce platelet activation and aggregation[2–5]. To maintain hemostasis, platelets are prominently involved in vascular repair, danger sensing and closely interact with other cell types, including endothelial cells and leukocytes[6–8]. However, the role of platelets is not limited to hemostasis, platelets can detect and respond to local danger signs, such as infectious pathogens[6,9]. Nowadays, the role of platelets in immune defense is increasingly being recognized. Platelets use Toll-like receptors (TLR) and adhesion receptors to mechanically probe the microenvironment[10]. In this manner, platelets can act as scavengers, scanning the surface to quickly detect and respond to pathogens and tissue injury[7,11]. This indicates a vital role for platelets in the first-line immune defense. Increasing knowledge demonstrates how platelets closely interact with neutrophils and the complement system during the innate immune response. Platelets express numerous complement components and receptors, such as cC1qR, gC1qR, C3aR and C5aR[12]. In addition, platelets contain plasmatic C3 and produce C3 that is different from plasmatic C3 in its electrophoretic mobility[12]. It is most likely that platelet born C3 is stored in their α-granules[1,13]. Platelet α-granules contain a large spectrum of pro-inflammatory/modulatory chemokines and cytokines, which allow platelets to act as immunomodulators to attract and prime immune cells[14].

In this thesis we will discuss the role of platelets, complement and neutrophils in immunothrombosis, infection and inflammation. We performed observational studies to gain insight into the dynamics of platelets, neutrophils, complement and DAMPs in multiple different clinical settings. We specifically focus on the role of platelets, complement and neutrophil extracellular traps (NETs) during COVID-19. Furthermore, we discuss the role of platelets, complement, neutrophils and DAMPs as drivers of acute

transfusion reactions after transfusion with platelet concentrates. Finally, we deliberate about the role of complement and neutrophil activation in atypical hemolytic uremic syndrome (aHUS) during therapeutic plasma exchange therapy and in solvent/detergent treated plasma.

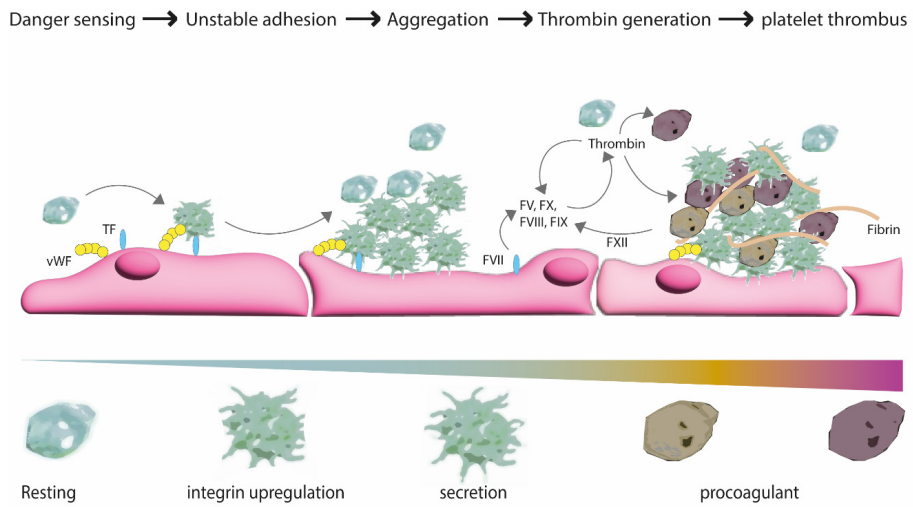

Figure 1. Platelet and coagulation activation. *Upon endothelial cell injury Tissue Factor (TF) and von Willebrand Factor (vWF) is released. Platelets sense damage and bind to TF and vWF leading to initial unstable binding and aggregation. Secondly, FVII forms a complex with exposed TF and initiates a series of proteolytic reactions leading to the formation of thrombin. Simultaneously, activated platelets express Phosphatidylserine (PS) which binds to FXII that also activates the coagulation pathway. Finally, thrombin creates a positive feedback-loop, activates platelets, and converges circulating fibrinogen into fibrin leading to stabilization of the platelet thrombus. Adapted from Versteeg* et al., *2013*[15]

THE COAGULATION SYSTEM

The coagulation system consists of a series of plasma zymogens, called coagulation factors, which are sequentially activated by proteolysis. Our understanding of the coagulation system has evolved considerably. It was originally described as a coagulation cascade, whereas currently the cell-based model of coagulation is commonly accepted[16]. While the second model still describes the cascade of proteolytic reactions, it places more emphasis on the interaction of these coagulation factors with cell membranes[17]. The cascade can be divided into three phases: initiation, amplification and propagation[17]. Classically, vascular tissue damage initiates a response of the coagulation system to maintain hemostasis. Tissue factor (TF), collagen and vWF are released from activated and injured endothelial cells. Following this exposure, platelets will bind to collagen and vWF, leading to firm adhesion, aggregation, and the formation of a hemostatic plug.

Secondly, complexation of TF with circulating activated factor VII (FVIIa) triggers the coagulation system. The TF-FVIIa complex (also known as tenase complex) converts FX into FXa. FXa together with FVa leads to the generation of thrombin from prothrombin. At this stage, thrombin creates a positive feedback-loop via activation of FXI that in turn activates more FX, activates platelets, and converges circulating fibrinogen to insoluble fibrin, which leads to amplification and propagation. The coagulation system can also be initiated, although insignificant, by the proteolysis of FXII to FXIIa upon contact with anionic surfaces, such as exposed endothelial collagen and platelet-derived phosphatidylserine (PS), called the contact phase. FXIIa initiates a chain of enzymatic reactions that also leads to the formation of a tenase complex. Inhibitors such as antithrombin and tissue factor pathway inhibitor (TFPI) provide tight regulation to prevent thrombosis, due to uncontrolled activation of the coagulation system.

THE COMPLEMENT SYSTEM

The complement system is an essential ancient part of the innate immune system. Complement acts as a crucial first-line defense mechanism in the rapid identification and clearance of pathogens and apoptotic cells. Complement activation results in opsonization, induction of inflammation and ultimately in the lysis of pathogens and infected cells. Like the coagulation system, the complement system consists of proteins that become sequentially activated upon proteolytic cleavage in a cascade-like manner. Initiation of complement activation occurs via three distinctive pathways: the classical, lectin and alternative pathway, that all converge into the generation of the C3 convertase. The C3 convertase cleaves C3 to generate the central effector molecule C3b and the anaphylatoxin C3a. C3b is deposited on the surface membrane of the target cell, e.g., a pathogen, which leads to opsonization. The classical pathway is mainly activated through ligation of antibodies to their target, thereby forming an immune complex to which C1q can bind. Activation of the lectin pathway occurs via the recognition of microbial carbohydrate structures and acetylated residues by mannose-binding lectin (MBL), collectin 11 and ficolins[18]. Initiation of the classical or lectin pathway results in activation of C4 and C2, which forms a C3 convertase (C4bC2b). The alternative pathway ensures a low continuous activity in the fluid phase and amplification of complement activation initiated by the classical and lectin pathway[19]. C3b deposition triggers activation of the alternative pathway in addition to spontaneous hydrolysis of C3, which leads to an amplification loop. As a consequence of continues activation of the alternative pathway, the complement system needs to be efficiently regulated by a set of membrane-bound and fluid phase proteins[20]. Amplification of the initial activation of complement is essential for the outcome of the terminal pathway[19]. Accordingly, activation of the terminal pathway leads to the cleavage of C5 into anaphylatoxin C5a and

C5b. Anaphylatoxins C3a and the more potent C5a are key effector molecules to attract and activate leukocytes and platelets to induce a robust inflammatory response[21,22]. Ultimately, activation of the complement system results in direct lysis of pathogens due to the formation of a membrane attack complex (MAC)[23].

NEUTROPHIL ACTIVATION AND NETs

Neutrophils are the most abundant leukocytes in the circulation and play a crucial role in the first-line defense of the innate immune system. They are short-lived and highly mobile cells. Due to their multi-lobed nuclei, neutrophils can enter tissue where other cells cannot. Neutrophils are equipped with stocks of innate defense armory that are packaged into different granule subsets. This armory includes NADPH oxidase to generate Reactive Oxygen Species (ROS), enzymes for digestive and bactericidal functions and cytotoxic molecules. The granules are released upon activation of the neutrophil by specific mediators, amongst others C5a. In addition, neutrophils can expel networks of extracellular fibers, primarily composed of nuclear and mitochondrial DNA decorated with DNA binding proteins (histones and HMGB1) and biologically active neutrophilic proteins (elastase and myeloperoxidase (MPO)), which bind and entrap pathogens[24]. NETs form a crucial mechanism of the innate immune defense to entrap pathogens such as bacteria (e.g., *S. aureus, Streptococcus sp., H. influenzae, K. pneumoniae, L. monocytogenes, M. tuberculosis and S. flexneri*) and viruses (e.g., respiratory syncytial virus, influenza, dengue, human immunodeficiency virus (HIV) and SARS-CoV-2), but also fungi and parasites (e.g., *C albicans,* malaria)[25–30]. NETs exert thrombogenic activity through DNA-binding proteins, such as histones and HMGB1, expression of TF, activation of FXII and inhibition of TFPI and are involved in the pathogenesis of DIC[31–34]. Moreover, NETs facilitate fibrin deposition, trigger platelet activation, disrupt local blood flow and protect thrombi against fibrinolysis[35]. Neutrophil extravasation and invasion of inflamed areas is platelet-dependent[36]. Activated platelets promote NETosis and subsequently, these NETs form a platform for additional platelets to bind histones and get trapped, thereby promoting the formation of a hemostatic plug[37–39]. Hence, NETosis plays an important role in the pathogenesis of microvascular complications[33]. Together this illustrates the close interaction of neutrophils with platelets in inflammation and coagulopathy.

SARS-CoV-2 INFECTION

Coronavirus Disease 2019 (COVID-19) is caused by an infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and is characterized by systemic inflammation. Clinical manifestations of COVID-19 begin with flu-like symptoms and

viral pneumonia, which may progress to acute respiratory distress syndrome (ARDS) and organ dysfunction caused by microvascular complications,[40–42]. Angiotensin converting enzyme-2 (ACE2) facilitates entry of the virus into the cell and is abundantly present in the lungs. The combination of SARS-CoV-2 and ACE2 on lung epithelium results in an increase in activity of angiotensin II[43]. Direct infection of endothelial cells with subsequent endotheliitis and endothelial cell dysfunction results in microvascular complications[44,45]. Hypercoagulability (as demonstrated by high D-dimer concentrations), platelet hyperactivation and endotheliopathy are well-recognized features in COVID-19 patients. Platelets of COVID-19 patients are pre-activated/hyperreactive, as evidenced by increased P-selectin expression on resting platelets[46–48]. In addition, uncontrolled complement activation is described as a distinct characteristic of COVID-19 and is linked to disease severity, respiratory failure and mortality[49–51]. SARS-CoV-2 infection induces elevated neutrophils counts and increased neutrophil influx in nasopharyngeal epithelium and lungs[52,53]. This suggests a prominent role for complement, platelets, and neutrophils in the pathogenesis of COVID-19. Together, SARS-CoV-2 induces hyperactivation of the innate immune system.

PLATELETS AND IMMUNOTHROMBOSIS IN COVID-19

Microvascular thrombotic complications are indicators of severe COVID-19 and are associated with organ dysfunction and mortality. Microvascular thrombosis and coagulopathy are major clinical manifestations of COVID-19. Hyperactivation of platelets, neutrophils, complement and coagulation may result in a process called immunothrombosis. This process describes the concurrent activation of the innate immune- and coagulation system to entrap and eliminate pathogens in the circulation. Immunothrombosis depends on synergism between platelets, complement, coagulation factors and neutrophils. It acts as an independent defense mechanism to identify pathogens, trap and prohibit pathogen survival and spreading throughout the circulation[33]. Immunothrombosis in COVID-19 is most likely driven by complement, TF, neutrophils and platelets[54–57]. During COVID-19 complement activation leads to an increase in anaphylatoxins C3a and the more potent C5a, which can activate neutrophils and platelets. Activated platelets subsequently promote the formation of TF-positive NETs by neutrophils and TF expression in monocytes[7,58,59]. Neutrophils scan the environment for activated platelets and require activated platelets for the formation of NETs[36,60,61]. NETs exert thrombogenic activity through bound TF, fibrin deposition, platelet activation and through citrullinated histone H3 exposure[35,60,62–64]. Furthermore, activated platelets secret extracellular vesicles (EV) or platelet-derived particles (PMP), which may also activate complement- and coagulation, via phosphatidylserine (PS) expression on the membrane surface[65–69]. It has been demonstrated that EVs, mostly platelet-derived,

are elevated in COVID-19 patients and that they are related to hypercoagulable states, including sepsis[70–73]. Further details on immunothrombosis in COVID-19 will be discussed in **Chapter 2**.

DAMAGE-ASSOCIATED MOLECULAR PATTERNS (DAMPS)

DAMPs, also known as alarmins, are endogenous molecules within cells which may elicit an innate immune response upon ligation of pattern recognition receptors (PRRs). DAMPs are mostly nuclear or cytosolic proteins/molecules, such as DNA (e.g., chromosomal, and mitochondrial (mt)DNA) and DNA binding proteins (e.g., histones and HMGB1). An overview DAMPs and their associated receptors and neutralizers is shown in **Table 1**. DAMPs are released from damaged or dead cells due to infection by a pathogen or trauma and serve as a warning sign for the immune system to signal and respond to damage or infection. Upon release DAMPs promote a non-infectious inflammatory response by binding to PRRs. This non-infectious inflammatory response helps to mitigate future damage to the tissue by promoting rapid removal of pathogens from the inflamed area and tissue repair. In contrast, pathogen-associated molecular patterns (PAMPs) trigger and perpetuate a pathogen-induced inflammatory response. Nucleosomes are considered to be DAMPs, even though they are not cytotoxic[74,75]. Nucleosomes induce danger signaling via ligation of TLR-9. Nucleosomes are composed of DNA wrapped around histones; due to their strong positive charge the latter are highly cytotoxic. In the nucleosome structure the electrostatic charges are balanced, however the slightest distortion of the structure may result in exposure of charges and hence cytotoxic parts of histones[76]. Histones and HMGB1 directly activate TLR-2 and TLR-4, which initiates danger signaling[77,78]. Mitochondria are thought to be descendant of the *Alphaproteobacterium Rickettsia prowazekii*[79]. As a result, mitochondria may potentiate proinflammatory signals to blood cells, such as neutrophils, when they are released in the circulation[80–83]. In addition, mtDNA are released from mitochondria during autophagy or active release by platelets and induce a potent proinflammatory response as cell free DNA via TLR9[84]. DNA binding protein HMGB1 can drive inflammation and tissue repair upon release into the circulation[85].

Table 1. Damage associated molecular patterns, their neutralizer, and receptors

DAMP	Neutralizer	PRR
Nucleosome	DNase[86]	TLR-9
mtDNA	hexadimethrine bromide (HDMBr)[87]	TLR-9
Histones	Factor VII-activating protease (FSAP)[76]	TLR-2, and-4
DNA-binding proteins (HMGB1)	Heparin[88]	TLR-2, and-4
Heme	Hemoxygenase (HO)-1[89]	TLR-4

PLATELETS CONNECTS IMMUNE SYSTEM, COMPLEMENT AND COAGULATION

In circulation there is an intensive crosstalk between complement, platelets, leukocytes, coagulation, and fibrinolysis. This is a well-orchestrated network in which plasma and cellular players together elicit an efficient immune response and provide tissue homeostasis. It has been demonstrated that intrinsic coagulation pathway may act as a node for the activation of complement (e.g., FXIa reduces the capacity of CFH to enhance C3b cleavage by factor I and degradation of C3bBb and FXIIa interacts with C1q and C1-inhibitor)[90,91]. This links coagulation to innate immunity and thus may contribute to the first-line immune response against bacterial infections[90]. Conversely, (surface-bound) complement interacts with multiple coagulation factors and are able to induce thrombin generation, platelet rolling and adhesion and clot formation[92–94]. Platelet granules contain a wide range of proteins, such as cytokines, complement components and coagulation factors, that exert numerous functions among hemostasis and inflammation[1,14]. Therefore, platelets are thought to be important immune modulators[14]. Platelets and neutrophils express different complement receptors on the cell surface and can therefore be activated by complement [12,21,95,96]. Secondly, neutrophils rely on platelets for adequate neutrophil extracellular trap (NET) formation[60]. Dysregulation with subsequent activation of complement, platelets and/or coagulation results in clinical manifestations and the progression of different diseases, such as sepsis and atypical hemolytic uremic syndrome (aHUS). aHUS is a thrombotic microangiopathy that is characterized by complement-induced endothelial cell damage and platelet activation. This becomes clinically overt by microangiopathic hemolytic anemia and thrombocytopenia with subsequent formation of thrombi most predominantly in the kidney leading to renal failure. This illustrates the complexity of interrelationships between the hemostatic and immune systems and platelets and complement in particular. Thus, not only does complement influence immunothrombosis during an infection, it also mediates thrombotic microangiopathies such as HUS[97].

PLATELET TRANSFUSION AND TRANSFUSION-INDUCED ADVERSE REACTIONS

Prophylactic or therapeutic platelet transfusions are administered to hematological patients with thrombocytopenia after treatment with intensive chemotherapy. The term “therapeutic transfusion” is used to refer to transfusion of platelets to treat active bleeding and transfusion of platelets in preparation for an invasive procedure associated with a substantial risk of bleeding in case of thrombocytopenia. Factors that could favor active bleeding include trauma, fever, infection, or inflammation, coagulopathy and acquired

or inherited platelet function defect. The term “prophylactic transfusion” is applied to platelet transfusions in pancytopenic patients with severe thrombocytopenia, e.g., after high dose and/or myoablative chemotherapy, to prevent bleeding. Collection of platelets for the preparation of platelets concentrates (PC) can be divided into two methods: whole blood collection and apheresis **(Fig. 2)**. Whole blood collection can be further divided into the platelet rich plasma (PRP)- and the buffy coat (BC) method. Whole blood collection is followed by a centrifugation step to obtain PRP or BC. To obtain a sufficient number of platelets PRP and BC of different donors are pooled. Subsequently, platelets are isolated from PRP or BC by additional centrifugation. Apheresis procedure separates platelets from other blood cells during the collection based on centrifugation technique and does not require pooling of PRP or BC of different donors. PCs are commonly stored at room temperature under gentle shaking, because lower temperatures may lead to vWF receptors clustering on the membrane surface and increased modification of vWF receptors with galactose-terminated glycans [98,99]. This subsequently would promote platelet clearance by spleen- and hepatic macrophages and hepatocytes and therefore results in reduced platelet survival in the recipient[98,99]. In addition, plasma as a storage solution for platelets was recently replaced by Platelet additive solution (PAS) to minimize platelet activation and therefore improves platelet storage.

Although platelet transfusions are important measures to treat or prevent bleeding in thrombocytopenic patients, they are associated with a high incidence of transfusion related adverse reactions (AR)[100–102]. The same holds for transfusion of solvent/detergent treated plasma during therapeutic plasma exchange therapy as applied in microangiopathic hemolytic anemias, such as thrombotic thrombocytopenic purpura (TTP) or atypical hemolytic uremic syndrome (aHUS). Although therapeutic plasma exchange is an effective method for the administration of plasma proteins, it is not complete free of risk for adverse reactions. During production platelets and plasma are separated from RBC and leukocytes. However, PC always contain a residual number of leukocytes, cell debris and RBC that can cause febrile nonhemolytic transfusion reactions (FNHTR), alloimmunization, or very rarely transfusion-associated graft-versus-host disease (TA-GVHD) in some patients. Therefore, the production process of PC includes a leuko-reducing filtration step (resulting in $<1.0 \times 10^6$ leukocytes/unit), to decrease the risk of AR[103,104]. Furthermore, storage at room temperature poses a risk for bacterial contamination. Accordingly, PC undergo extensive bacterial culture testing during storage and/or pathogen reduction methods[104,105]. As mentioned above, plasma as a storage solution for PC was replaced by platelet additive solution (PAS), to besides improve storage conditions also reduce the incidence of AR. Indeed, PAS reduced the risk of AR, such as allergic reaction. However, the incidence of FNHTR remained unchanged. Several studies suggested that DAMPs, including high mobility group box 1 (HMGB1), cell-free DNA and DNA-binding proteins, are related to adverse transfusion reactions, in

particular FNHTR, by initiating a systemic inflammatory response[106–109]. Platelets convey various DAMPs, such as mtDNA and HMGB1[109–111]. Upon platelet activation HMGB1 and mtDNA are released from platelets to promote systemic inflammation[110,111]. Complete mitochondria can also be released in the circulation directly or inside microvesicles during activation of platelets to induce inflammation[111]. Furthermore, a link between increased concentrations of complement and the incidence of AR was proposed[112,113].

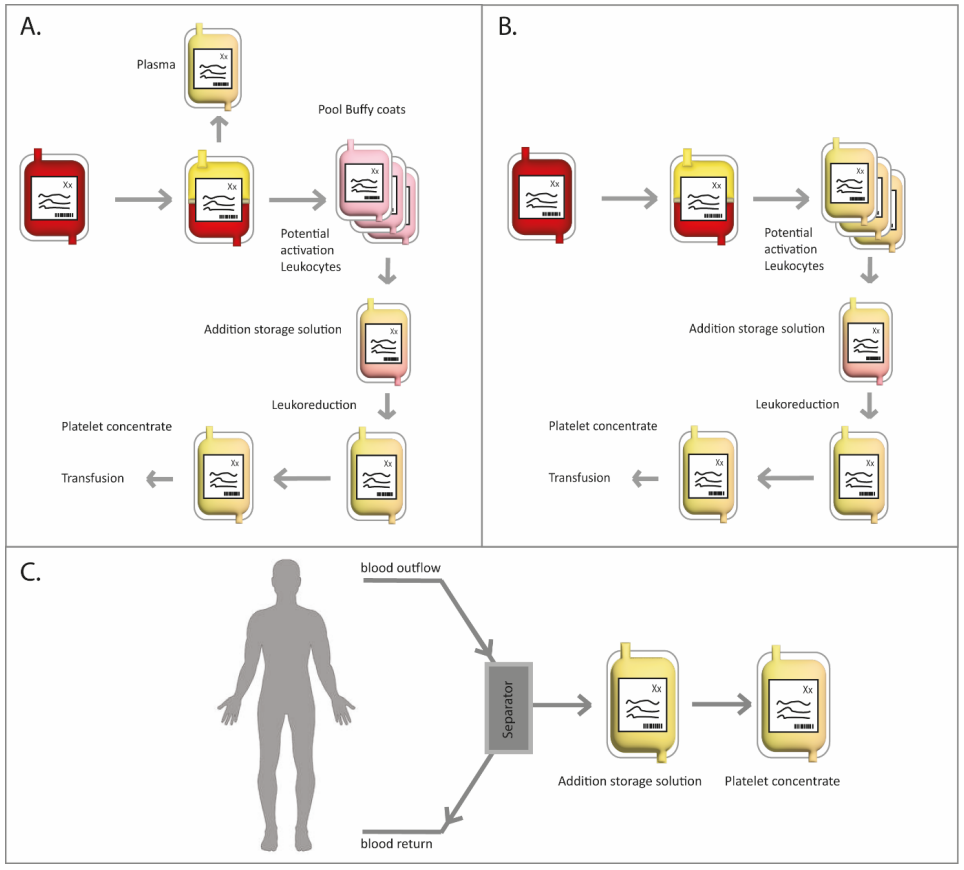

Figure 2. Schematic overview of platelet concentrate production. *Collection of platelets for the preparation of platelets concentrates (PC) can be divided into two methods: whole blood collection (A and B) and apheresis (C). Whole blood collection can be further divided into the buffy coat A)- and the platelet rich plasma (PRP) method(B).*

OUTLINE

As described in this introduction, complement, neutrophil and platelets closely collaborate in inflammation. The scope of this thesis is to study markers of inflammation and immunothrombosis in patients suffering from COVID-19 and in the context of prophylactic and therapeutic administration of blood products with a special focus on both, the blood product itself and the recipient.

Many studies have been performed on COVID-19 within the first two years of the pandemic. Thrombotic complications, because of an over-active innate response of neutrophils and platelets in the process of immunothrombosis, are described as pathological features of COVID-19. The crosstalk between platelets, complement and neutrophils in the process of immunothrombosis is reviewed in **Chapter 2**.

In **Chapter 3** we studied COVID-19 patients during the disease among different disease severities. We observed elevated neutrophil- and complement activation in severe COVID-19 patients, whereas a in mild and moderate disease activation of neutrophils and complement decreased during remission.

In **Chapter 4** we determined an increase of platelet, complement- and neutrophil activation products, and DAMPs in pooled buffy coat-derived PC during storage.

Subsequently, In **Chapter** 5 we determined that the levels of DAMPs, complement- and neutrophil activation products and platelet activation differ among several types of PC. The results of chapter 4 and 5 raised new questions regarding the pro-inflammatory capacities of complement, neutrophil- and platelet activation products and DAMPs in inducing AR in recipients. In **Chapter 6** we observed that increased levels of complement activation products, DAMPs and platelet activation are associated with AR. The results of chapters 4 to 6 combined give more insight into the role of complement, neutrophils, platelets, and DAMPs in the pathology of AR.

In **Chapter 7** we performed a pilot study that indicated that SD-treated plasma contains elevated levels of complement activation products and that after PEX aHUS patients have increased complement activation products in their circulation.

Finally, in **Chapter 8** the results of this thesis are summarized and discussed.

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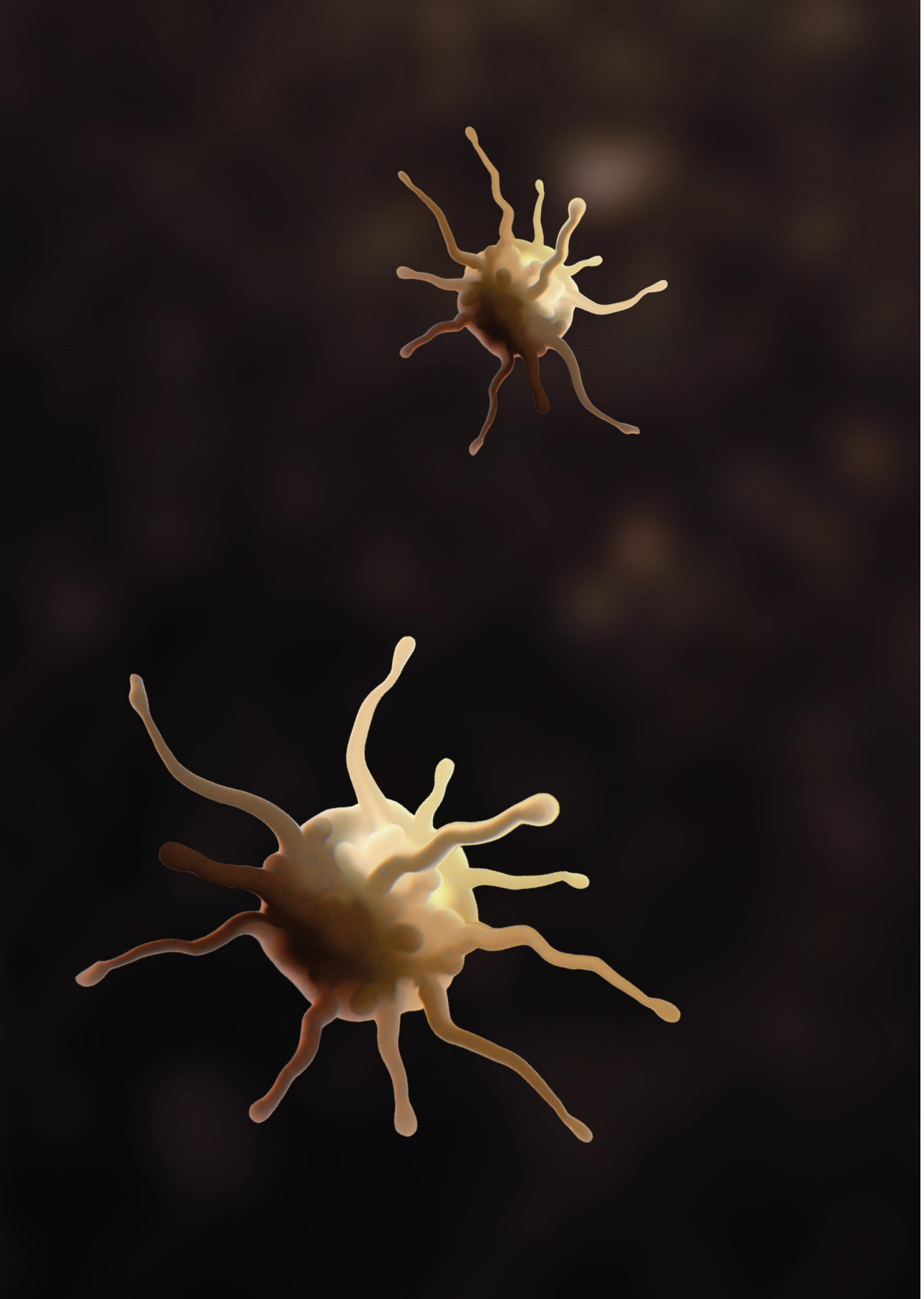

CHAPTER 2

Menage a trois in COVID-19: Platelets, Complement and NETs in immunothrombosis

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SUMMARY

Platelets are important mediators of hemostasis. Nowadays, it is increasingly acknowledged that next to their role in coagulation, platelets play a significant role in the first-line defense of the immune system. Platelets are the second-most abundant cell in the circulation and with the use of pattern recognition receptors (PRR) they can recognize pathogens and respond to it. In addition to recruiting neutrophils and other leukocytes to the area of inflammation, platelets promote coagulation and NETosis to locally confine the infection in a process called immunothrombosis. In this process there is a tight interaction between platelets, complement, neutrophils and the coagulation system. If immunothrombosis is dysregulated, it may result in thrombotic complications. Microvascular thrombotic complications are frequently observed in COVID-19 and are considerably associated to mortality. Therefore, there is a need to understand the role of platelets, complement and neutrophil extracellular traps (NETs) in immunothrombosis during SARS-CoV-2 infection. This review aims to explore the tight interaction between platelets, complement and NETosis in the setting of immunothrombosis in COVID-19.

INTRODUCTION

Platelets play a pivotal role in hemostasis and innate immunity. As part of the innate immune system they are one of the first responders to external threats[1]. Platelets contain a large range of effector proteins in their granules, such as cytokines, complement and coagulation factors, that exert numerous functions in both inflammation and coagulation[2]. Moreover, neutrophils rely for multiple functions on platelets for an effective response, such as neutrophil extracellular trap (NET) release[3]. Close and orchestrated interactions between platelets, complement, coagulation, and neutrophils are crucial for an effective first-line innate immune response. One of the mechanisms involved in trapping ("wall-off") and eliminating pathogens from the circulation is immunothrombosis, a process integrating activation of innate immune cells, plasma coagulation and platelets[4]. Because platelets are effector cells in both hemostasis and inflammation, and provide crosstalk between coagulation, complement and leukocytes, they could potentially be of concern in infectious diseases characterized by systemic inflammation, such as corona virus disease 19 (COVID-19). An important clinical feature of severe COVID-19 are microvascular complications due to immunothrombosis in the end causing organ dysfunction[5].

In this review we will first discuss insights in structure and function of platelets, and regulation of immunothrombosis, followed by the complex interaction of platelets with complement and neutrophils in COVID-19.

PLATELETS

Platelet structure and granules

Platelets are the second most abundant type of blood cell in the circulation with a concentration of 150.000-400.000 per microliter[6,7]. Platelets are anucleated cell-fragments with a diameter of 2-4 micrometer composed of granular cytoplasm[6,7]. Megakaryocytes in the bone marrow and occasionally in the lung release platelets by fragmentation. This process, called thrombopoiesis, leads to a release of over one thousand platelets per megakaryocyte. During thrombopoiesis megakaryocytes make high amounts of cytokines and growth factors, which are subsequently packaged into separate granules and distributed heterogeneously into the cytosol of produced platelets[8,9]. Platelets have three major types of granules: alpha (α) granules, dense granules and lysosomes[10]. The three types of granules undergo different patterns of release during platelet activation[10]. Platelet α-granules are the most abundant type and contain a wide variety of proteins, including adhesion proteins, coagulation factors, chemokines, and growth factors[2,10,11]. They derive their protein content by endocytosis

and biosynthesis[10]. Dense (δ) granules are involved in hemostasis, and are abundant in ADP, ATP, calcium, and serotonin[11]. Platelet lysosomes store glycohydrolases that are able to degrade glycoproteins, glycolipids and glycosaminoglycans[10]. A non-comprehensive selection of well-known platelet granule proteins is shown in ***Table 1***. Within a specific type of granule the composition of proteins is heterogenous due to uneven distribution of proteins and molecules over granules during thrombopoiesis[10]. It is believed that these heterogenous granules undergo different release kinetics during platelet activation[10]. Circulating, resting platelets have a discoid or biconvex form and their surface is smooth, which allows them to flow smoothly through veins, arteries and capillaries[7,12]. Upon activation platelets transform from discoid form into compact spheres with long filaments and protrusions with their surface covered with adhesion molecules[12]. These structural changes facilitate adhesion and aggregation of platelets[12].

Table 1 Granule content of platelets.

Name protein/molecule	Function
Adhesion	
P-selectin	Plays an essential role in the initial recruitment of leukocytes to the site of injury during inflammation.
Thrombospondin	Plays a role in angiogenesis, apoptosis, activation of TGF-beta and Immune regulation.
Von Willebrand factor	Binds to other proteins, in particular FVIII, and it is important in platelet adhesion to sites of injury.
Fibronectin	Plays a crucial role in wound healing. Along with fibrin, plasma fibronectin is deposited at the site of injury, forming a blood clot.
Growth factor	
Insulin-like growth factor	Plays a role in cell proliferation and the inhibition of apoptosis.
Transforming growth factor beta	Was first identified in platelets with a potential role in wound healing and controlling the immune system.
Platelet-derived growth factor	Plays a significant role in blood vessel formation, angiogenesis, mitogenesis, proliferation, as well as chemotaxis.
Platelet factor 4	Plays a role in wound repair and inflammation.
Hemostasis	
ADP	Interacts with a family of ADP receptors found on platelets (purinergic receptors), which leads to platelet activation and aggregation.
ATP	Regulates platelet reactivity by interacting with platelet purinergic receptors or by hydrolysis to adenosine diphosphate (ADP), which leads to platelet activation and aggregation
Calcium	Elevation in cytosolic Ca2+ concentrations is essential for platelet activation in hemostasis and thrombosis.
serotonin	Serves as a vasoconstrictor or a vasodilator while regulating hemostasis and blood clotting.

Platelet function

Platelets are commonly known for their role in coagulation and hemostasis. The classical concept of hemostasis is that upon vascular damage platelets become activated, adhere

to the site of injury, and aggregate through interactions of adhesive receptors with extracellular ligands and soluble proteins[13]. Vascular damage leads to the exposure of subendothelial collagen, von Willebrand factor (vWF) and tissue factor (TF). This leads to the generation of trace amounts of thrombin, which engages other coagulation factors and platelets. During activation (e.g., by thrombin), platelets upregulate membrane-bound receptors/adhesive glycoproteins (GP) and release soluble factors, such as fibrinogen, fibronectin, and vWF, that are stored in granules (*summarized in* ***Table 1***). These receptors and adhesive glycoproteins allow platelets to bind to coagulation factors and exposed collagen matrix on subendothelial matrix[14]. An important GP receptor on the platelet surface is the GPIb-IX-V complex, which contains the subunit GPIbα[15]. This subunit bears the binding site for vWF, P-selectin, thrombin and leukocyte integrin macrophage-1 antigen (Mac-1)[15,16]. In addition, activation by thrombin promotes upregulation of P-selectin, a receptor that plays an essential role in the recruitment of neutrophils[17]. The upregulation of these proteins leads to recruitment of leukocytes and firm adhesion of platelets to which additional platelets start to aggregate and form an initial hemostatic plug, also known as platelet thrombus. Secretion of vasoconstricting molecules, such as serotonin results in contraction of the damaged vessel. Multiple positive feedback loops of the coagulation system and in platelet activation result in a marked increase in thrombin generation with subsequent cleavage of fibrinogen to fibrin resulting in a hemostatic plug[13].

Platelet defense mechanisms

Platelets can detect and respond to local danger signals such as pathogen-associated molecular patterns (PAMPs) of infectious pathogens introduced into the bloodstream at the wound site[1,18]. To sense danger, platelets use pattern recognition receptors (PRR), such as Toll-like receptors (TLR))[1,18,19]. Platelets express a variety TLRs, such as TLR2, -3, -4, -7, and -9, enabling a quick response to danger signals, e.g. PAMPs and damage-associated molecular patterns (DAMPs)[1,18–21]. Platelets use adhesion receptors to mechanically probe their local microenvironment in order to identify adhesive substrates[22]. After firm adhesion to circulating substrates, platelets migrate and form a thrombus with the adhesive substrate and bound coagulation factors. In this manner, platelets can function as cellular scavengers, scanning the vascular surface to identify and catch potential pathogens in the circulation. Circulating platelet-pathogen aggregates activate and perpetuate polymorphonuclear leukocyte (PMN) and other immune cells, thereby promoting an inflammatory response[22]. Platelets closely interact with the complement system. Among their surface proteins, platelets contain various complement receptors, such as cC1qR, gC1qR, C3aR, and C5aR[23,24]. In addition, platelets contain plasmatic C3 and can produce C3 that is different from plasmatic C3 in its electrophoretic mobility[23]. Platelet α granules contain a large variety of pro-inflammatory/modulatory chemokines and cytokines, which allows platelets to attract and prime immune cells[25]. For example, in dengue infections, platelet-monocyte interactions skew monocyte cytokine profiles towards a proinflammatory pattern[26]. Similar

observations have been made for platelet-monocyte interactions in sterile inflammation processes, such as peripheral artery disease, and aging[27,28]. Furthermore, platelets directly interact with human immunodeficiency virus (HIV)-1 through different mechanisms such as binding, engulfment and internalization[29]. Platelets express Dendritic Cell-Specific ICAM3-Grabbing Non-integrin (DC-SIGN) and C-type lectin receptor 2 (CLEC-2), which enables interaction with HIV-1[30]. Also, platelet factor 4 (PF4) suppresses HIV-1 infection of T-lymphocytes by modulating viral attachment[31]. Although platelets are anucleate they do contain DAMPs. Mitochondrial (mt)-DNA and DNA binding proteins such as, high mobility group box 1 (HMGB1) are present in resting platelets[32–34]. Upon activation, platelets release HMGB1 and mtDNA which may induce systemic inflammation[33,34]. Mitochondria as a whole can be released into circulation or packed into micro vesicles (MV) generated during platelet activation[34]. Furthermore, platelets express HLA class I molecules for antigen presentation, costimulatory receptors (such as CD40L), and FcγRIIa receptors, which may link to the adaptive immune response [35–37]. In summary, platelets in immune defense do have an important role to recognize, bind, and eliminate infectious invaders.

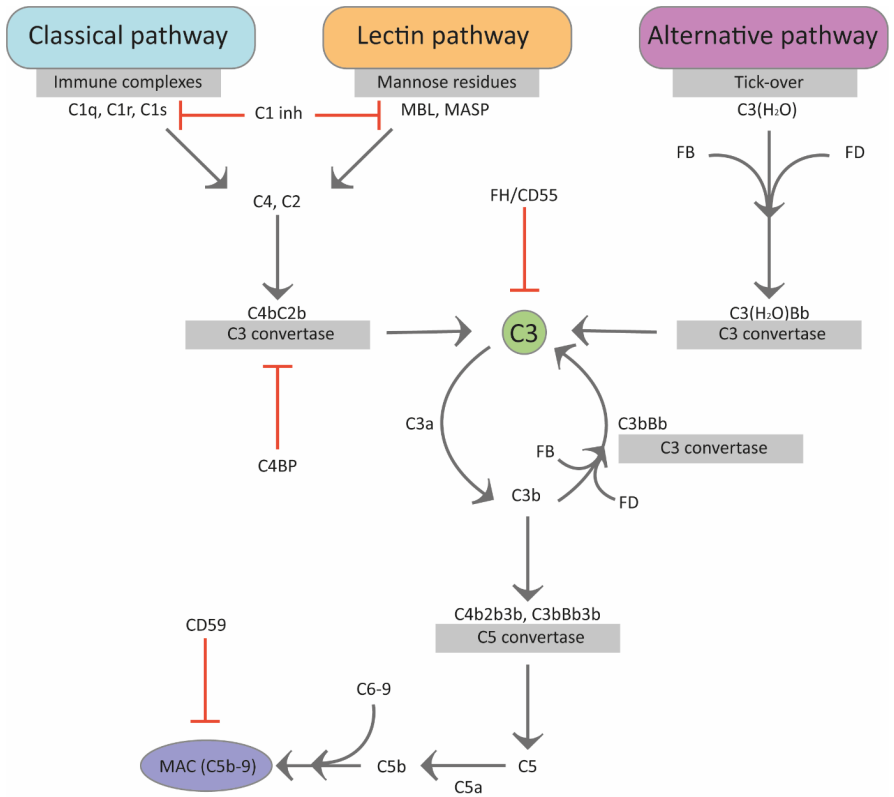

Figure 1. The complement system. *Schematic overview of the complement cascade illustrating the three activation pathways (classical, lectin, and alternative) and the membrane attack complex (MAC).*

COMPLEMENT SYSTEM

The complement system is phylogenetically an old innate defense system. Complement participates in the identification and clearance of pathogens and death cells/cell debris. Activation occurs via three distinctive pathways: the classical, the lectin and alternative pathway, that all converge in the formation of the key C3 convertase (illustrated in **Fig. 1**). The classical pathway is initiated by antibody immune complexes, to which C1q can bind with subsequent activation of the associated proteases C1s and C1r, respectively. Activated C1 cleaves both C2 and C4, creating the C3 convertase C4bC2a[38]. The lectin pathway is activated by the recognition of microbial carbohydrate structures and acetylated residues by mannose-binding lectin (MBL), collectin 11, and ficolins, which form a complex with MBL-associated serine proteases 1 and 2 (MASP-1 and MASP-2), which subsequently cleaves C2 and C4, to form the C4bC2a C3 convertase[39].

The alternative pathway maintains a low level of spontaneous activity in the fluid phase as evidenced by low level of spontaneous hydrolysis of C3 resulting in formation of C3(H_2O). Factor B binds C3b or C3(H_2O) and is subsequently cleaved by Factor D to form the C3 convertase, C3bBb[38]. This spontaneous low-level activity of the alternative pathway may finally support amplification of complement activity initiated by the classical and lectin pathways[40]. Amplification seems to play a leading role for the outcome of initial specific activation of the classical and lectin complement pathways on the terminal pathway. Recently it was shown that the alternative pathway amplification contributed to 80–90% of C5 activation after initiation by the classical pathway[40]. Due to the spontaneous low-level activity of the alternative pathway and its capacity for amplification a tight regulation is mandatory. Factor H (FH) is a key regulator of the alternative pathway in fluid phase and limits complement activation on endogenous cell surfaces[41]. In addition, C4b binding protein (C4BP) and C1 inhibitor are major inhibitors of the classical and lectin pathways[38]. Human cells express a range of complement regulators on their membrane, such as CD46, CD55 and CD58, that protect cells from complement lysis by preventing formation of the membrane attack complex (MAC)[42].

Complement acts as a first line defense against pathogens by opsonization, promoting inflammation by the generation of vasoactive and chemo attractive anaphylatoxins, as well as by direct lysis of pathogens and infected cells. Initiation of complement activation leads to cleavage of C3 to C3b and C3a by the C3 convertase. Activation is followed by opsonization, through C3b deposition on the membrane surface of the pathogen or infected/dying cell. Further activation results in the cleavage of C5 to C5a and C5b. Anaphylatoxins C3a and the more potent C5a are vasoactive and known to attract leukocytes to the site of infection and to induce downstream proinflammatory effector functions[43,44]. By opsonization and attracting immune cells, complement facilitates rapid recognition and efficient clearance

of pathogens through complement-receptors by phagocytes. Ultimately, complement activation leads to direct destruction of the pathogen or infected cell via the formation of a membrane attack complex (MAC) by C5b-9[45].

COAGULATION SYSTEM

The coagulation system comprises numerous plasma zymogens, called coagulation factors, which are sequentially activated by proteolysis finally resulting in the generation of thrombin[13]. The coagulation system consists of two distinct pathways: the extrinsic and intrinsic pathway. Classically, the coagulation system is triggered upon tissue, -or vascular damage. Circulating platelets bind to collagen and von Willebrand factor (vWF), which are released from injured endothelial cells[13]. This initiates platelet adhesion, aggregation and the formation of the hemostatic plug at the site of injury, the so called "primary hemostasis"[13]. The coagulation cascade is activated by binding of tissue factor (TF) that is released from injured cells to FVIIa (called the initiation phase)[13]. The TF-FVIIa complex proteolytically cleaves FX into FXa, to form a prothrombinase that converts prothrombin into thrombin[13](**Fig. 2**). The contribution of the classical "intrinsic pathway" to thrombin formation is still debated. It is now demonstrated that FXII plays an important role in thrombosis, but less in hemostasis[46,47]. The latter is evidenced by the fact, that congenital FXII deficiency is not associated with a bleeding tendency[47,48]. The intrinsic pathway through contact activation occurs through autoactivation of FXII exposed to anionic surfaces or endothelial collagen. Similarly, to the extrinsic pathway, FXIIa lead to the generation of FXa through the activation of FXI to FXIa with subsequent cleavage of FIX to FIXa[49]. The slowly accumulating amounts of thrombin activate platelets that have adhered at the site of injury[13]. Additionally, thrombin converts (platelet derived) FV into FVa, and FVIII into FVIIIa, which acts as a cofactor to FIXa. In addition, as a positive feedback loop thrombin converts FXI into FXIa boosting FXa and finally thrombin formation (so called amplification phase)[13,49](**Fig. 2**). Similar to the complement system, coagulation needs to be tightly regulated. Antithrombin (AT) and tissue factor pathway inhibitor (TFPI) are major inhibitors of coagulation. AT targets FVIIa, FXa, FIXa, FXIa, FXII and, to a greater extent, thrombin[50]. TFPI primarily inhibits initiation of coagulation, dampening procoagulant stimuli before thrombin is generated, via inhibition of FVIIa-TF complex and FXa[50].In addition, thrombin induces activation of protein C, which in turn - bound to thrombomodulin or endothelial-cell protein C receptor inactivates FVa and FVIIa, respectively, thereby downregulating thrombin generation[51].

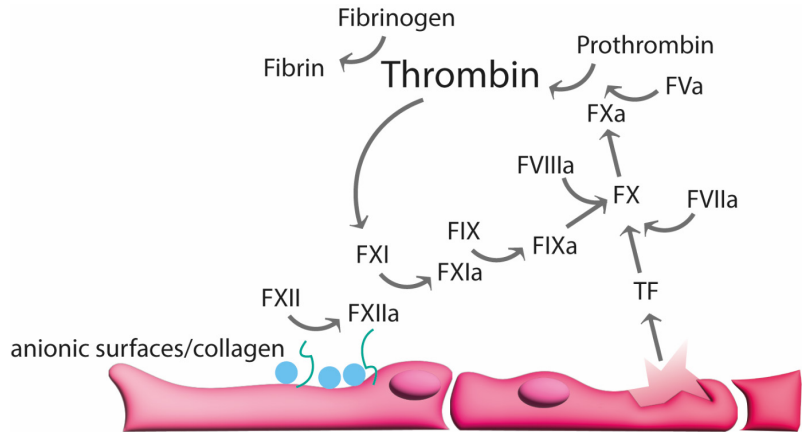

Figure 2. Simplified scheme of the coagulation cascade. *Endothelial cell damage leads to the release of TF or expression of anionic surfaces and endothelial collagen, which initiates the coagulation cascade. Following the initiation phase, prothrombin is cleaved by FXa generating small amounts of thrombin. Subsequently, thrombin forms a positive feedback loop (amplification phase).*

IMMUNOTHROMBOSIS

General aspects of immunothrombosis

Immunothrombosis defines the concurrent activation of the innate immune system, including innate immune cells, such as monocytes and neutrophils, coagulation and complement to locally confine an infection and eliminate pathogens in the circulation during infection[4]. This process requires synergistic action of platelets, NET formation upon neutrophil activation and coagulation activation triggered by TF-FVII on monocytes resulting in the release of platelet-derived microparticles (PMP) and in thrombin generation with subsequent fibrin formation. Activated neutrophils release NETs, which consist of modified chromatin fibers decorated with histones, and neutrophilic bactericidal proteins from granules and cytoplasm (a "toxic meshwork"), during a dynamic process called NETosis. NETosis plays an important role in host protection by immobilizing pathogens, including RNA-stranded viral pathogens, such as respiratory syncytial virus, influenza, dengue, and HIV[52–55]. NETs exert thrombogenic activity through the expression of TF, activation of FXII, and proteolytic neutralization of TFPI in various inflammatory diseases[56,57]. More specifically, NETs mediate fibrin deposition, promote platelet activation, disrupt local blood flow, and protect thrombi against fibrinolysis and thereby are key in the pathogenesis of immunothrombosis[58].

Role of platelets in immunothrombosis

Platelets modulate immune responses such as leukocyte migration, cytokine and chemokine secretion and TF expression by neutrophils and monocytes[19,26,59–62]. The

interaction with activated platelets facilitate neutrophil extravasation and invasion of inflamed tissues[63]. Platelets play an indispensable role during NETosis through their ability to bind to and to form aggregates on neutrophils resulting in NET release[3,59,64]. Depletion of platelets with anti-thrombocyte serum prevented the release of NETs within sinusoids in endotoxemic and septic mice[65]. Neutrophils scan the environment for activated platelets. Platelets activated by agonists, such as thrombin, stimulate NETosis in humans and mice[66]. In various thrombo-inflammatory disorders, including acute myocardial infarction, venous thromboembolism and autoimmune diseases, the formation of NETs requires platelet-neutrophil interactions[56,59]. Activated platelets and platelets trapped inside NETs release PF4, which binds to NETs, making them compact and offering protection against degradation by DNases[67].

Role of complement in immunothrombosis

Complement activation requires the presence of foreign or endogenous activating cellular surfaces for initiation of the activation pathways as part of the innate defenses against external threats[68]. Crosstalk between the complement system and neutrophils is well established[69]. During infection complement activation leads to an increase in anaphylatoxins C3a and the more potent C5a, that can activate neutrophils and platelets. Therapeutic inhibition of complement results in reduced neutrophil activation and disrupted TF expression[70–72]. Moreover, C5a promotes NET formation via induction of mitochondrial reactive oxygen species (ROS) production by neutrophils[73]. Blocking the C5a receptor, C5aR1, attenuated platelet-mediated NETosis[72]. In addition, C3 and C5 can be proteolytically activated by coagulation factors such as thrombin[74]. This may lead to amplification of complement dependent neutrophil and platelet activation. This illustrates how immunothrombosis is driven by the complement-TF-neutrophil-platelet axis (**Fig. 3**).

Complement-platelet activation

Complement can induce platelet activation. Binding of C1q, C3a (and to a lesser extent $C3a^{desArg}$) and C5a induces platelet activation[75–77]. In the other way around, platelet activation with subsequent expression of P-selectin leads to complement activation on the level of C3 and further downstream propagation through C5 activation[78]. These findings point to an important role for complement activation at the site of platelet adhesion and aggregation, to support local NETosis and hence immunothrombosis. In addition, activated platelets release extracellular vesicles (EV), e.g. PMP, which may activate complement, coagulation, and inflammation in a phosphatidylserine (PS)-dependent manner by exposure of PS on the surface[79–82]. Extracellular vesicles (EVs) released upon activation of immune cells and platelets during inflammation induce a hypercoagulable state, as seen in sepsis and thrombosis[83,84]. Given the negative charge of PS calcium-dependent coagulation factors can assemble on the EV surface, thereby forming a tenase and/or prothrombinase complex with subsequent thrombin formation[85]. In addition, complement C3 and C1q may bind to

PS resulting in the activation of the alternative and classical complement pathways[80,86,87]. In addition, complement activation resulting in the formation of C5b-9 on platelets induce the release of PMPs from platelets, thereby reinforcing coagulation as PMPs provide a surface for the conversion of prothrombin to thrombin[88].

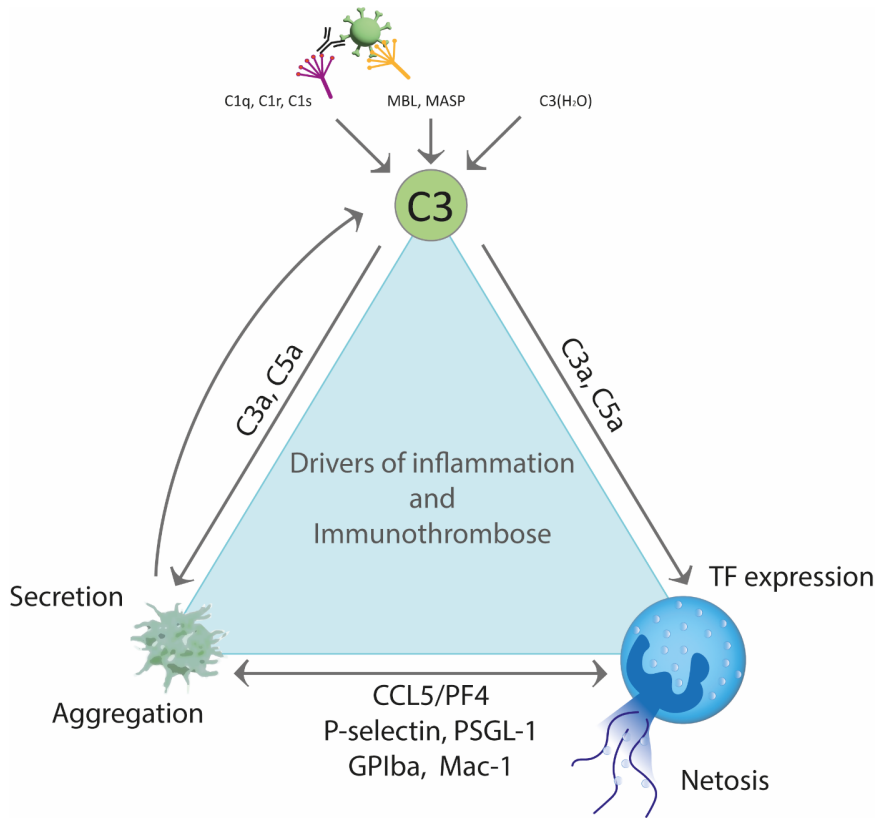

Figure 3 Menage a trois: interaction between complement, platelets and neutrophils. *The interaction of complement, platelets and neutrophils and the consequences of this interaction. Shown are the major receptor–ligand couples involved in the platelet–neutrophil interaction (P-selectin–PSGL1 and GPIbα–Mac-1) and cytokines that enhance leukocyte activation (CCL5 and PF4). Downstream effects of the platelet–neutrophil interaction include increased activation of coagulation via tissue factor (TF), subsequent platelet aggregation, and generation of neutrophil extracellular traps (NETs).*

Dysregulation of immunothrombosis detrimental to disease outcome

Immunothrombosis has a significant role as a first defense of innate immunity to protect the organism from invading pathogens, e.g., bacteria, fungi, and viruses by walling-off[89,90]. The process of immunothrombosis by walling-of and lysis results in elimination of the invading microorganism. However, immunothrombosis may also harm, especially

when the process is initiated at unwanted sites and/or by non-infectious stimuli as seen in diseases with systemic inflammation, e.g., autoimmune diseases (e.g., Systemic Lupus Erythematosus), acute lung injury (e.g., respiratory distress syndrome ARDS) and sickle cell disease[91–93]. Way too much immunothrombosis and/or dysregulation of the resolution of immunothrombosis may cause harm as well. Host DNases preserve microvascular patency and prevent vascular occlusion by degrading intravascular NETs[94]. A defect to degrade NETs is associated with microvascular occlusions in patients with severe bacterial infections[94]. Excessive NET-induced coagulation during sepsis may exacerbates tissue injury by triggering and promoting microvascular occlusion and Disseminated intravascular coagulation (DIC) with subsequent organ hypoperfusion resulting in organ dysfunction[95]. Neutrophils recruited at the site of inflammation due to bacteria or viruses modulate lung pathology through the release of NETs and extracellular histones, which results in turn in the activation of platelets with the subsequent formation of pulmonary microvascular thrombosis[96, 97].

CORONA VIRUS DISEASE 2019 (COVID-19)

General aspects

Corona virus disease 2019 (COVID-19) is caused by an infection with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The disease is characterized by flu-like symptoms and viral pneumonia, but occasionally may progress to acute respiratory distress syndrome (ARDS) and multiple organ dysfunction syndrome (MODS)[98]. Vascular complications are considered to cause organ dysfunction, since postmortem analysis revealed microvascular thrombosis in organs of patients died from COVID-19[99,100]. Microvascular complications are also caused by direct invasion of endothelial cells by SARS-CoV-2 with subsequent lysis, propagating microvascular thrombosis in the microcirculation.[101–103].

Platelet dysfunction in COVID-19

Severe thrombocytopenia without bleeding complications together with increased levels of D-dimers (a fibrin degradation product after a blood clot is degraded by fibrinolysis) are frequently observed in patients with severe COVID-19 and associated with poor prognosis[104–106]. In COVID-19 patients with progressive disease, as reflected by systemic inflammation, P-selectin and CCL5 levels are increased[107,108]. PF4, which is able to protect NETs from degradation by DNase I, is greatly elevated in COVID-19[96,109]. The increase in P-selectin and PF4 are considered indicators of poor prognosis in COVID-19[110]. Interestingly, platelets in COVID-19 patients show a procoagulant phenotype as characterized by assemblage of coagulation factors on their surface and having catalytic activities[111]. This procoagulant phenotype of platelets is either acquired in the peripheral

circulation or upon release from proinflammatory megakaryocytes[8,112]. COVID-19 patients exhibit significantly higher levels of circulating PMP and EVs as compared to SARS-CoV2 negative hospitalized patients and healthy controls, respectively [113,114]. Half of the total circulating EVs in COVID-19 patients were derived from platelets[114].

Plasma proteomic analysis in COVID-19 patients uncovered numerous alterations of plasma proteins[115]. Unbiased pathway-enrichment analysis identified platelet degranulation and complement activation as pathophysiological pathways that likely contribute to the pathogenesis of SARS-CoV-2 infection[115]. A retrospective observational study revealed that history of macular degeneration and coagulation disorders (thrombocytopenia, thrombosis and hemorrhage) are risk factors for SARS-CoV-2-associated morbidity and mortality[116]. Furthermore, candidate-driven genetic association analysis demonstrated that putative complement and coagulation-associated loci were correlated with COVID-19 severity[116]. Together, these results indicate that platelets in concert with the complement system play a critical role in inflammatory processes and that platelet dysfunction is key to the pathogenesis of severe COVID-19.

Hyperactivation and hyperinflammation of platelets

Hypercoagulability, and platelet activation are well-recognized features in COVID-19 patients. Autopsies on lungs of COVID-19 patients exhibited severe coagulation abnormalities, immune cell infiltration, and platelet activation[117]. Platelets in COVID-19 patients are pre-activated and hyperreactive[109,118–120], as evidenced by increased P-selectin expression on circulating platelets in COVID-19 patients[111,118]. In addition, plasma from severe COVID-19 patients induced activation of platelets from healthy volunteers *ex vivo*[121]. Plasma levels of platelet-derived sCD40L are significantly elevated in the early stages of the disease[122]. Interestingly, the plasma levels of sCD40L decreased overtime while that of sP-selectin significantly increased, indicating sCD40L to be a specific marker for early-stage severe COVID-19 [122]. Even though P-selectin is already expressed on circulating platelets, activation can still lead to additional expression of proteins that are stored in α-granules[109,111,118]. In addition, some cytokines/chemokines (e.g. IL-5, IL-13, IL-22, and IL-31) are only found in releasate when stimulated *ex vivo* and not in plasma of COVID-19 patients[123]. This demonstrates that circulating platelets in COVID-19 patients are only partly degranulated and that these platelets may still be able to locally deliver cytokines and chemokines.

Besides the hyperactivated state platelets from COVID-19 patients exhibit a more pro-inflammatory phenotype, as compared to platelets of healthy controls, evidenced by the transcriptional profile assessed by transcriptomics[118]. Platelets from COVID-19 patients are hyperresponsive to *in vitro* agonist stimulation due to altered gene expression[118]. Furthermore, platelets show increased interaction with leukocytes during a SARS-CoV-2

infection[118]. Upon stimulation with thrombin, platelets of COVID-19 patients released increased amounts of cytokines (IL-1α, IL-1β, IL-1RA, IL-4, IL-10, IL-13, IL, 17, IL-27, IFN-α, and IFN-γ), chemokines (MCP-1/CCL2), and growth factors (VEGF-A/D) as compared to controls[111]. Thus, platelets are primed to conduct proinflammatory and procoagulant activities in the circulation during COVID-19 pneumonia. Moreover, high levels of sP-selectin at hospital admission correlated with IL-6, CRP and LDH, which are clinically used as indicators of pneumonia severity and disease progression[124].

The finding that platelets in COVID-19 patients have a more proinflammatory phenotype may also be the result of inflamed megakaryocytes. A substantial proportion of the platelet biogenesis takes place in the lung[9]. A high density of alveolar megakaryocytes are found in inflamed areas of SARS-CoV-2 infected lungs[125]. An infected megakaryocyte or a megakaryocyte in an inflamed area may therefore produce platelets with a different transcriptome[123]. Moreover, detailed postmortem histology analysis identified a marked increase of naked megakaryocyte nuclei in the bone marrow and lungs in critically ill patients[126]. This confirms that increased platelet biogenesis takes place in the lungs of patients with severe COVID-19. Most probably, megakaryocytopoiesis is promoted by elevated levels of IL-6 as seen during severe systemic inflammation. IL-6 is causally linked to increased thrombopoiesis through promoting thrombopoietin (TPO) transcription[127]. A peak in IL-6 levels is observed in critically ill COVID-19 patients at the time of intubation/ intensive care unit admission and this is 5 days later followed by an increase in platelet count[106]. The local production of hyperactivated platelets in inflamed areas of the lungs, may induce a hypercoagulability state prone to abnormal immunothrombosis.

Platelets interact with SARS-CoV-2

The activation and hyperresponsiveness of platelets during infection is multifactorial: (1) excessive systemic inflammation due to complement activation and/or cytokine release may occur and activate platelets[128]. (2) Viruses, such as Dengue virus, influenza virus and HIV, have also been identified to activate platelets directly via TLR[20,129,130].

In some patients, the presence of SARS-CoV-2 RNA in platelets is documented[119]. This suggests that SARS-CoV-2 can at least interact with and occasionally invade platelets. However, which platelet receptors is engaged in this interaction remains debated. SARS-CoV-2 requires binding to the angiotensin converting enzyme-2 (ACE2) to enter cells. Although some studies report ACE2 expression on platelets and megakaryocytes, most studies fail to detect the presence of this receptor on protein- or mRNA level[118,119]. For the fusion and entry of SARS-CoV-2 cleavage of the S protein of SARS-CoV2 by the cellular serine protease TMPRSS2 is needed. TMPRSS2 expression was also not detected in platelets, although this has not been extensively studied so far[118]. Meanwhile, there have been studies that identified CD147 (basigin) as a potential receptor for SARS-CoV-2 in platelets[131–133]. CD147 is believed to play an important role in other viral infections, such as HIV-1, hepatitis C virus, hepatitis B virus, Kaposi's sarcoma-associated herpesvirus, measles, and SARS-CoV since it mediates the activation of platelets during infection[133,134]. Once viral RNA is present inside the platelet endosomal compartments it may activate platelets via TLR-7[20]. Another possible explanation for the presence of SARS-CoV-2 RNA in platelets is pinocytosis. Platelets continuously sample their surrounding thereby adsorbing or internalizing plasma proteins, such as fibrinogen. It is plausible that platelets, passively adsorb viral particles as part of a first-line surveillance independent of receptor binding. At the same time, the ACE2 receptor can be shed from cells and might also be taken up by platelets[135]. This could explain why some studies have detected the ACE2 receptor in platelets and others have failed.

SARS-CoV-2 infection significantly modifies the coding and non-coding transcriptional profile and function of platelets[136]. SARS-CoV-2 alters the energy metabolism of platelets and thereby promotes thrombus formation[136]. Integrative network biology analysis of circulating platelets from COVID-19 patients revealed a transcriptional landscape, in which four key subnetworks and 16 regulators underly SARS-CoV-2 infection[136]. Platelet activation, immune response pathways, and four risk genes (upstream binding transcription factor, RNA polymerase II, I and III subunit L, Y-box binding protein 1 and yippee like 2) were significantly affected by SARS-CoV-2 and associated with severe COVID-19[136].

NETs and complement during immunothrombosis in COVID-19

One distinct characteristic of SARS-CoV-2 infection is a severe systemic inflammatory response, characterized by an exuberant innate response and a delayed adaptive immune response[137]. Activation of the complement system, as evidenced by marked expression of C3, sC5b-9, and C3aR in lung epithelial cells, is a frequently observed feature of COVID-19 and the intensity of activation correlates to disease severity[138]. Furthermore, increased levels of anaphylatoxins C3a and C5a and decreased levels of C1 inhibitor are correlated with increased levels of D-dimers in COVID-19 patients[139]. This increase in anaphylatoxins in combination with the direct damage due to the invasion of endothelium by SARS-CoV-2 may lead to excessive platelet and neutrophil activation contributing to the subsequent formation of microvascular thrombosis in the lung (**Fig. 4**).

High neutrophil levels measured in the peripheral blood are an early predictor of a moderate to severe ARDS in SARS-CoV-2 infection[140]. In severe SARS-CoV-2 pneumonia, dysregulated immunothrombosis is key in the development of ARDS and systemic hypercoagulability[106]. In pulmonary autopsies, the presence of inflammatory NET-containing microthrombi with platelets and fibrin was demonstrated[96,106]. Elevated plasma levels of Myeloperoxidase (MPO)-DNA complexes and citrullinated histone H3 as markers for NETs are present in COVID-19 patients and correlate with disease

severity[96,141,142]. Neutrophil-platelet aggregates and a distinct neutrophil and platelet activation pattern are identified related to the stage and disease severity in COVID-19 patients[106]. As mentioned earlier NETs have high procoagulant potential through platelet-dependent mechanisms which may promote pulmonary thrombosis in SARS-CoV-2 infection[143]. Formed NETs and pulmonary microthrombi can further exacerbate the respiratory insufficiency in SARS-CoV-2 infection, as a result of disturbance of the blood flow and additional damage to tissue. Therefore, hyperactivation of neutrophils and platelets may result in NETosis-induced coagulation and platelet aggregation[106].

Elevated levels of HMGB1 are observed in systemic conditions with coagulation activation, such as sepsis and COVID-19[144,145]. Upon platelet activation HMGB1 is expressed on the cell surface and secreted[146]. Activated platelets are an important source of HMGB1 within thrombi[33]. Besides activated platelets, HMGB1 promotes also the induction of NETs with subsequent microvascular thrombosis, thereby serving as a link between inflammation and coagulation[147,148].

Autopsy reports identified significant amounts of intravascular TF in the lungs of COVID-19 patients[149]. Furthermore, plasma levels of NETs, TF and sC5b-9, which are key drivers of immunothrombosis, are also increased in patients[150]. Neutrophils express high levels of TF and release NETs bearing active TF[150]. *In* vitro treatment of control neutrophils with COVID-19 platelet-rich plasma promoted the formation of NETs carrying TF, which holds thrombotic activity[150].

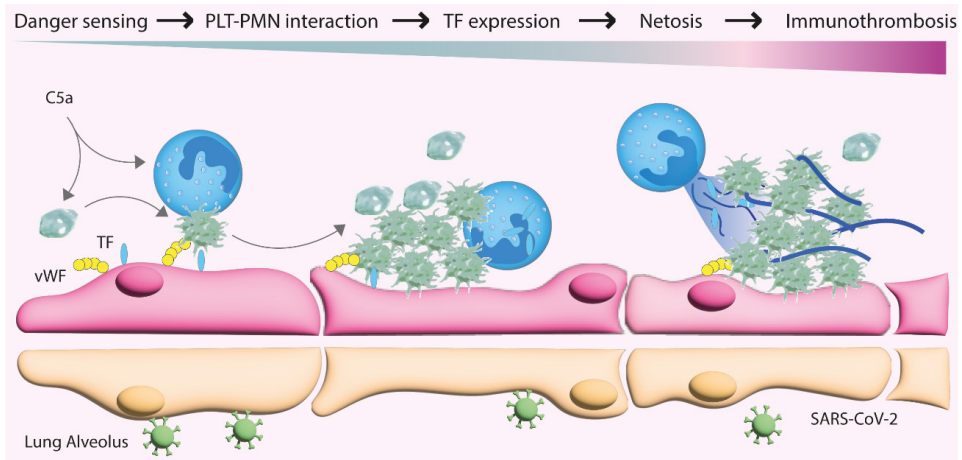

Figure 6 Immunothrombosis induced by SARS-CoV-2. *SARS-CoV-2 infection of alveolar endothelial cells results in complement and platelets activation and simultaneously vWF and TF are secreted from the damaged cells. Complement and platelets activate Polymorphonuclear neutrophils (PMN), which form aggregates with platelets on the surface of damaged endothelial cells. Neutrophils stimulated by complement increase TF expression and activated platelets promote neutrophils to form NETs. NETs then activate additional platelets and bind fibrin, resulting in the formation of a robust immunothrombus.*

Role of platelets immunothrombosis in COVID-19

Platelets can release effectors of immunothrombosis, including PF4 and HMGB1[33,151]. Considering the above, a platelet-NET-TF or complement-platelet-NET-TF axis as a potential mechanism for immunothrombosis may prevail and may be responsible for subsequent sepsis-induced lung injury[66]. In COVID-19 patients levels of sP-selectin, sE-selectin and sL-selectin increase with disease severity[124]. sP-selectin levels positively correlated with D-dimers in these patients[124]. Additionally, sP-selectin levels correlate with VWF levels in COVID-19 patients[124]. Notably, high sP-selectin levels at hospital admission have shown to be early predictors of thrombosis in COVID-19 patients[124]. These data suggest an important role of SP selectin in immunothrombosis. Previously, it was shown *in vitro* that thrombin-activated platelets induced TF-enriched NET formation and subsequent immunothrombosis in ARDS patients[152]. In addition, TF-enriched NETs in ARDS patients could induce thrombin generation[152]. Given the indispensable role of platelets in the process of NETosis and the presence of hyperactive, procoagulant platelets in COVID-19 patients, these platelets may accelerate NET formation and through PF4 release prevent NET degradation by DNase I, resulting in progressive immunothrombosis.

CONCLUDING REMARKS

Severe COVID-19 is characterized by an overwhelming innate immune response with the activation of innate immune cells and plasma protein systems resulting in vascular complications. Direct infection of endothelial cells and microvascular thrombosis finally lead to organ dysfunction. This menage a trois between complement, platelets and neutrophils forms the base for immunothrombosis, which is considered to play a significant role in this process including an orchestrated interplay of neutrophil activation in the form of NETs, hyperactivated platelets, complement activation and coagulation activation paralleled by the neutralization of anticoagulants. Hyperactivated platelets with an “pro-inflammatory” signature are key in this process- catalyzing NETosis and promoting the procoagulant state in the microvasculature. Furthermore, hyperactivated platelets offer a template for coagulation- as well as complement activation and release procoagulant mediators, e.g., PMP and inhibitors of DNAse1, thereby preventing NET degradation. Therapeutic strategies to “calm” hyperactive platelets and to neutralize their proinflammatory mediators should the focus of future research.

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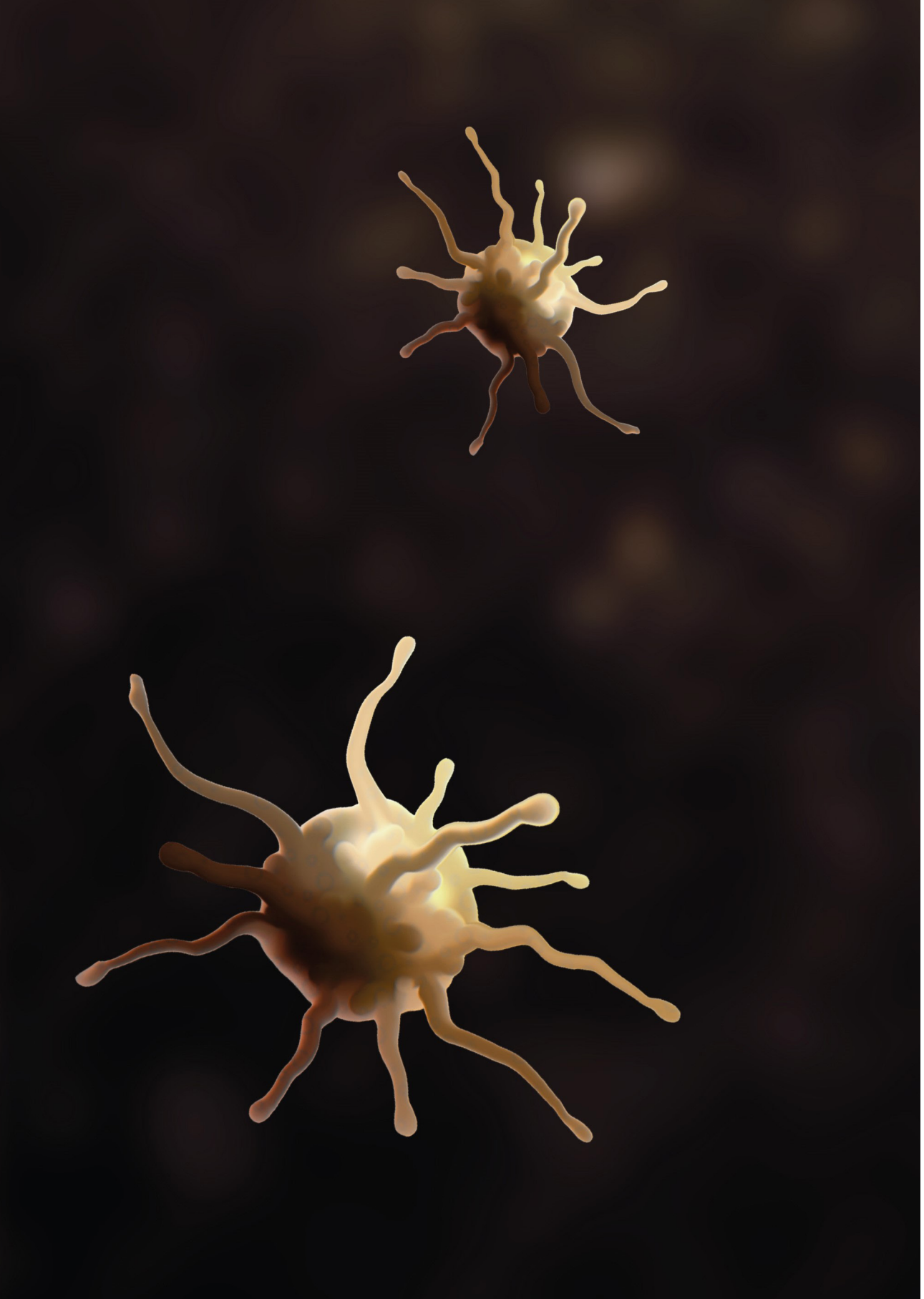

CHAPTER 3

Devils Dance: Complement, NETs, and Thrombosis in COVID-19

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ABSTRACT

Severe COVID-19 is characterized by systemic inflammation and multiple organ dysfunction syndrome (MODS). Arterial and venous thrombosis are involved in the pathogenesis of MODS and fatality in COVID-19. There is evidence that the complement- and neutrophil activation in the form of neutrophil extracellular traps are the main drivers to develop microvascular complications in COVID-19. During the two first waves of COVID-19, before the availability of SARS-CoV-2 vaccination, plasma and serum samples were collected from 83 patients infected by SARS-CoV-2 with differing severity of disease (ranging from mild to severe) at enrollment day, day 11, and day 28. In this comprehensive study, we measured cell-free DNA, neutrophil activation, deoxyribonuclease 1 activity, complement activation, and D-dimers in longitudinal samples of COVID-19 patients. We show that all the above markers, except deoxyribonuclease 1 activity, increased with disease severity. Moreover, we provide evidence that in severe disease there is continued neutrophil and complement activation, as well as D-dimer formation and nucleosome release, whereas in mild and moderate disease all these variables decrease over time. These findings suggest that neutrophil and complement activation are important drivers of microvascular complications and that they reflect immunothrombosis in these patients. Neutrophil activation, complement activation, cell-free DNA and D-dimer levels are reliable biomarkers for disease severity and fatality in COVID-19. They might also serve as suitable markers with which to monitor the efficacy of therapeutic interventions in COVID-19.

Keywords

Cell-free DNA**,** Complement activation, COVID-19, Neutrophil extracellular traps, Thrombosis.

INTRODUCTION

Infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes corona virus disease 2019 (COVID-19). The disease presents with flu-like symptoms and viral pneumonia, which may progress to acute respiratory distress syndrome (ARDS) and occasionally to multiple organ dysfunction syndrome (MODS)[1]. Arterial and venous thrombosis contribute to ARDS, MODS, and fatality in COVID-19[2-4]. Before the availability of SARS-CoV-2 vaccine, specific anti-viral drugs and therapeutic neutralizing monoclonal antibodies, 14.5% of COVID-19 patients developed severe systemic inflammation with subsequent organ failure despite virus clearance[1]. Based on postmortem analysis, microvascular complications are a main driver of the pathophysiology of organ dysfunction in COVID-19[5,6]. This is illustrated by the finding that SARS-CoV-2 may directly invade endothelial cells, with subsequent lysis, and induce microthrombosis, especially in the lungs[7-9]. Other studies have linked systemic complement activation in COVID-19 patients to respiratory failure[10] and described this as a distinct feature of COVID-19[11]. Furthermore, uncontrolled complement activation at the level of C3 has been shown to correlate with disease severity and mortality[12,13]. Indeed, postmortem analyses of lung specimens of patients who died from COVID-19 demonstrate deposition of MASP-2, C4d, C3d, and C5b9 in the intra-alveolar septa and in the microvasculature, consistent with the activation of the lectin pathway and alternative pathway, respectively[14]. Interestingly, these patients' biopsy specimens of purpuric lesions showed thrombogenic vasculopathy with deposition of C4d and C5b9[14].

High neutrophil:lymphocyte ratio and D-dimer levels are predictors of death in patients with severe COVID-19[15]. In addition, SARS-CoV-2 infection induces neutrophil influx in the nasopharyngeal epithelium and the lungs and increases their count in peripheral blood[16-18]. These data suggest a prominent role for neutrophils in the pathogenesis of microvascular complication with subsequent organ dysfunction. Indeed, neutrophil activation in the form of neutrophil extracellular traps (NETs) plays an important role in the pathogenesis of microvascular complications, since NETs are a main driver of immunothrombosis[19,20]. NETs are present in obstructed vessels in lungs from autopsies of COVID-19 patient studied by immunohistochemistry[21]. In addition, elevated markers of NETs can be detected in the plasma of COVID-19 patients, and these markers significantly correlate with D-dimers, pointing to a role for NETosis in the pathogenesis of ARDS and thrombosis[22,23]. Markers for NETs in COVID-19 patients increase with disease severity[24] and are involved in several affected organs[25]. Interestingly, C3-deficient mice infected with SARS-CoV have less respiratory distress, with a significant attenuation of the inflammatory response and less neutrophil infiltration, emphasizing not only the role of complement and neutrophils in COVID-19, but also linking complement activation to neutrophil activation [26]. NET degradation by deoxyribonuclease I (DNase

1) is an essential step in maintaining microvascular patency during inflammation[27-29]. Compared with healthy controls, DNase 1 activity was found to be significantly increased in COVID-19 patients; nevertheless, COVID-19 patients with sepsis had decreased DNase 1 activity compared with healthy volunteers. In the same study, COVID-19 patients' plasma cell-free DNA levels and NET formation were reduced by adding DNase 1[30].

The strength of our current study is that we comprehensively investigate the role of complement, neutrophil and coagulation activation in the pathogenesis of systemic inflammation in a unique cohort of COVID-19 patients included during the first two waves of COVID-19 before any vaccination was available. In detail, we studied the main drivers of the innate immune response involved in the development of microvascular complication with these markers: neutrophil activation in the form of NETs, DNase 1 activity, cell-free DNA (e.g. nucleosome and mitochondrial DNA forms), complement activation and D-dimer levels.

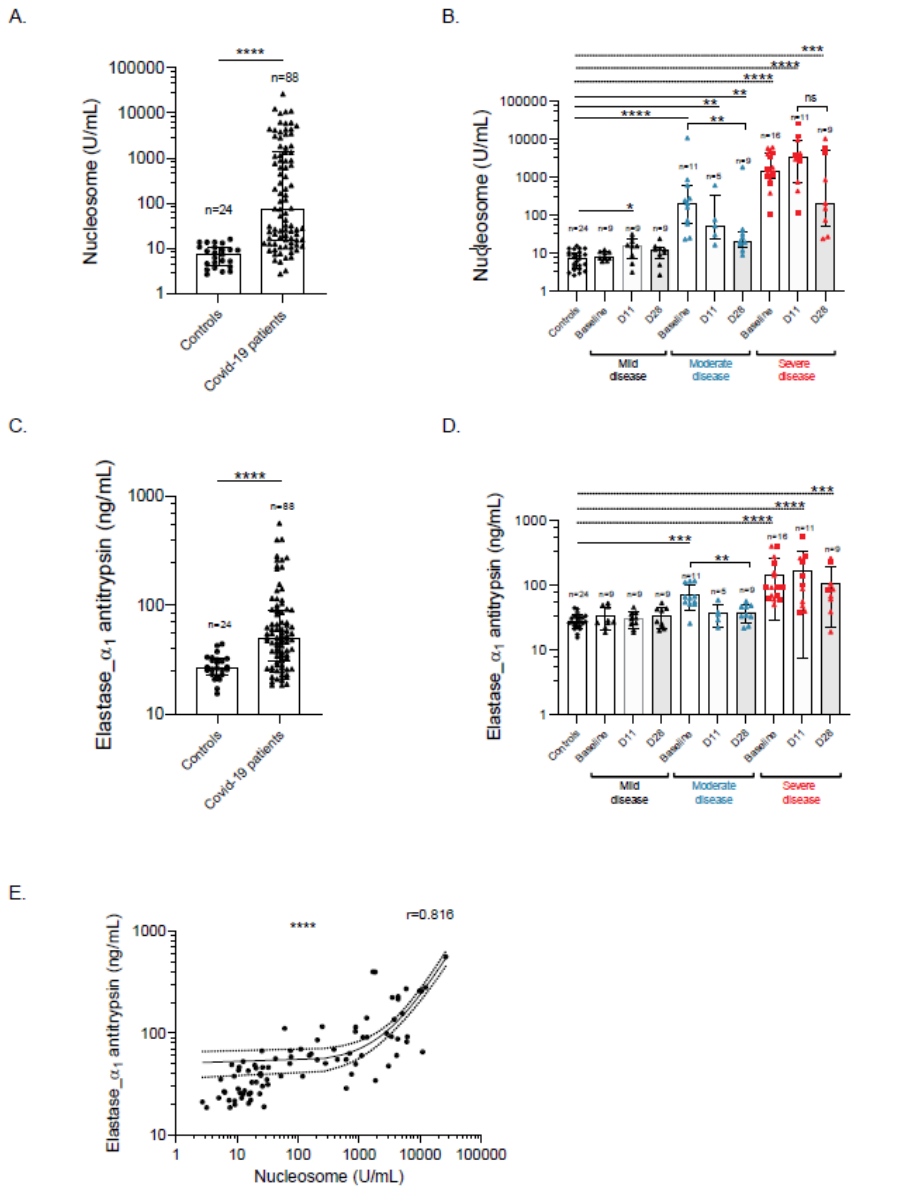

Fig. 1. Nucleosome release and neutrophil activation in COVID-19 patients. *Nucleosome levels at baseline in COVID-19 patients (n=36) and in controls (n=24) (a), Nucleosome levels in patients with different severity levels of COVID-19 at different time points and in controls (b), Elastase–α1-antitrypsin complex levels in patients with COVID-19 included at the baseline (n=36) and in controls (n=24) (c), Elastase–α1-antitrypsin complex levels in patients with different levels of COVID-19 disease severity at different time points and in controls (d), Correlation between elastase–α1-antitrypsin complexes and nucleosome levels (e). Plasma from healthy donors (controls) and from SARS-CoV-2–infected patients with different disease severities (mild, moderate, and severe) were analyzed at different time points: baseline corresponding to the enrollment day; 11 ± 3 days after enrollment (D11); and 28 ± 7 days after enrollment (D28). Squares represent patients who died. Data are presented as median ± interquartile range. The Spearman rank correlation was determined. Statistical significance was set at p<0.05.*

METHODS

Study Design and Participants

We prospectively included 87 patients with PCR-confirmed SARS-CoV-2 infection in the two first waves of COVID-19 before vaccination against SARS-CoV-2 was available from March 5, 2020, to December 15, 2020, at the Inselspital in Bern. Blood samples were collected from 83 patients at the time of enrollment (baseline), at day 11 ± 3 (D11), and at day 28 ± 7 (D28). Using this approach, the patients served as their own control. It is important to specify that samples were not collected at all time points for all the patients. COVID-19 disease severity was categorized according to the *COVID-19 WHO Ordinal Scale for Clinical Improvement* (WHO COVID-19 synopsis 2020)[31]. Patients with a score of 1–2 (outpatients) were defined as having mild disease, whereas patients with scores of 3–4 (admitted patients with or without oxygen by mask of nasal prongs) and 5–8 (non-invasive ventilation, high-flow oxygen, mechanical ventilation, extracorporeal membrane oxygenation, death) were defined as having moderate or severe disease, respectively. This study is part of a larger COVID project (NCT04510012) and data of some patients were previously analyzed regarding immune functionality and neuro-axonal damage[32,33]. The study was approved by the Ethics Committee of the Canton of Bern, Bern, Switzerland, Nr. 2020-00877 and registered at clinicaltrials.gov (NCT04510012). Patients were included after provision of informed consent. In case of lack of capacity and/or inability to provide consent, enrollment followed the procedures for research projects in emergency situations according to Swiss law.

Blood Collection from patients with COVID-19

Blood was collected from 37 patients, with median aged at 58 [36-72] years involving 8 females and 29 males, in S-monovette® EDTA tubes (2.7 mL tubes, Sarstedt, Germany) and in S-monovette® citrate tubes (3 mL tubes, Sarstedt, Germany) using a butterfly needle, from patients infected by SARS-CoV-2. For one COVID-19 patient at the baseline point, corresponding to enrollment day, the blood was not collected whereas it has been at D11 and D28. The tubes were centrifuged once for EDTA tubes, and twice for citrate tubes, for 15 minutes at 2500g. Plasma was stored in aliquots at-70°C.

For serum samples, blood was collected from 72 patients, with median aged at 49 [31-63] years involving 26 females and 48 males, in S-Monovette® serum tubes (9 mL, Sarstedt, Germany) and left at room temperature for 30 min for complete coagulation. The tubes were then centrifuged at 2000g for 10 min at room temperature to collect the serum. Serum was stored in aliquots at-70°C. See the samples repartition in the study in Fig. 1.

Table 1. Clinical features of patients with COVID-19

	All COVID-19 patients (n=87)		**Mild disease** (n=34)		**Moderate disease** (n=24)		**Severe disease** (n=29)	
	Total number	%	Total number	%	Total number	%	Total number	%
Gender								
Female	27/87	31	16/34	47.1	5/24	20.8	7/29	20.6
Male	60/87	69	18/34	52.9	19/24	79.2	23/29	79.3
Age group								
18-49 yr	38/87	43.7	29/34	85.3	6/24	25	3/29	10.3
≥50 yr	49/87	56.3	5/34	14.7	18/24	75	26/29	89.6
Median age – range *(years)*	*54 - [22 to 86]*		*31 - [22 to 71]*		*62.5 - [22 to 84]*		*67- [32 to 86]*	
Symptoms								
Fever	50/83	60.2	13/34	38.2	17/24	70.8	19/25	76
Rhinorrhea	33/72	45.8	23/34	67.60	6/24	25	4/14	28.6
Sorethroat	26/73	35.6	20/34	58.80	4/24	16.7	2/15	13.3
Cough	61/80	76.3	22/34	64.70	20/24	83.3	20/23	86.90
Dyspnea	34/83	41	7/34	20.60	8/24	33.3	19/23	82.6
Myalgia	40/72	55.5	25/34	73.5	8/24	33.3	7/14	50
Nausea	4/72	5.5	1/34	2.9	1/24	4.2	2/14	14.3
Diarrhea	18/73	24.6	8/34	23.50	7/24	29.20	3/15	20
Anosmia	2/2	100	2/2	100	-	-	-	-
Comorbidities								
All comorbidities	47/88	53.4						
Diabetes	15/87	17.2	0/34	0	5/24	20.8	10/29	34.5
Cardiovascular	25/87	28.7	0/34	0	9/24	37.5	15/29	51.7
Hypertension	26/87	29.9	0/34	0	10/24	41.7	16/29	55.2
Pulmonary	10/87	11.5	5/34	14.7	3/24	12.5	2/29	6.9
Immune	04/87	4.6	0/34	0	1/24	4.2	3/29	10.3
Malignancy	8/87	9.2	1/34	2.9	2/24	8.3	5/29	17.2
Kidney	7/87	8.0	0/34	0	0/24	0	7/29	24.1
Other	19/87	21.8	0/34	0	10/23	43.5	9/29	31.0
At least 2 comorbidities	33/88	37.5	1/34	2.9	12/23	50	19/29	65.5
Other information								
Hospitalized	54/87	62.1	1/34	2.9	24/24	100	29/29	100
ICU at enrollment	28/87	32.2	0/34	0	2/24	8.3	26/29	89.6
ICU at any time	32/87	36.8	0/34	0	3/24	12.5	29/29	100
Death	10/87	11.5	0/34	0	0/24	0	10/29	34.5

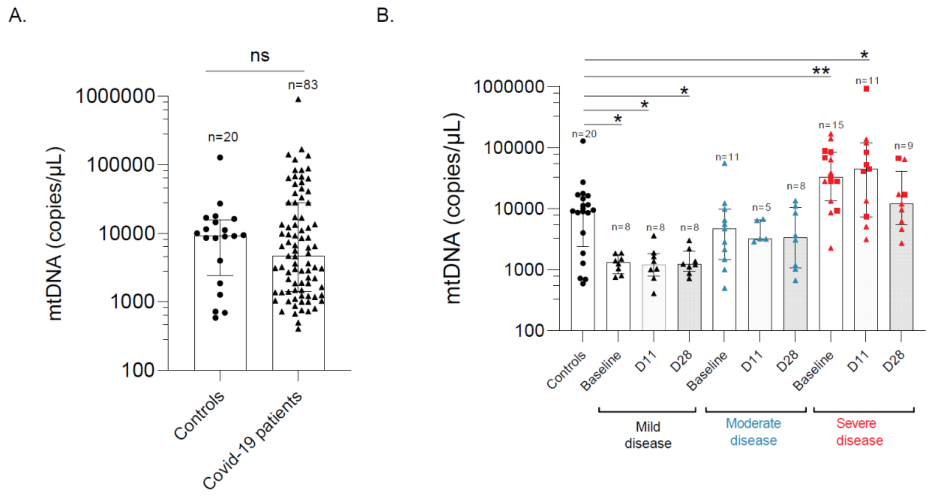

***Fig. 2. Cell-free mtDNA in COVID-19 patients**. Mitochondrial DNA (mtDNA) amount in patients with COVID-19 included at the baseline (n=34) and in controls (n=20) (a), mtDNA amount in patients and in controls with different severity levels of COVID-19 at different time points (b). Plasma from healthy donors (controls) and from SARS-CoV-2-infected patients with different disease severities (mild, moderate, and severe) were analyzed at different time points: baseline corresponding to the enrollment day; 11± 3 days after enrollment (D11); and 28 ± 7 days after enrollment (D28). Squares represent patients who died. Groups with non-dependent values were compared by use of the Mann-Whitney test. Data are presented as median ± interquartile range. Statistical significance was set at p<0.05.*

Blood Collection from healthy donors

In a first enrollment, control samples were collected anonymously from 24 healthy blood donors at the blood donation center SRK in Bern (project P357) from which no demographic data were available, using K3 EDTA vacuette® tubes (4 mL tubes, Greiner Bio-One, Switzerland) and vacuette® citrate tubes (3.5 mL tubes, citrate 3.2%, Greiner Bio-One, Switzerland). Since blood collection using vacutainer systems may activate complement, a second enrollment phase of 14 volunteers donors have been done, for complement measurement products, blood was drawn in S-monovette® EDTA (2.7 mL tubes, Sarstedt, Germany) using a butterfly needle. The tubes were centrifuged once for EDTA tubes, and twice for citrate tubes, for 15 minutes at 2500g. Plasma was stored in aliquots at-70°C.

For serum sample, a third enrollment phase of 29 healthy donors at the blood donation center SRK in Bern (project P357) have been done. Blood was collected in S-Monovette® serum tubes (9 mL, Sarstedt, Germany) or vacuette® tubes (9 mL tubes, Greiner Bio-One, Switzerland) and left at room temperature for 30 min for complete coagulation. The tubes were then centrifuged at 2000g for 10 min at room temperature to collect the serum. Serum was stored in aliquots at-70°C.

The number of samples from healthy donors was different between S-monovette EDTA with 14 healthy volunteers used only for complement activation and 24 healthy donors from SRK to measure nucleosome, Elastase–α_1-antitrypsin complexes and mitochondrial DNA levels, citrated tubes and among them 15 samples were used to measure D-dimer, and finally serum of 29 donors from SRK to measure DNase I activity.

Enzyme-linked immunosorbent assay for activated C3 and C4

Activated C3 and C4 was determined by detection of complement activation products C3b/c and C4b/c as described previously, using a sandwich ELISA with EDTA samples[34,35].

Quantification of mitochondrial DNA

Mitochondrial DNA (mtDNA) was purified from plasma using a QIAamp DSP Virus kit (Qiagen). Patient DNA samples were subsequently diluted (1:5) in DNase-free water. A digital droplet PCR (ddPCR) was performed according to the manufacturer's instructions: the ddPCR system included an automated droplet generator and reader from Bio-Rad, (QX200 Droplet Digital PCR, Bio-Rad, Hercules, California, USA) and a T100 thermal cycler (Bio-Rad). For mtDNA quantification, primers and probes targeting the mitochondrial DNA encoded NADH dehydrogenase 1 (MT-ND1), NADH dehydrogenase 1 (ND1), Human (FAM) (Bio-Rad, unique assay ID dHsaCNS669425578) were used. Results were analyzed using QuantaSoft software (Bio-Rad), and absolute values of mtDNA (ND1) (copies/µL) were calculated for each DNA sample.

Nucleosome measurement

Nucleosome levels were determined with an ELISA as previously described[36]. Briefly, ELISA plates were coated with monoclonal anti–histone H3 antibody (CLB/ANA-60) and the samples were added and incubated for 1 hour at room temperature. After washing, biotin-labelled F(ab')2 fragments of monoclonal anti-nucleosome antibody (CLB/ANA-58) were added and incubated for another hour at room temperature. Binding of biotin-labelled antibodies was detected with streptavidin–horseradish peroxidase using tetramethylbenzidine as a substrate. The reaction was stopped with 2 M H_2SO_4 and the absorbance was measured at 450 nm.

Neutrophil activation

Elastase–α_1-antitrypsin complexes were measured by ELISA as described[36].

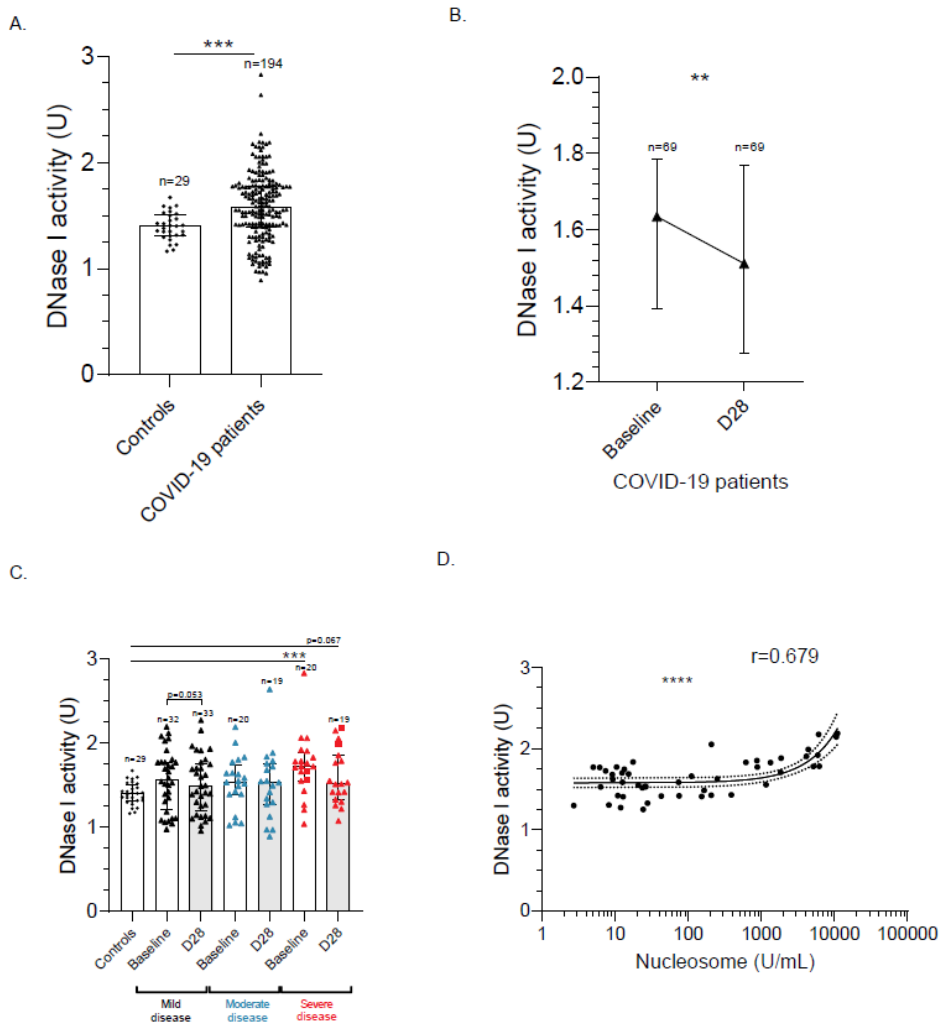

Fig. 3. DNase I activity in COVID-19 patients. DNase I activity in patients with COVID-19 included at the baseline (n=72) and in controls (n=29) *(a), DNase I activity in patients with COVID-19 at the baseline and D28 time points (b), DNase I activity in patients with different levels of COVID-19 disease severity at different time points and in controls (c), Correlation between DNase I activity and nucleosomes levels in all patients with COVID-19 (n=48 pairs) (d). Serum from healthy donors (controls) and from SARS-CoV-2-infected patients with different disease severities (mild, moderate, and severe) were analyzed at different time points: baseline corresponding to the enrollment day; and 28 ± 7 days after enrollment (D28). Squares represent patients who died. Squares represent patients who died. The Spearman rank correlation was determined. Data are presented as median ± interquartile range. Statistical significance was set at p <0.05.*

DNase activity

DNase I activity was quantified with a Quant-iT PicoGreen® dsDNA Assay Kit (Invitrogen). Serum samples were diluted 1:10 and incubated with TE buffer (10 mM Tris-HCL in UltraPure DNase/RNase-free distilled water) without or with 10 µL of double-stranded calf thymus DNA solution (Invitrogen, ref 15633-019) at 5 µg/mL final concentration in a 96-well plate. The wells with plasma samples without added DNA provided the background signal. For the standard curve, different DNase I (#EN0521, Thermo Scientific) concentrations were used and added to wells containing only TE buffer and dsDNA. The total volume was adjusted to 100 µL before incubation. The plates were incubated for 15 min at 37°C. Samples were stained with PicoGreen according to the manufacturer's instructions. After 2 to 5 minutes, the reduction in PicoGreen staining by fluorescence was measured using TECAN. Before using samples from our cohort, we assessed our assay of DNase I activity (suppl. Fig. 1).

D-dimer measurement

D-dimer were measured using immunoturbidimetry with INNOVANCE® (Siemens) Sysmex CS-S100 (Sysmex Europe GmbH, Germany). Polystyrene particles covalently was coated with a monoclonal antibody (8D3) then samples were added. The cross-linking region of the D-dimers has a symmetrical structure, which means that the epitope for the monoclonal antibody is present twice. Therefore, a single antibody is sufficient to trigger an aggregation reaction, which is detected by an increase in turbidity. Values below 500 g/L allow acute venous thromboembolism to be ruled out with high specificity[37].

Statistical Analysis

All statistical analyses and figures were computed with GraphPad Prism software version 9.3 (GraphPad Software, La Jolla, CA, USA). Results are indicated as median ± interquartile range. For the same patients at different time points, statistical significance was determined by using the Wilcoxon test. For all other experiments, statistical significance was determined by using the non-parametric Mann-Whitney test to compare two groups, or the Kruskal-Wallis test followed by Dunn's multiple comparisons test. Data are presented as median with interquartile range. Correlation has been calculated using Spearman rank testing. Statistical significance was defined as p <0.05.

RESULTS

Clinical features of patients infected by SARS-COV-2

Eighty-seven patients infected by SARS-CoV-2 were included in the cohort at the Inselspital in Bern (Table 1). Among them, the median age was 54 years old (range 22 to 86 years), 56% of the patients were more than 50 years old (Table 1), and 69% were males. The

common symptoms at enrollment were fever (60%), cough (76%), and myalgia (55%). Thirty-nine percent (n=34) of the patients suffered from mild, 28% (n=24) from moderate, and 33% (n=29) from severe disease. Among the patients suffering from severe disease, 90% of patients were more than 50 years old (median age 67 years). Among these patients suffering from severe disease who were older than 50 years old, 67% had at least two comorbidities, including mainly hypertension, cardiovascular disease, or diabetes. Ten patients died during the study, for an overall case-fatality rate of 11%.

Nucleosome levels, neutrophil activation, mtDNA, and DNase 1 activity in COVID-19 patients

EDTA and citrated plasma were available in 42% (37/87) of patients. In this subset of patients, we longitudinally measured nucleosome levels, neutrophil activation and mtDNA at baseline, D11 and D28. Nucleosome levels in patients with COVID-19 were significantly higher than those in healthy controls (Fig. 1a). In patients with mild disease, the nucleosome levels were not significantly different from those in controls (Fig. 1b). In contrast, in patients with moderate and severe disease, nucleosome levels were significantly higher than in controls. In patients with moderate disease, nucleosome level significantly declined over time. In contrast, in patients with severe disease, nucleosome levels remained high after 28 days and were highest in the non-survivors.

Next, we measured neutrophil activation as evidenced by elastase–α1-antitrypsin complex (EA) levels in our study patients. (Fig. 1c,d). Overall patients with COVID-19 had significantly higher EA levels than controls. In patients with mild disease, EA levels were similar to healthy controls and remained unchanged over time. In patients with moderate and severe disease, EA levels were significantly higher than they were in controls. Whereas EA levels significantly decreased in patients with moderate disease, in patients with severe disease the levels remained high over time. There was a strong and significant correlation between nucleosome and EA levels (Fig. 1e).

Using a specific ddPCR, mtDNA was measured in the COVID-19 patients and controls (Fig. 2a,b). Interestingly, mtDNA was significantly lower in patients with mild and moderate disease as compared with the controls. In severe disease, mtDNA was significantly higher at the baseline and after 11 days than it was in the controls. We found a strong correlation between neutrophil activation and the concentration of mtDNA ($r = 0.74$ & $P < 0.0001$, data not shown).

Finally, DNase I activity was assessed longitudinally in serum of 72 COVID-19 patients and of 29 healthy controls, at baseline and D28. DNase I activity was significantly higher in patients with COVID-19 as compared with the controls (Fig. 3a). DNase I activity decreased over time in patients with COVID-19 (Fig. 3b) and patients with severe disease

had higher DNase I activity in comparison with the controls (baseline vs D28; Fig. 3c). In COVID-19 patients, DNase I activity was correlated with nucleosome level (Figure 3D).

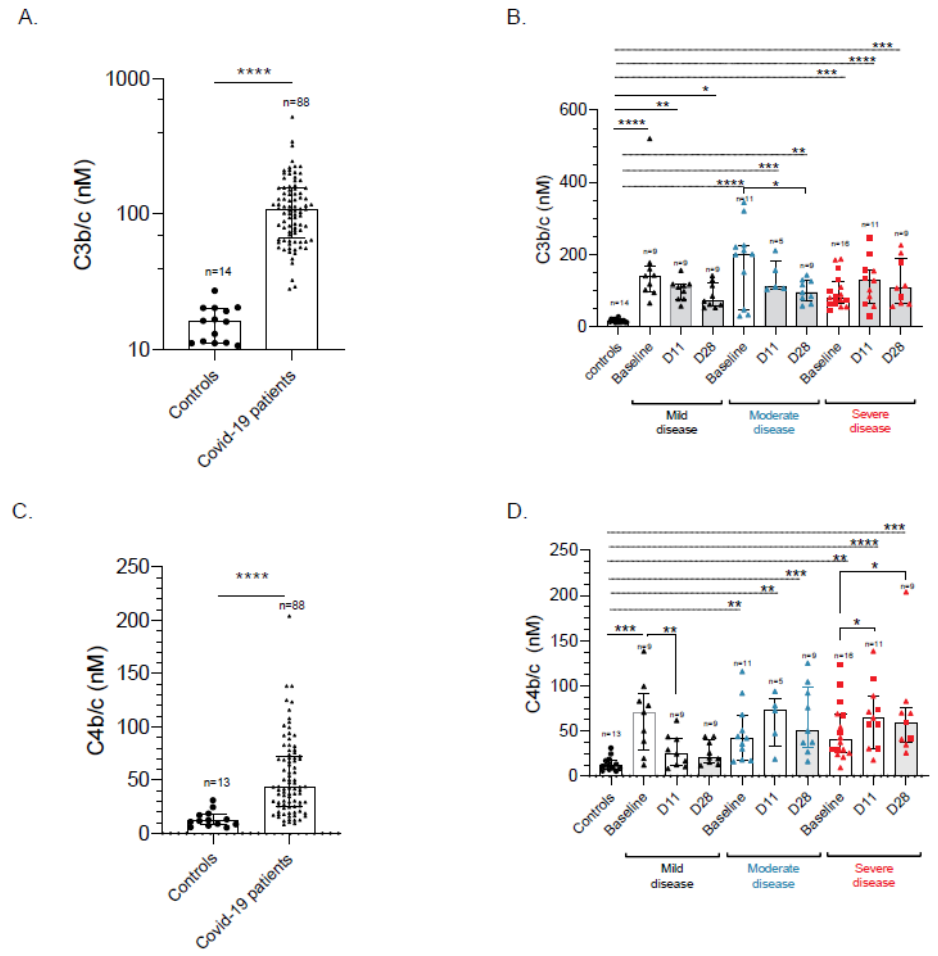

Fig. 4. Complement activation in COVID-19 patients. *C3b/c levels in patients with COVID-19 included at the baseline (n=36) and in control (n=20) (a), C3b/c level in patients with different severity levels of COVID-19 at different time points and in controls, with Wilcoxon test *p=0.0391 (b), C4b/c levels in patients with COVID-19 included at the baseline (n=36) and in controls (n=20) (c), C4b/c levels in patients with different severity levels of COVID-19 at different time points and in controls (d). Plasma from healthy donors (controls) and from SARS-CoV-2–infected patients with different disease severities (mild, moderate, and severe) were analyzed at different time points: baseline corresponding to the enrollment day; 11 ± 3 days after enrollment (D11); and 28 ± 7 days after enrollment (D28). Squares represent patients who died. Data are presented as median ± interquartile range. Statistical significance was set at p <0.05. #1 control dropout for C4b/c because of technical issue.*

Complement activation in COVID-19 patients

We longitudinally measured complement activation products of C3 and C4 in the EDTA plasma of the 36 COVID-19 patients and in 14 healthy controls, at the baseline, D11 and D28. Overall, C3b/c and C4b/c levels were significantly higher in COVID-19 patients than controls (Fig. 4a, c). At the baseline, C3b/c and C4b/c concentrations were increased in all patients. However, in mild and moderate diseases, C3b/c levels decreased over time, whereas in severe disease the levels remained high (Fig. 4b). In C4b/c levels, such a decrease was observed exclusively in patients with mild disease (baseline vs D11). Interestingly, in moderate and severe cases, C4b/c levels increased over time (baseline vs D11). Then in moderate disease, C4b/c levels had a tendency to decrease between D11 and D28 but remained higher than healthy controls (baseline vs D28). Whereas, in severe cases the C4b/c concentration remained high at D28 (Fig. 4d).

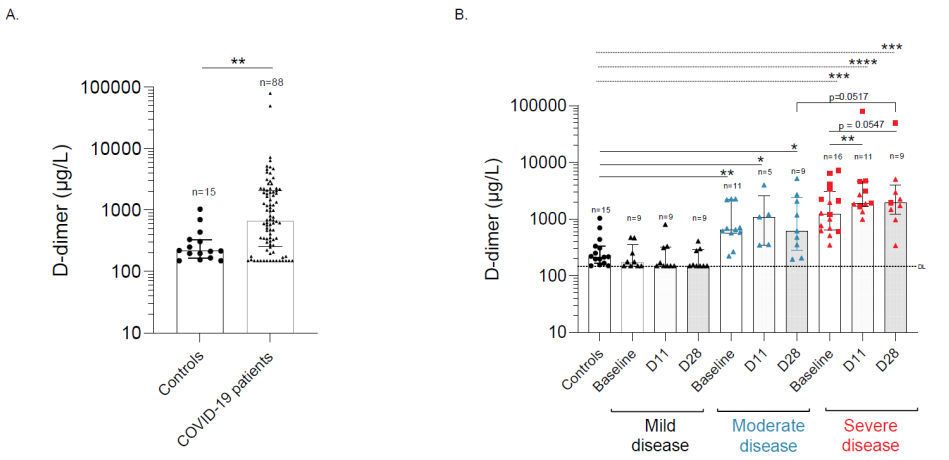

Fig. 5. D-dimer production in COVID-19 patients *D-dimer levels in COVID-19 patients included at the baseline (n=36) and in controls (n=15) (a), D-dimer levels in patients with different severity levels of COVID-19 at different time points and in controls (b). Plasma from healthy donors (controls) and from SARS-CoV-2–infected patients with different disease severities (mild, moderate, and severe) were analyzed at different time points: baseline corresponding to the enrollment day; 11 ± 3 days after enrollment (D11); and 28 ± 7 days after enrollment (D28) .Squares represent patients who died. The dotted line represents the detection limit (DL). Data are presented as median ± interquartile range. Statistical significance was set at p <0.05.*

D-dimer production in COVID-19 patients

As a measure of the procoagulant state, we measured D-dimer levels and followed their course in 36 patients at the baseline, D11 and D28 (Fig. 5). D-dimer levels in patients with COVID-19 were significantly higher as compared with controls (Fig. 5a). In most patients with mild disease, D-dimer levels were low below the detection limit (<155 µg/L). Nevertheless, in both moderate and severe disease, D-dimer levels were significantly higher than controls. In moderate disease, the D-dimer level increased until D11, and by D28 we observed a downward trend. In severe disease, D-dimer levels increased significantly between baseline and D11, and remained high at D28.

DISCUSSION

COVID-19 is a disease characterized by systemic inflammation, as evidenced by complement activation, neutrophil activation, and the release of cfDNA. Markers for complement and neutrophil activation, as well as cfDNA, increase with disease severity in systemic inflammation, and have been proven to be reliable markers for disease severity in sepsis[38-40]. In this comprehensive study, we measured cfDNA (in the form of nucleosomes and mtDNA), neutrophil activation (in the form of EA), DNase I activity, complement activation (C3bc, C4bc), and D-dimers in longitudinal samples of COVID-19 patients having different disease severities (ranging from mild to severe). We showed that neutrophil activation, complement activation, cfDNA, and D-dimer levels increased with disease severity. Moreover, we have provided evidence that in severe disease there is continued neutrophil and complement activation, as well as D-dimer formation and nucleosome release, whereas in mild and moderate disease all these variables of immunothrombosis decrease over time.

Activation of the complement system is a well-recognized process of the innate immune response during sepsis[41-43]. C3a and C4a levels are increased in patients with sepsis and septic shock, and plasma concentrations of C3a correlate with mortality rate[44]. Complement activation reflecting systemic inflammation could also be found in our patients suffering from COVID-19. The circulating levels of C3b/c and C4b/c were significantly elevated in patients with COVID-19 and were associated with disease severity. These results are coherent with a recent study that reported significantly increased levels of soluble C5b9 in patients with moderate and severe COVID-19 as compared with healthy controls[45]. Interestingly, C5a levels were increased in patients with moderate and severe COVID-19 disease as compared with healthy controls. However, the levels did not differ between the two patient groups[45]. Another study reported an association between mortality and the level of overactivation of C3, as evidenced by the C3a:C3 ratio, pointing to a possible central role of C3[13]. In contrast, autopsy specimens of lung tissue from deceased COVID-19 patients showed strong deposition of MASP-2 but only weak C1q deposition, suggesting a prominent role of the lectin pathway in COVID-19[46]. The measurement of complement protein level does not necessarily reflect “activation”. However, the measurement of complement activation in plasma is troublesome and strongly dependent on pre-analytic conditions, since freezing and thawing may activate

complement in vitro. Moreover, we observed that the procedure to draw blood may affect complement activation and interestingly the butterfly needle seems to prevent it (Supplemental Figure 2). Anaphylatoxins, such as C3a and C5a, are susceptible to cleavage and degradation by plasma carboxypeptidases[47]. We have now demonstrated, using an assay for C3b/c and C4b/c, that complement activation in plasma samples is increased in COVID-19 patients and correlates with disease severity and fatality.

Our group identified cfDNA in the form of nucleosomes as a surrogate marker for systemic inflammation, predicting severity and fatality in sepsis[39, 40]. Analogously, nucleosome levels increased with disease severity in COVID-19 patients. CfDNA in the form of nucleosomes is the product of chromatin degradation by endonucleases and consists of an octamer of two copies of each of the four core histones (H2A, H2B, H3, and H4) wrapped by 145–147 base pairs of helical DNA. Our findings are in line with other studies demonstrating that cfDNA is a potentially useful marker to monitor COVID-19 progression and severity[48-50]. In this study, we report that mtDNA levels were also correlated with disease severity. This result is in line with a previous study demonstrating high circulating mtDNA levels in COVID-19 patients as a predictor of ICU admission, intubation, vasopressor use, and fatality[51]. Together, these data suggest cfDNA to be a marker of systemic inflammation, correlating with disease severity and fatality in COVID-19.

In our cohort of COVID-19 patients, neutrophil activation increased with disease severity and decreased during disease resolution. This is analogous to sepsis, where neutrophil activation is a reliable surrogate marker of severity and fatality[2]. Neutrophil infiltration in the inflamed lung is a hallmark of ARDS[3]. Indeed, studies have reported increased numbers of neutrophils in patients with severe COVID-19 and in COVID-19 patients who died[52]. In addition, high neutrophil infiltration in pulmonary capillaries and extravasation into the alveolar space characterized by neutrophil mucositis of the trachea and fibrin deposition were observed in autopsy specimen of the lungs of COVID-19 patients[17].

Microvascular complications in systemic inflammation are a result of immunothrombosis. Neutrophil activation in the form of NETs has a central role in the pathogenesis of immunothrombosis[19, 20]. In our patients, we demonstrated that neutrophil activation, nucleosome levels, and mtDNA increase with disease severity. The strong correlation between neutrophil activation and cfDNA in the form of nucleosomes and mtDNA may suggest the presence of neutrophil activation in the form of NETs. Several studies report a role of NETs in the pathogenesis of COVID-19 and have demonstrated high levels of NETs as evidenced by circulating markers for NETs to be associated with thrombotic complications[50, 53-56]. One has to keep in mind that measurement of citrullinated histone 3–DNA or elastase–DNA complexes might be troublesome, since in vitro formation of these complexes after sampling cannot be excluded. To circumvent this issue, we assessed neutrophil activation using an elastase-complex assay as well as an assay for nucleosomes. Recent data show that the release of cfDNA early in inflammation occurs from hematopoietic cells and that only in later stages do parenchymal cells also release cfDNA[57]. Therefore, we also cannot rule out that the release of nucleosomes is caused by cell damage or by activated neutrophils.

NET degradation by deoxyribonuclease I (DNase1) is essential in maintaining microvascular patency[28]. Interestingly, we found increased DNase I activity in all COVID-19 patients, independent of disease severity, as compared with healthy controls. In contrast, a previous study reported increased DNase activity in COVID-19 patients, but only in severe forms of COVID-19[30]. In a recent study, despite high levels of DNase activity, the impaired DNase was not enough efficient in NET degradation contributing to disease severity, and was more associated to the elderly male patients [Buhr et al. 2022].

Interestingly, in our patients suffering from mild COVID-19 with no evidence of neutrophil activation and circulating cfDNA, DNase I activity increased, whereas patients with moderate and severe COVID-19 with strong systemic inflammation had DNase I activity similar to that seen in mild disease. One may speculate that in mild disease, increased DNase I activity ensures microvascular patency, but that DNase activity reaches a maximum in moderate to severe disease, with a relative DNase I insufficiency resulting in microvascular thrombosis. Unfortunately, we could not follow the dynamics of DNase activity as no serum from day 11 was available.

Elevated concentrations of D-dimers are associated with inflammation, organ injury, and poor outcome in SARS-CoV-2 infection[58, 59]. Our findings corroborate the results of other groups, who have reported that D-dimer levels are associated with disease severity in COVID-19[60]. We observed a similar pattern of increasing levels of D-dimer, neutrophil activation, and complement activation, which are soluble markers of immunothrombosis, correlating with disease severity. Altogether, these results point to immunothrombosis as a main driver of microvascular complication in COVID-19.

Many parallels can be drawn between non-COVID sepsis and COVID-19 sepsis that suggest how the disease might be managed. For example, it has been proposed that blocking complement with therapeutics targeting C5 or the mannan-binding lectin-associated serine protease-2 benefits the clinical outcome[61]. Blocking complement activation inhibits NETosis[62, 63]. Our study implicates complement activation occurs via the classical pathway and/or the lectin pathway. Interestingly, in sepsis patients C1-inhibitor treatment attenuated complement-and neutrophil activation, as well as improved organ function and survival[38, 64, 65]. There are a couple of case reports and case series

on the effect of complement inhibitors targeting different levels of complement, which seem promising. However, results from randomized controlled trials are lacking. The open questions remain on when such a therapy should be initiated and which pathway to target. Our data suggest that complement activation starts early and therefore a timely start of treatment with complement inhibitors should be chosen in clinical trials evaluating such compounds to prevent downstream actions of complement, such as neutrophil activation.

To the best of our knowledge, this is the first study that measured the dynamic in timeline of all biomarkers of the crosstalk between neutrophil activation, complement system and thrombosis in patients with mild, moderate or severe disease of COVID-19. However, we also have to point out the limitations of our study. The patients were included in the first and second COVID-19-waves, before large parts of the population had either vaccine- or infection-elicited adaptive immunity to SARS-CoV-2. Even though pre-existing adaptive humoral and cell-mediated immunity to SARS-CoV-2 may not uniformly protect from re-infection, it is likely that it affects the extent of the innate immune response, such as complement activation, NETosis and immunothrombosis. Therefore, our findings may not directly translate to a pre-immunized population. Finally, we exclusively focused on the innate immune axis. We acknowledge that the adaptive immune response may also play a role in pathogenesis of thromboembolic events in COVID-19. Finally, we only have anonymous healthy blood donors as control, and we miss sex and aged matched controls.

In summary, we have shown that complement and neutrophil activation, D-dimers, and cfDNA increase with disease severity, decrease in patients with disease resolution, and remain high in non-survivors. Therefore, neutrophil activation, cfDNA, and complement activation are reliable biomarkers for disease severity and fatality in COVID-19. They may also serve as suitable markers for monitoring the efficacy of therapeutic interventions in COVID-19.

Conflict of Interest Statement

The authors declare no competing financial interests.

Statement of Ethics

Human blood samples were collected from healthy donors with the blood donation center SRK in Bern (project P357) and the help of a physician in agreement with the local Ethical Board. The study was approved by the Ethics Committee of the Canton of Bern, Bern, Switzerland, Nr. 2020-00877 and registered at clinicaltrials.gov (NCT04510012). Patients were included after provision of informed consent. In case of lack of capacity and/or inability to provide consent, enrollment followed the procedures for research projects in emergency situations according to Swiss law.

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Author Contributions

Contributions: T.R. and Y.dW. performed the experiments, analyzed and interpreted the data, wrote, edited and reviewed the manuscript and figures; N.S. performed the experiments and analyzed the data; G.M. assisted with the experiment and reviewed the manuscript; A.A-S analyzed the data and reviewed the manuscript; J.B. collected plasma samples from COVID-19 patients and was involved in data analysis; J.C.S. helped with patients, reviewed the manuscript, C.H. wrote the ethical approval, established the cohort, collected clinical data from patients and reviewed the manuscript; I.J. was involved in data analysis and reviewed the manuscript; S.Z. designed and supervised the work.

Author notes

* T.R. and Y.dW. contributed equally to this study

S.Z., I.J. and C.H. share senior authorship

Data Availability Statement

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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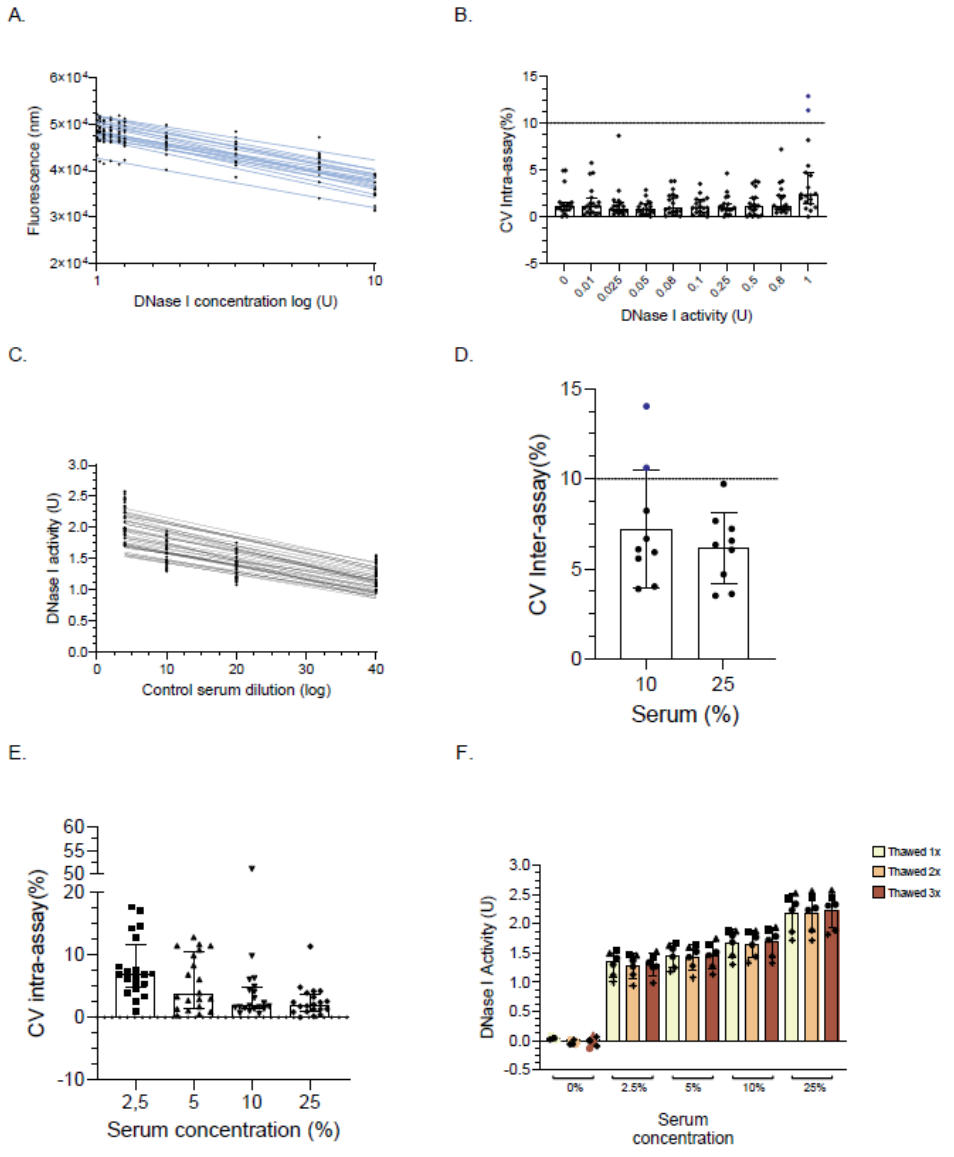
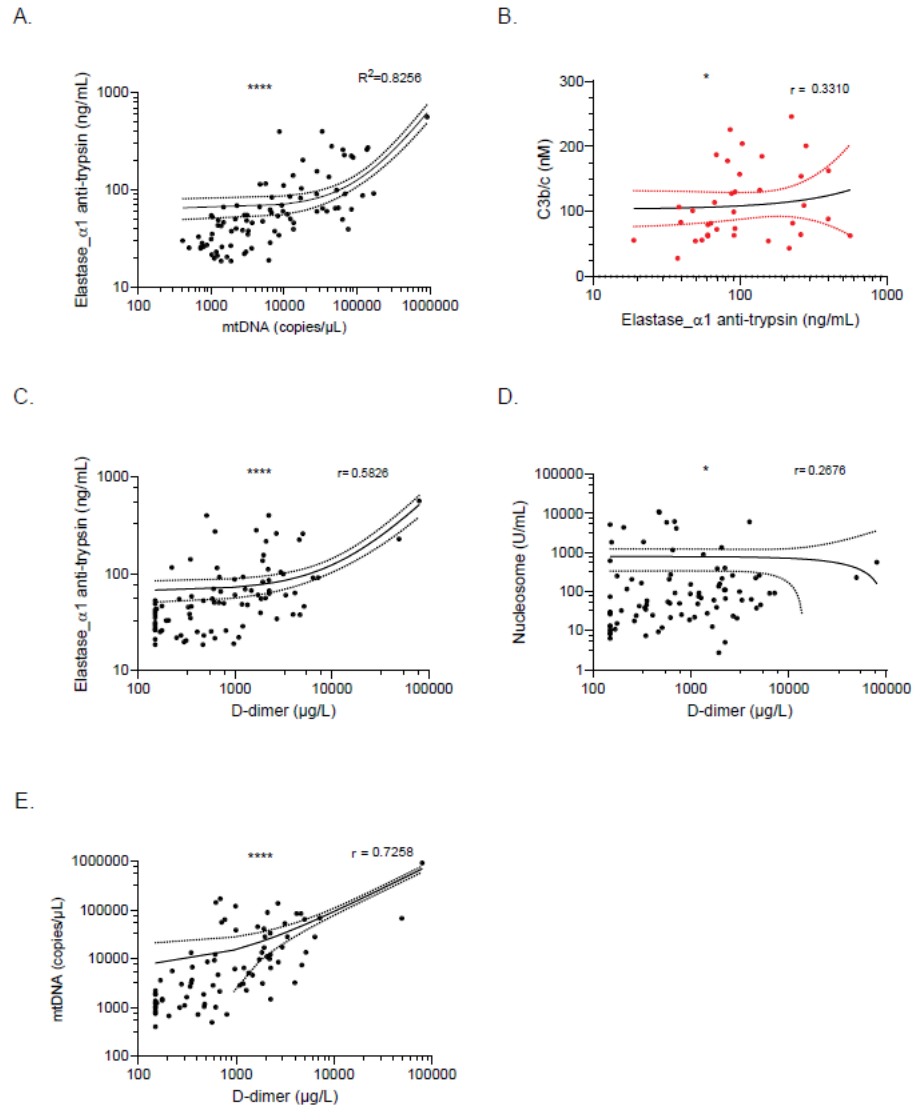
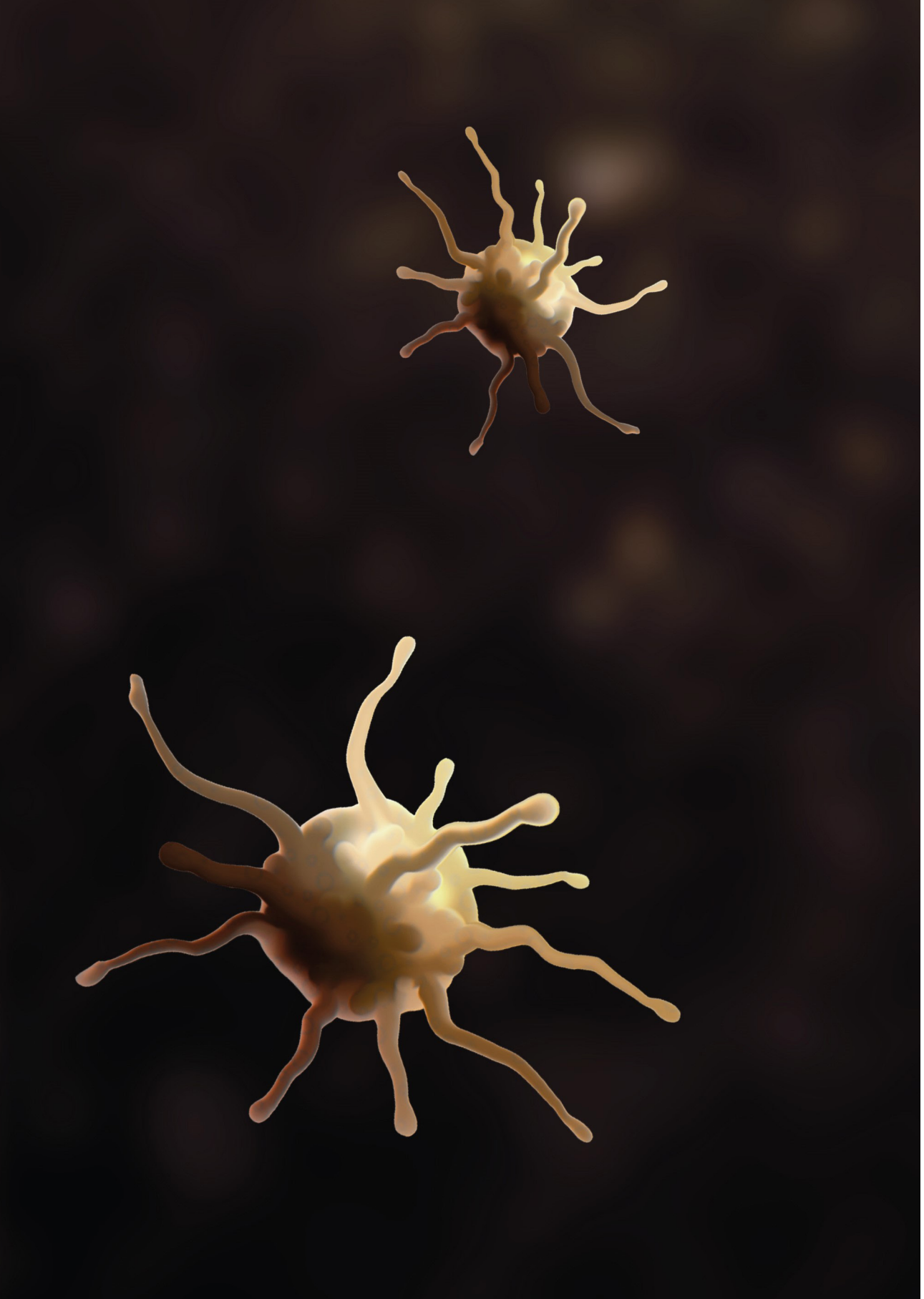
SUPPLEMENTARY FILES



Suppl. Fig. 1. Standard curves of DNase I activity and serum DNase I concentration from healthy volunteers *Standard curves of DNase I concentration (a), Standard curve of intra-assay coefficients of variation (CV) of assays from the same plate (b), DNase I activity assessment in different concentrations of control human serum (c), Inter-assay coefficients of variation (CV) for different concentrations of the same serum samples measured in different plates (d), Intra-assay coefficients of variation (CV) for different serum concentrations in the same plate (e), DNase I activity dependence on the concentration of serum that was thawed once (1x), twice (2x) or thrice (3x) (f). Each sample was used in triplicate. The experiment was repeated three times. The blue points correspond to values above 10%. Data are presented as median ± interquartile range.*



Suppl. Fig. 2. Complement activation in controls from different drawing system *C3b/c levels in controls Vacuette (n=30) or S-monovette (n=14) (a), C4b/c levels in controls Vacuette (n=23) or S-monovette (n=13) (b). Plasma from healthy donors (controls) was collected in Vacuette EDTA tubes corresponding to vacutainer system, or with butterfly needle in S-monovette EDTA tubes. Groups were compared by use of the Mann-Whitney test. Data are presented as median ± interquartile range. Statistical significance set at p <0.05.*



CHAPTER 4

Platelet concentrates in Platelet additive solutions generate less complement activation products during storage than platelets stored in plasma

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Blood Transfus, 2022

ABSTRACT

Background

Platelet transfusions can be associated with adverse reactions, such as febrile nonhemolytic transfusion reaction (FNHTR). Damage-associated molecular patterns (DAMP) and complement have been suggested to play a role in FNHTR. This study investigated the nature of DAMPs and complement activation products contained in platelet concentrates during storage with a specific focus on different platelet storage solutions.

Material and methods

Buffy coats (BC) from healthy donors were pooled (15 BC per pool) and split into three groups of same volume. After addition of different storage solutions, plasma, Platelet additive solutions (PAS)-C or PAS-E (n=6 for each group), BC pools were processed to platelet concentrates (PC). Leukoreduced PCs were stored on a shaking bed at 20-24°C and sampled on day 1, 2, 6 and 8 after collection for various quality parameters, platelet activation, DAMPs (High Mobility Group Box 1 (HMGB1), nucleosomes) and complement activation products.

Results

During storage equal levels of free nucleosomes and increasing concentrations of HMGB1 were present in all groups. Complement activation was observed in all PC. However, the use of PAS reduced C3b/c levels by ~90% and C4b/c levels by ~65% at day 8.

Discussion

Nucleosomes and HMGB1 were present in PCs prepared in plasma and PAS. Complement was activated during storage of platelets in plasma and in PAS. The use of PAS is associated with a lower amount of complement activation products due to the dilution of plasma by PAS . Therefore, PC in PAS have less complement activation products than platelets stored in plasma. These proinflammatory mediators in PC might induce FNHTR.

INTRODUCTION

In patients with hematological malignancies low platelet counts, as a consequence of myeloablative chemotherapy, are associated with an increased morbidity and mortality due to bleeding complications. Prophylactic platelet transfusions have been shown to be effective in order to prevent or at least reduce the frequency of bleeding complications[1]. However, platelet transfusions can also be associated with immunological and infectious adverse reaction (AR). Immunological AR include allergic reactions, febrile nonhemolytic transfusion reactions (FNHTR), transfusion-related acute lung injury (TRALI) and platelet refractoriness due to anti-HLA and -HPA antibodies, respectively[2–4]. Infectious complications include mainly systemic infections due to bacterial product contamination, and in very rare cases infection by blood born viruses[5–7]. Altogether, these immunological and infectious AR are responsible for an increased morbidity with subsequent increase of hospital admission duration and potential fatality after platelet transfusion[5,6,8]. With recent implementation of safety measures, such as Hepatitis C virus (HCV) and Human immunodeficiency virus (HIV) nucleic acid test (NAT) donor testing, donor screening for antibodies to hepatitis B core antigen (anti-HBc), and improved bacterial detection methods, platelet transfusion-transmitted infections have been significantly decreased[7,9,10]. Leukoreduction has shown to effectively reduce the incidence of transfusion reactions after platelet transfusions[11–13] . Furthermore, Identification of IgA- and haptoglobin-deficient recipients reduced the occurrence as well as the severity of allergic reactions in the context of platelet transfusions[14,15].

The role of storage solution in the pathogenesis of transfusion reactions after platelet transfusion has been extensively studied[16,17]. Plasma as storage solution for platelets has widely been used. Plasma may induce allergic reactions and contain cytokines, chemokine, other soluble immunomodulatory factors, anti-HLA and -HPA antibodies resulting in TRALI[18]. Therefore, storage solutions with a significant reduced amount of plasma may reduce AR after platelet transfusion. Plasma has recently been replaced by platelet additive solution (PAS) to improve storage lesions. This results in a significantly reduced amount of plasma (approximately 35% plasma remaining) in the platelet concentrate[16]. PAS has been optimized during the past years to improve platelet storage properties and reduce AR caused by storage lesions[19,20]. Indeed, platelets stored in PAS are associated with a decreased incidence of AR such as allergic reactions as compared to platelet products with plasma[16,17,21]. No significant reduction in TRALI has been observed between platelets stored in PAS or plasma[16, 17]. This is most probably due to low incidence of these complications[16,17]. Interestingly, the incidence of FNHTR remained unchanged after the introduction of first generations PAS[2].

PCs used for transfusion contain a variety of secretory products that have impacts on

hemostasis as well as innate immunity. Biological response modifiers in transfusion grade PCs supernatants increase during storage. HMGB1, which exerts a potent inflammatory role through TLR2 and TLR4[18], is released by platelets, and illustrates the central role of platelets in bridging stress as well thrombotic and immune responses. Damage associated molecular patterns (DAMPs), such as HMGB1, cell-free DNA and DNA-binding proteins, have been suggested to play a role in AR and particularly the pathogenesis of FNHTR by inducing a systemic inflammatory response[22–27]. Additionally, recent findings propose complement activation to play a role in the pathogenesis of FNHTR[28,29]. DAMPs and complement activation products may be produced during the collection and production process and/or may accumulate during storage of the product[28–33]. Administration of complement activation products and DAMPs contained in the platelet product may lead to complement activation and may perpetuate complement activation in the recipient. This may result in a systemic inflammation, which will negatively impact effectiveness of platelet transfusion.

In this study we investigated the concentration of nucleosomes, HMGB1 and complement activation products in platelet concentrates (PC) made from pooled buffy coats and stored in plasma, PAS-C (the former standard storage solution in the Netherlands) and PAS-E (the current standard storage solution in the Netherlands), shortly after production and during subsequent room temperature storage. In addition, as white blood cells are present in the buffy coats and the fact that the PCs are leukoreduced but not completely leukodepleted, neutrophil activation may occur and constitute a source of DAMPs. Accordingly, we analyzed neutrophil activation in these PCs during storage.

MATERIALS AND METHODS

Blood collection

Approximately 500 mL of whole blood was collected from non-remunerated, informed donors in quadruple bags, bottom-and-top collection systems containing 70 mL of citrate-phosphate-dextrose (CPD) anticoagulant (Fresenius Kabi, Bad Homburg, Germany). Blood collections were performed in accordance to the institution's guidelines and practices, with the aid of calibrated blood collection balances equipped with a mixing platform allowing mixing at regular intervals, monitoring of blood flow and bleeding time, a final check of the weight of the donation and cooling of the blood to 20-24°C on butane-1, 4-diol cooling plates (Compocool, Fresenius Kabi) immediately after collection. The day of blood collection was designated as day 0 of the study. This study was approved by the institutional medical ethical committees, in accordance with the standards laid down in the 1964 Declaration of Helsinki.

Blood processing and Preparation of PCs

Donations meeting the Dutch regulatory criteria of volume (500 ± 50 mL of blood) and bleeding time (<12.5 min) were selected for further processing after overnight hold according to the routine buffy coat procedure as previously described[34]. Briefly, blood processing started with centrifugation in a Sorvall RC12BP centrifuge at 20°C at 4793g for 8 min. The centrifuged blood was separated in plasma, buffy coat and red cell suspensions, using an automated blood component separator (Compomat G5, Fresenius HemoCare). A schematic overview of the process from BC to PC is represented in figure 1. A total of 90 BCs were included in the study for the preparation of PC. Six pools of fifteen BCs of the same blood group were formed. After mixing, the pool was divided over 3 pooling bags from C5000 platelet pooling and storage systems (Fresenius Kabi) to get 3 pools of equal composition and volume. These 3 pools were respectively diluted with a plasma unit from one of the corresponding 15 donations, 300 mL of PAS-E (TPAS+, Terumo BCT) (69mmol/L NaCl, 5mmol KaCl, 1,5mmol $MgCl_2$, 10mmol Na_3-citrate, 26mmol NaH_2PO_4/Na_2HPO_4, 30mmol Na-acetate), or 280 mL of PAS-C (Intersol, Fresenius Kabi) (77mmol NaCl, 10mmol Na_3-citrate, 26mmol NaH_2PO_4/Na_2HPO_4, 30mmol Na-acetate). After a soft spin, platelet concentrates (PC) were prepared. During the preparation of PC from the pooled buffy coats on the Compomat G5, the PCs were filtered directly using a leukoreduction filter (Compostop CS, Fresenius HemoCare), with an empty 1.3-L PVC-BTHC storage container (Compoflex F730, Fresenius HemoCare) connected to the outlet of the filter.

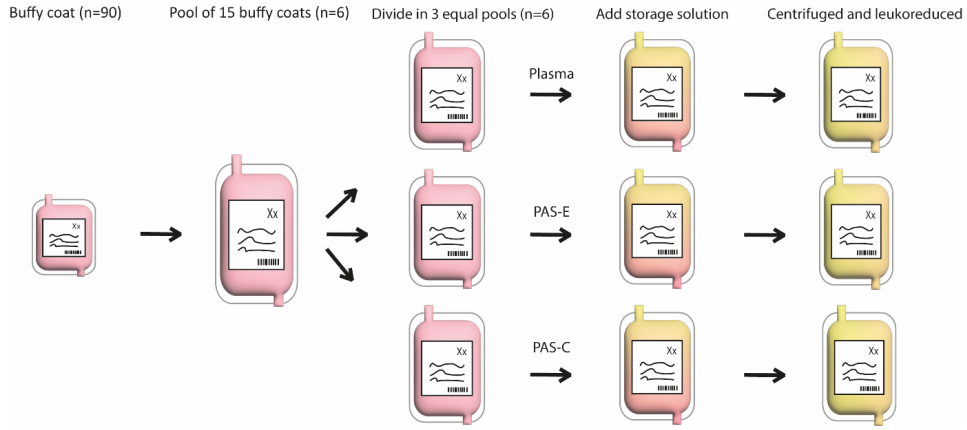

Figure 1 Schematic overview of study design. *A total of 90 BCs were collected. Six pools were created of each 15 BCs. Every pool was divided in three equal pools and supplemented with storage buffer (plasma of one of the 15 donors, PAS-E or PAS-C). Afterwards the PCs were centrifugated and filtered over a leukoreducing filter.*

Storage and sampling

Samples of the different storage solutions (plasma [n=6], PAS-C [n=1] and PAS-E [n=1] were taken prior to addition to the buffy coat pool. Leukoreduced PCs were stored on a shaking bed, 1 cycle per second, at 20-24°C and sampled on day 1, 2, 6 and day 8 after collection. Samples (10 mL) were taken using a swan-lock adapter, allowing needle-free aseptical sampling. The adapter was decontaminated with 70% IPA before sampling for the measurements of various quality parameters and soluble factors. The day of blood collection was designated as day 0 of the study. At the end of the storage period, PCs were checked for sterility, as previously described[35]. In short, both aerobic and anaerobic culture bottles were inoculated with a 7.5 mL sample under aseptic conditions in a laminar airflow cabinet. Culture bottles were incubated at 35°C in the BacT/Alert system until a positive reaction was detected or for 7 days if negative.

Table 1. Storage properties of PC. PCs were stored in plasma, PAS-E or PAS-C until day 8 after whole blood collection at 22±2°C, horizontally shaking with 1 cycle per second. Samples were taken aseptically at days 2, 6 and 8 for analysis of quality parameters.

		Plasma n=6		PAS E n=6		PAS C n=6		Range European and Dutch guideline blood products
	Day	median	IQ	median	IQ	median	IQ	
Volume (mL)	2	371	360-377	354	353-355	330	311-337[a]	250-400
	6	360	347-364	342	340-343	317	298-324[a]	
	8	349	337-353[d]	331	329-333[d]	307	287-314[a,d]	
Platelet concentration (pltx10^9/L)	2	1111	994-1214	1054	949-1095[a]	1048	946-1140	700-1800
	6	1099	995-1193	940	856-1038	862	771-957[a]	
	8	1072	983-1171	920	833-1001[d]	824	737-924[a,d]	
Total platelet count (pltx10^9/ unit)	2	402	368-452	375	335-387	345	301-376[a]	250-500
	6	389	353-431	321	293-354	273	233-308[a]	
	8	365	340-412[d]	303	278-331[d]	254	215-285[a,d]	
Mean platelet volume (fL)	2	8.2	8.1-8.6	8.5	8.4-86	8.6	8.4-8.8	-
	6	9.2	8.5-9.7[c]	9.1	8.6-9.4	9.0	8.8-9.4	
	8	9.1	9.0-9.2[d]	9.0	8.8-9.1	8.9	8.6-9.1	
pH at 37°C	2	7.21	7.18-7.22	7.13	7.13-7.15	7.13	7.11-7.15[a]	6,3-7,5
	6	7.23	7.22-7.25	7.21	7.20-7.22[c]	7.03	7.03-7.05[a]	
	8	7.16	7.13-7.17	7.19	7.18-7.20	6.94	6.91-6.95[b,d]	
Glucose (mmol/L)	2	18.1	17.7-18.8	6.7	6.7-7.0[a]	6.7	6.7-6.9[a]	>0
	6	14.9	14.7-15.5	4.9	4.8-5.0	3.1	3.0-3.2[a]	
	8	13.2	12.8-13.7[d]	3.6	3.6-3.7[d]	1.0	0.9-1.4[a,d]	

Table 1. Continued.

		Plasma n=6		PAS E n=6		PAS C n=6		Range European and Dutch guideline blood products
	Day	median	IQ	median	IQ	median	IQ	
Glucose consumption (mmol/ day/10^12PLT)	6	0.74	0.66-0.80	0.53	0.46-0.57	1.08	0.96-1.19[b]	-
	8	0.79	0.69-0.87	0.59	0.52-0.65	1.12	1.03-1.32[b]	
Lactate (mmol/L)	2	7.1	6.8-8.0	4.4	4.2-4.5[a]	4.9	4.5-5.1	-
	6	12.1	10.7-12.9	7.9	7.5-8.7[a]	10.9	10.5-11.2	
	8	15.4	13.7-16.2[d]	10.0	9.4-10.7[a,d]	14.5	14.0-15.1[d]	
Lactate production (mmol/ day/10^12PLT)	6	1.05	0.95-1.22	1.01	0.83-1.09	1.64	1.55-1.94[a,b]	-
	8	1.19	1.02-1.45	1.04	0.92-1.16	1.91	1.72-2.25[b]	

aP<0.05 vs. Plasma, bP<0.05 vs. PAS E, cP<0.05 day 2 vs. day 6, dP<0.05 day 2 vs. day 8

Routine parameters

To determine the *in vitro* quality of the PCs, the PCs were weighed and sampled using a swan-lock adapter (Codan, Lensahn, Germany). The swirling effect was judged and pH, blood gasses, glucose and lactate concentrations were determined with a blood gas analyzer at 37°C (Rapidlab 1265, Siemens Healthcare Nederland BV, Den Haag, NL. Platelet count, and mean PLT volume (MPV) were determined with a blood cell analyzer (Advia 2120, Siemens).

Flow cytometric analyses

After sampling of the PCs, platelets were stained for CD62p-FITC (P-selectin, Beckman Coulter, Immunotech art no. A07790) in Isotone, and Annexine V-FITC (VPS-Diagnostics, artno. A705) in HEPES buffer (20mM HEPES, 132mM NaCl, 6mM KCl, 1mM MgSO4, 1,2mM KH2PO4, 2,5 mM CaCl2, and 5 mM Glucose, pH 7.4), After staining, the samples were fixed using 0,5% PFA, and analyzed within two hours by 3 Laser FACS Canto II+HTS (BD Biosciences, Erembodegem, Belgium).

Quantification of sCD62p and CD40L

The levels of soluble CD40L (sCD40L - HCYTOMAG-60K - minimum detectable concentrations: 5.1 pg/mL) and CD62P (sCD62P- HCVD2MAG-67K- minimum detectable concentrations: 0.244 ng/mL) were quantified in PC supernatants using Luminex technology (Millipore, Molsheim, France), according to the manufacturer's instructions, using a Bioplex 200 system (BioplexManager software; Biorad, Marnes-la-Coquette, France) as described previously[36].

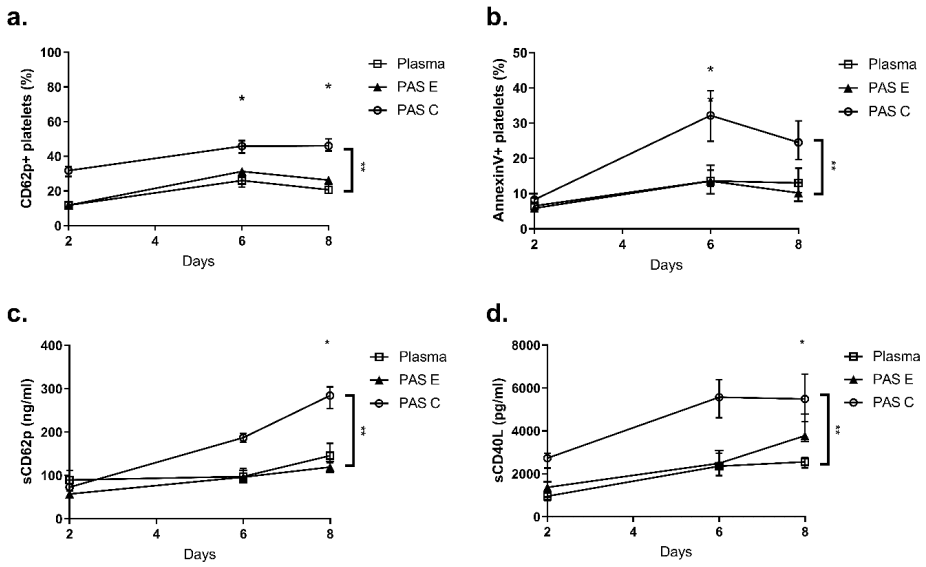

Figure 2 More Platelets express activation markers in PAS- C than in PAS-E or plasma. *PCs (n=6) stored in plasma, PAS-E, or PAS-C were sampled at multiple timepoints and the percentage of a: CD62p+; b: AnnexinV+ platelets and c: sCD62p; d: sCD40L levels was measured over time. The median and interquartile range are shown. Significant differences between day 2 and 6 or 8 are shown above the trendlines. The differences between groups are indicated by the capped line on the side. *p<0.05; **p<0.01.*

Neutrophil activation

Elastase, Elastase-α1-antitrypsin complexes[37], Lactoferrin[38], and nucleosomes were measured by ELISA as described before[39].

Complement activation

Levels of C3b/c, and C4b/c were assessed by ELISA as previously described[40]. IgM concentrations were measured by ELISA as described[41].

Quantification of high mobility group box 1 (HMGB1)

The levels of soluble HMGB1 were quantified using ELISA technology (IBL International GmbH, Hamburg, Germany) - minimum detectable concentrations: 0.313 ng/mL. Absorbance at 450 nm was determined with an ELISA reader (Magellan software Sunrise; Tecan group Ltd., Lyon, France) and data was expressed in ng/ml as described previously[27].

Statistical analysis

All statistical analyses and figures were computed with GraphPad Prism software v 9.1.1 (GraphPad Software, La Jolla, CA, USA). Results are represented as median with interquartile range. The Friedman test was performed between the groups at different

timepoints, and Dunn's test was used for *post hoc* multiple testing. The Wilcoxon test was performend to determine statistic differences between timepoints for glucose consumption and lactate production as there were only two timepoints tested. The results were considered significant at $p<0.05$.

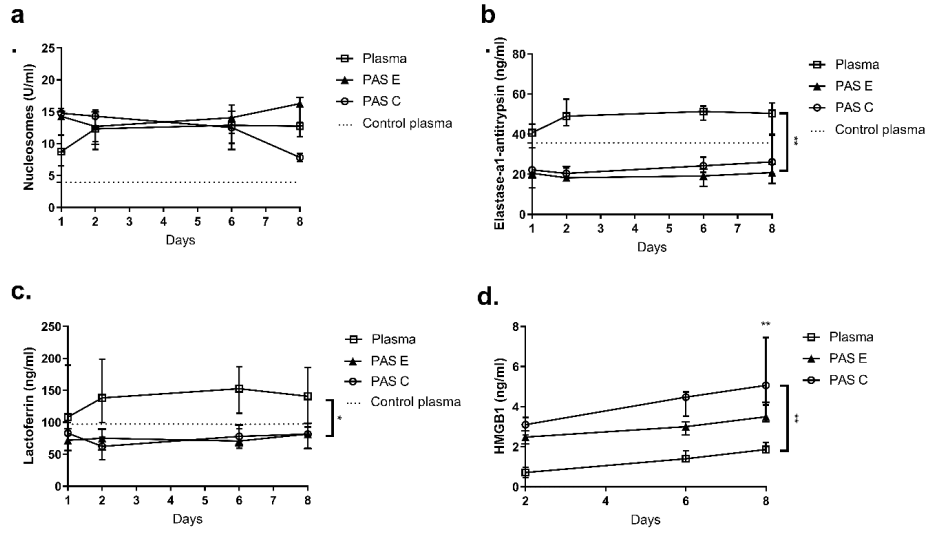

Figure 3 Nucleosomes and HMGB1 are contained in PCs. *PCs (n=6) stored in plasma, PAS-E, or PAS-C were sampled at multiple timepoints and a: nucleosome levels; b: elastase-a1-antitrypsin complexes; c: lactoferrin levels; and d: HMGB1 contained in the product were followed over time. The dotted line represents the level in the plasmas of donors that were used as storage solution in these products. The median and interquartile range is shown (a-c). Significance between day 1 and 6 or 8 are shown above the trend lines. The differences between groups are indicated by the capped line on the side. *p<0.05; **p<0.01.*

RESULTS

Platelet stored in PAS-E show equal or improved storage properties as platelet stored in plasma

First, we analyzed quality parameters, representative for platelet integrity and metabolic activity during storage, comparing PAS-E and PAS-C to plasma (table 1). Over time, PC stored in PAS-C showed more deterioration of PLT quality, compared to platelets in plasma, as evidenced by a significant decrease in platelet concentration, a relatively high glucose consumption and lactate production resulting in a decrease in pH (table 1). It should be noted that because of the plasma reduction in PC stored in PAS, there is less glucose present in the PC stored in PAS. Notably, the pH of PC stored with PAS E stays stable during the whole storage period (table 1). Overall, both PC in PAS-E and

PC in plasma demonstrate suitable storage properties. Moreover, PC in PAS-E have a lower glucose consumption and lower lactate production than PC stored in plasma (table 1). Lower lactate production prevents the lowering in pH. Thus, PC stored in PAS-E demonstrated equal or even improved metabolic parameters as compared to PC stored in plasma. The were no bacterial contaminations in the PC included in the study as determined at the end of the storage period.

Expression of platelet activation markers increased most in platelets stored in PAS-C

To evaluate platelet activation status and apoptosis during storage, migration of CD62p (P-selectin) to the membrane and the binding of Annexin V as a marker for apoptotic cells, were measured. In all PC, a significant increase in CD62p positive and Annexin V positive platelets during storage was observed (figure 2a and 2b), which was most pronounced during storage in PAS-C. In addition, activation markers sCD62p and sCD40L significantly increased during storage in all groups (figure 2c and 2d). In figure 2d, PC stored in plasma have lower levels of sCD40L than PC stored in PAS-C and PAS-E. Additionally, in figure 2c, it can be observed that PC stored in plasma have lower levels of sCD62p than PC stored in PAS-C and only slightly higher than PC stored in PAS-E.

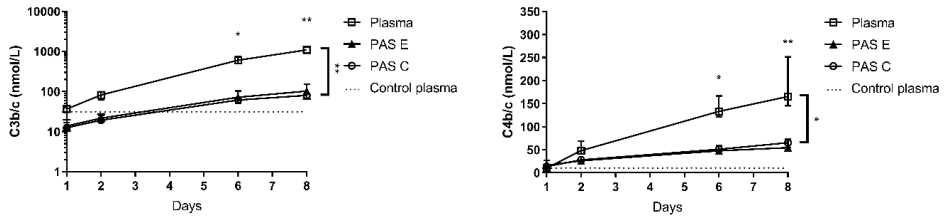

Figure 4 Complement activation products are detected in PC's. *PC's (n=6) stored in plasma, PAS E, or PAS C were sampled at multiple timepoints and a: C3b/c levels; b: C4b/c levels contained in the product were followed over time. The dotted line represents the level in the plasmas of donors that were used as storage solution in these products. The median and interquartile range are shown. Significance between day 1 and 6 or 8 are shown above the trendlines. The differences between groups are indicated by the capped line on the side. *p<0.05; **p<0.01.*

Cell-free DNA and DNA binding proteins: Nucleosomes and HMGB1 are both present in PCs prepared in plasma and PAS

In order to study the influence of storage solution on DAMP release, we measured cell-free DNA in the form of nucleosomes, the basic structural unit of DNA where a segment of DNA is wrapped around histone proteins, and HMGB1 in the supernatants of the concentrates. First nucleosomes, which can be released by every nucleated cell upon activation and/or after cell death, were measured. Regardless of the additive solution,

approximately the same amount of nucleosomes in the supernatants of the concentrates were observed. The level of nucleosomes in the PCs were above the median value of the donor plasmas used for these PCs (figure 3a). In order to investigate whether neutrophils might be a source of the nucleosomes measured in the supernatant, neutrophil activation has been assessed by measuring human neutrophil elastase (HNE) α1-antitrypsin complexes and lactoferrin levels. Although these markers of neutrophil activation increased in platelets after the first day of storage in plasma, no significant changes in these markers over time could be observed in platelet supernatant after storage (figure 3b and 3c). However, we observed a significantly difference between PC stored in plasma and PC stored in PAS-C and PAS-E (figure 3b and 3c). Finally, we measured HMBG1 in the supernatants of PCs at day 2, 6 and day 8 of storage. In the nucleus HMGB1 interacts with nucleosomes, transcription factors, and histones. In all PCs the concentration of HMGB1 that is secreted in the product significantly increased between day 2 and day 8. Additionally, at all timepoints the concentration HMGB1 was significantly higher in PAS-C than in plasma (figure 3d). Although HMGB1 levels were also higher in PAS-E, this was not significant (figure 3d).

Complement is activated during storage of platelets

Previously, it has been described that complement activation occurred in PCs prepared from apheresis during storage in plasma [29]. Therefore, we examined activation of complement in PCs prepared from pooled buffy coats stored in plasma, PAS-C and PAS-E. In PCs prepared in plasma complement activation was observed during storage as evidenced by an increase in C3bc and C4bc levels (figure 4a and 4b). In PCs stored in PAS, levels of C3b/c were ~65% lower as compared to plasma on day 2 and ~90% lower on day 8, while C4b/c levels were ~65% lower at day 8. In order to exclude changes in volume during storage as a cause of the increase of complement activation products or the differences between storage media, IgM and C1-inhibitor concentrations during storage were measured. Both protein concentrations remained stable during storage in the products with different storage solutions (figure S1a and S1b). Together, this data demonstrated that thrombocytes prepared with plasma as storage solution resulted in significant higher complement activation as compared to PCs stored in PAS.

DISCUSSION

Due to potential adverse effects, such as allergic reactions, plasma as a storage solution for platelet concentrates has recently been replaced by PAS. PCs stored in PAS contain a significant reduced amount of plasma (35%), and hence are associated with a decreased incidence of allergic reactions. In this study we demonstrate that PAS-E (similar to PAS C with the addition of potassium and magnesium), is either equal or superior to plasma

as storage solution in maintaining quality characteristics of platelets during storage. In addition, we demonstrate that platelet storage results in an increase of complement activation products and HMGB1 in the platelet products. However, the increase of complement activation products was less prominent in products using PAS as compared to plasma as storage solution.

Although the rate of allergic AR to platelet transfusion significantly decreased after the introduction of PAS, recent data has shown that the incidence of FNHTR after platelet transfusion using platelets stored in PAS remains unchanged. Although multiple factors have been proposed to play a potential role in adverse transfusion reactions, the mechanism behind FNHTR remains unclear. It has been suggested that DAMPs, such as cell free DNA, may be involved in the initiation of FNHTR[22–26]. We now demonstrate that cell-free DNA in the form of nucleosomes and HMBG1 is present in the PCs independent of the storage solution and that the levels of nucleosomes are higher than in plasma of healthy individuals. Given the stable concentration of nucleosomes during storage, and the fact that the PCs are leukoreduced, white blood cells present in the buffy coats seem the most probable source to release nucleosomes. Neutrophil activation in PCs has been assessed by measuring human neutrophil elastase (HNE) α1-antitrypsin complexes and lactoferrin levels. We observed a slight increase in neutrophil activation in PC stored in plasma, whereas this increase was absent in PC stored in either PAS. Given the fact that especially elastase complexes and nucleosomes in PC stored in plasma have a comparable lapse, neutrophil activation as an additional source for nucleosomes cannot be excluded. The release of nucleosomes will therefore most likely mainly take place between blood collection and processing, whereas release during storage from the few contaminating white blood cells seems less likely. Additionally, we demonstrate that HMGB1 levels were significantly higher in PCs stored in PAS-C and increased during storage. This suggests that HMGB1 is released as platelets became activated upon storage, which is supported by an increase of CD62p expression in PC stored in PAS C. Considering that HMGB1 is a DNA binding protein, it would additionally be possible that the source of HMGB1 are PMNs or dead cells. However, as we did not observe an increase in nucleosomes and together with the increase in CD62p, we hypothesize that the main source of HMGB1 are platelets.

There is a lot of controversy over the proinflammatory properties of nucleosomes and their causal pathogenic role in systemic inflammation[42]. It has been demonstrated that nucleosomes themselves are not cytotoxic, as evidenced both *in vivo* by a mice study and *in vitro* with cultured endothelial cells[43,44]. Nonetheless, nucleosomes are composed of DNA wrapped around histones, the latter considered highly cytotoxic, even at low concentrations, mostly due to their strong positive charge. The lack of cytotoxicity of nucleosomes is explained by the structure resulting in balanced electrostatic charges,

especially neutralizing cationic effects of histones. However, only slight distortion of the nucleosome structure, e.g. by bacterial DNases, induces exposure of potentially toxic parts of attached histones resulting in cytotoxicity[45]. In addition, the measurement of histones in plasma is difficult, since cell-free and DNA-free histones are immediately degraded by factor VII-activating protease[45]. Since most of the cell-free histones circulate bound to cell-free DNA, nucleosome measurement seems to be a reliable marker for histones[42].

HMGB1 as a DNA-binding protein, is often bound to cell-free DNA and may act as a proinflammatory mediator due to its cytokine-like function[22,46,47]. Activated platelets upregulate and release HMGB1 in various inflammatory conditions[48]. Moreover, nucleosomes, protein-free DNA as well as HMGB1 may ligate Toll-like receptors 2, 4 and 9, thereby inducing and/or modulating an inflammatory response[42,49]. Platelets also have a major inflammatory and immune function, essentially through their Toll-like Receptors (TLRs) and Sialic acid-binding immunoglobulin-type lectin (SIGLEC)[50]. Autocrine activation of platelets from PCs due to DAMPS release and Pattern Recognition Receptor expression cannot be excluded, as it is the case for CD40/CD40L[51].

Together, this suggests that potentially cell-free DNA in the form of nucleosomes and HMGB1 might be involved in the pathogenesis of FNHTR. Although the amount of nucleosomes contained in the PC is rather low as compared to the levels measured during systemic inflammation in patients, it cannot be excluded that they may induce an inflammatory response. In addition, patients receiving PC transfusion often suffer from neutropenic fever, which may camouflage clinical signs of an inflammatory response induced by platelet transfusion.

It has previously suggested that complement activation plays a role in the initiation of FNHTR[29]. Interestingly, we found complement activation products in all PC products independent of the storage solution, albeit in lower concentrations in PCs with PAS as compared to plasma. The lower concentration in the PAS products is most likely due to dilution of plasma by PAS. Nonetheless, complement activation products accumulated during storage in PC prepared in both plasma and PAS. Although we measured complement activation by the generation of C4bc and C3bc, also C3a and, considering downstream activation of C5, C5a will be released as well[31]. The anaphylatoxins, C5a and to a lesser extent C3a, although rapidly degraded through carboxypeptidases in plasma, may activate platelets in the products[52–56]. C3a and C5a are key mediators of inflammation and exert their function through binding to their receptors[57]. In addition, the C3a and C5a receptors are expressed by many leukocytes as well as by endothelial cells. Upon platelet transfusion, the complement activation products and probably anaphylatoxins contained in the product are administered to the patient. One could hypothesize that these transfused complement

activation products may induce further complement activation in the patient's circulation. This may subsequently induce an inflammatory response in the recipient through the activation of immune cells and exert vasoactive effects on endothelial cells. Here it is important to stress on the fact that also cleaved forms of anaphylatoxins, e.g. C5adesArg, may retain their vasoactive properties. However, the effects on vascular permeability are 100 times weaker as compared to the non-cleaved forms. In summary, these active complement products contained in the PC may induce an inflammatory response in the recipient, becoming clinically overt as FNHTR.

An advantage of the current study design was that the platelets from the PC designated to the different groups (plasma, PAS-E and PAS-C) all originated from the same buffy coat pool. With this design we could eliminate confounding factors arising from different donor-pool compositions. A limitation of this study is the limited number of clinical products, which decreases the power of the study. Another limitation is that we did not study the effects of these products in recipients. Finally, for this design 15 buffy coats were needed to produce a pool large enough to supply the three study arms. Under standard practice In the Netherlands 5 donors are used to produce one buffy-coat PC. Thus, this enlargement of the pool may have concealed effects of a donor which would have affected the outcome in a smaller pool.

CONCLUSIONS

To summarize, we demonstrated that cell-free DNA in the form of nucleosomes and HMGB1 is present in platelet concentrates. We also established that HMGB1 levels increase over time and is significantly higher in PC stored in PAS-C. In addition, we observed a marked increase of complement activation products in the concentrates. Both DAMPs, such as nucleosomes and HMGB1, and complement may potentially induce systemic inflammation in the recipient upon transfusion and hence be involved in the pathogenesis of FNHTR. Further research is needed to investigate the role of these complement activation products in the pathogenesis of FNHTR.

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Author Contributions
All Authors contributed to the design of the study, acquisition, analysis, and interpretation of data. Y.W. wrote the manuscript and J.L., D.K., F.C., A.B., and S.S. revised the work critically for important intellectual content. All authors approved the final version to be published and agreed to be accountable for all aspects of the work in ensuring that

questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

The Authors declare no conflict of interest

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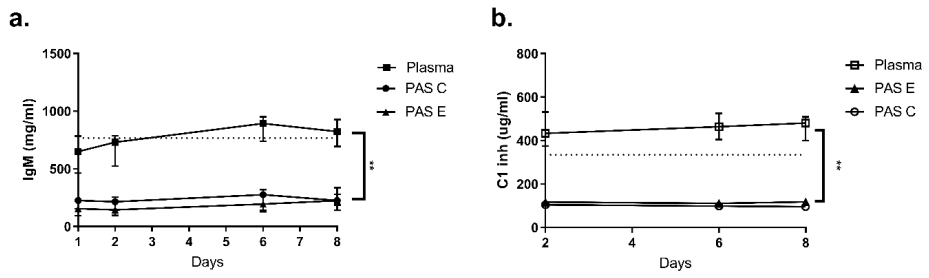
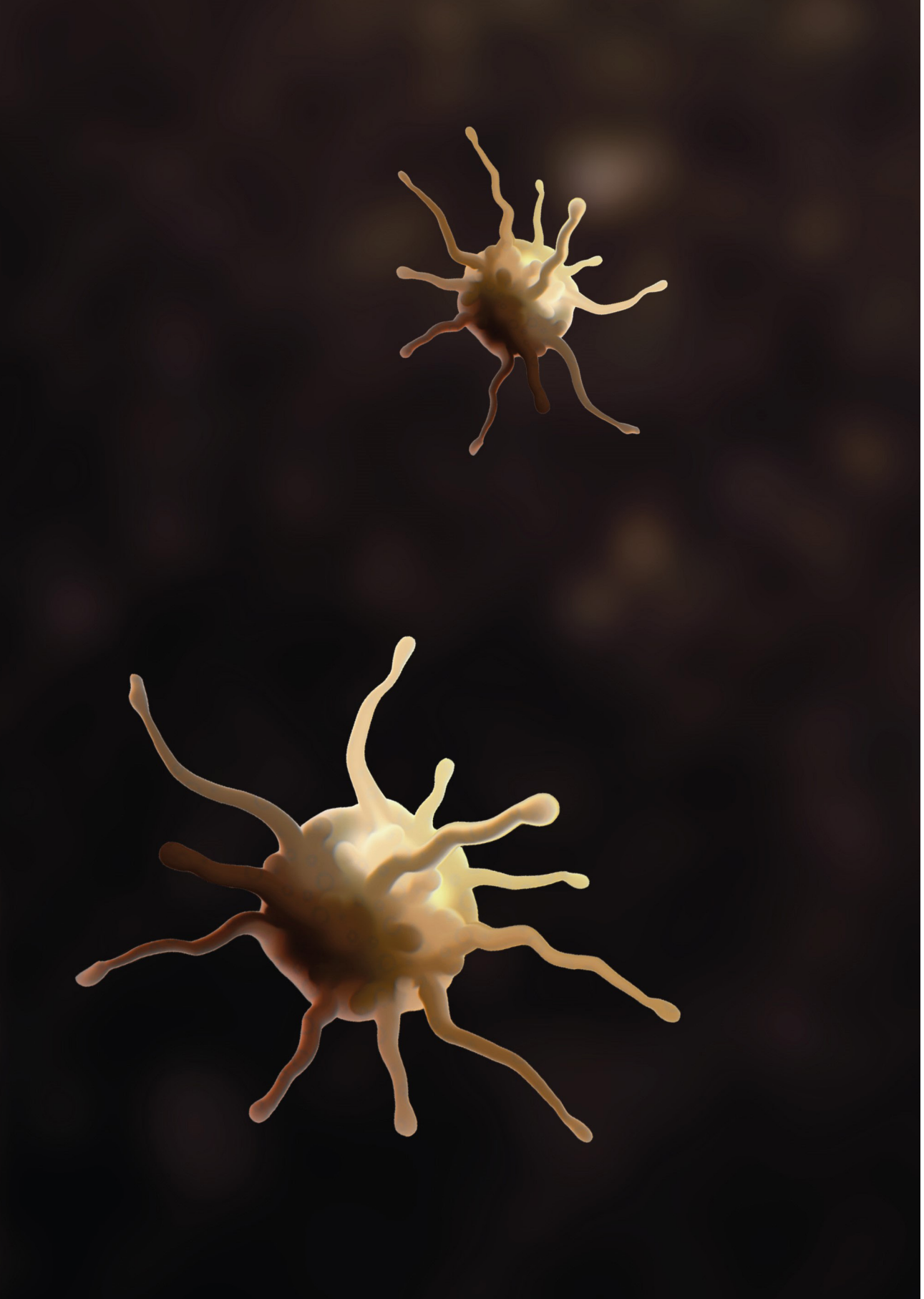
SUPPLEMENTARY



Supplemental Figure 1 Changes in volume during storage were not the cause of the increase of complement activation products or the differences between storage media*. PC's (n=6) stored in plasma, PAS E, or PAS C were sampled at multiple timepoints and a: IgM levels; b: C1-inhibitor levels contained in the product were followed over time. The dotted line represents the level in the plasmas of donors that were used as storage solution in these products. The median and interquartile range are shown. The differences between groups are indicated by the capped line on the side. **p<0.01.*



CHAPTER 5

Blood collection technique and processing impacts the contents of damage-associated molecular pattern (DAMP) contents of platelet products

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Manuscript in preparation

ABSTRACT

Background

Platelet concentrates (PCs) available for transfusion are prepared either from whole blood donations or by platelet apheresis procedures. The whole blood donations method can be further divided in the platelet rich plasma (PRP) method and buffy-coat (BC) method. The predominant method has globally shifted from PRP towards BC. This has led to the production of mostly apheresis-PC and BC-PC.

Study design and methods

Single donor apheresis-PC and BC-PC were produced in a regional setting of the Dutch Blood Establishment. After production samples were collected from PC and platelet activation markers, damage-associated molecular patterns (DAMPs) and complement activation products were measured. A comparison was made between apheresis-PC and BC-PC.

Results

Higher levels of CD62p+ platelets were observed after stimulation in apheresis-PC than BC-PC. The levels of nucleosomes, and C4b/c were significantly increased in BC-PC as compared to apheresis-PC. In addition, the levels of elastase-α1-antitrypsin complexes (EA) were higher in BC-PC from morning blood collections than in apheresis-PC or BC-PC from afternoon blood collections, which indicates increased neutrophil activation.

Conclusion

Apheresis-PC can be better activated than BC-PC. BC-PC contain higher levels of nucleosomes and EA, most likely due to the presence of neutrophils in the BC. In addition, higher levels of complement activation products were observed in BC-PC.

INTRODUCTION

To maintain hemostasis, an adequate number of functional active platelets is required. In general, platelet counts below <10.000 /µL go in concert with a significant risk of bleeding. In thrombocytopenic patients, platelet transfusion are administered to treat active bleeding as well as in preparation for an invasive procedure that could cause bleeding. Prophylactic platelet transfusion are routinely administered, in patients suffering from hematological malignancies, such as acute myeloid leukemia, treated with intensive chemotherapy. Platelet concentrates (PC) can be either prepared from whole blood collection and apheresis. Apheresis is a procedure where blood is taken from an individual and returned after platelet-rich plasma is separated from it. Two methods of preparing PCs from whole blood are in use of which the predominant method in Europe is the buffy-coat (BC) method and in the United States the platelet rich plasma (PRP) method[1]. Whole blood collection is followed by centrifugation, to isolate platelets from red blood cells, afterwards PRP or the BC is used for further processing. For the latter, BC of five donors need to be pooled to get the right amount of platelets for the concentrate, hence the name BC-pooled PC. A global shift from PRP-derived PC toward the use of BC-derived PC has been made. BC-derived PC is widely applied, especially in the European countries, since 1995[2]. Nowadays, platelet preparation by BC pooling method is used in Europe, United States, South America and in some countries in Asia[3]. Previous data indicates that viability and metabolic function of platelets are maintained in both, BC-PC and apheresis derived PC, respectively, as reflected by swirling, glucose, lactate levels, and pH during the 5-day storage period[2,4]. In addition, the average increment in platelet count 2 hours after transfusion of one unit of BC-derived PC is similar to a unit of single donor (SD)-apheresis[2]. Previously, our group demonstrated the presence of nucleosomes and complement activation products in BC-PC and SD-apheresis[5,6]. In addition, we established an association between increased levels of DAMP/complement activation products and adverse reactions (AR) to the product[6]. We hypothesized that the release of nucleosomes will probably, occur between blood collection and processing and is affected by the presence of neutrophils. During production of BC-PC neutrophils are present until leukofiltration, which takes place after pooling of donors. In apheresis-derived PC neutrophils are already separated from platelets during collection, which limits the time for nucleosome release. This may result in lower nucleosome concentrations in apheresis-derived PC compared to BC-PC.

In this study we aimed to analyze platelet activation, complement, DAMPs and neutrophil activation in different types of PC. Quality control samples of PCs were collected from PCs derived from apheresis and BC, respectively. Apheresis-derived PC stored in plasma were compared to apheresis-PC stored in PAS-E, and BC-derived PC stored in PAS-E from morning blood donations were compared to afternoon donations.

MATERIALS AND METHODS

Blood collection and processing

In this study samples collected from single-donor apheresis (SDA)-PC stored in plasma and PAS-E ((TPAS+, Terumo BCT) (69mmol/L NaCl, 5mmol KaCl, 1,5mmol $MgCl_2$, 10mmol Na_3-citrate, 26mmol NaH_2PO_4/Na_2HPO_4, 30mmol Na-acetate), and BC-derived PC stored in PAS-E from morning and afternoon whole blood collections have been analyzed. SDA-PCs and BC-derived PC were produced in accordance to the institution's guidelines and practices (Sanquin). All PCs were leukoreduced (<10^6 white blood cells/bag). A schematic timeline is shown in Figure 1A. Note that morning whole blood donations are processed to BC on the same day and stored as BC overnight till further processing. Afternoon whole blood collection are stored overnight as whole blood until further processing.

Sample preparation

During the production of PC a small volume (~10mL) of the product is collected in a small blood bag (~10mL) that is sampled for quality assessment and bacterial contamination. Extra samples were taken from this small bag for the analysis of this study within 3h after production. One part of each sample was subsequently subjected to slow centrifugation (490g, 10min at RT), and PRP was subsequently subjected to fast centrifugation (4800g, 10min, RT) to collect the supernatant of the product for analysis of soluble factors. The other part of the sample was prepared for flow cytometry analysis as followed: 100µL PC was added to 100µL storage buffer (10mM HEPES, 140mM NaCl, 3mM KCl, 0.5mM $MgCl_2.6H_2O$, 0.5 mM $NaHCO_3$, and 10 mM D-Glucose, pH 7.4) and subjected to slow centrifugation (490g, 10min at RT). Supernatant was removed and the platelet were resuspended in 150µL storage buffer for analysis by flow cytometry.

Flow cytometry analysis of platelet activation

Platelets were activated by 1 µM thrombin receptor-activating peptide 6 amide (TRAP-6) (Bachem, Bubendorf, Switzerland) and stained with anti-CD61-FITC (platelet glycoprotein GPIIIa, Beckman Coulter, Immunotech art no 555753 and anti-CD62p-PE (P-selectin, Beckman Coulter, Immunotech art no. A07790) in storage buffer. After staining, the samples were fixed using 0,5% PFA, and analyzed within two hours by 3 Laser FACS Canto II+HTS (BD Biosciences, Erembodegem, Belgium).

Quantification of soluble mediators

Mitochondrial (mt)DNA was purified from the supernatants of PC using the QIAamp DSP Virus kit (Qiagen). A digital droplet PCR was performed according to manufactures instructions: the ddPCR system included automated droplet generator and reader from Bio-Rad, (QX200 Droplet Digital PCR, Bio-Rad, Hercules, California, USA) and a T100 thermal cycler (Bio-Rad). For mtDNA quantification, a primer and probe targeting the mitochondrially encoded NADH dehydrogenase 1 (MT-ND1) NADH dehydrogenase 1 (ND1), Human (FAM) (Bio-Rad, unique assay ID dHsaCNS669425578) was used. Results were analyzed using the Quanta soft software, and absolute values of mtDNA (ND1) (copies/µl) were calculated for each DNA sample. Nucleosome levels and elastase-α1-antitrypsin complexes (EA) were measured by ELISA, as previously described[7]. Complement activation was determined by measuring complement activation products C3b/c and C4b/c by ELISA[7]. Heme was detected in the supernatant of PC using QuantiChromTM Heme Assay Kit (BioAssay Systems art no. DIHM007. In addition, the pellet of PCs was lysed using H_2O for 5min at RT and heme was subsequently measured.

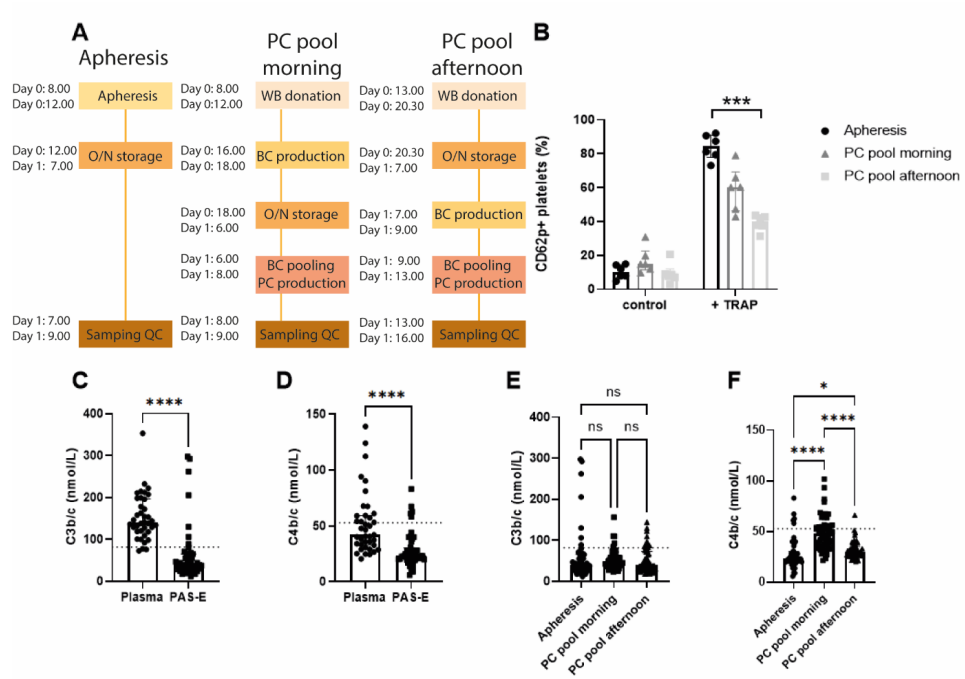

Figure 1 CD62p expression on platelets isolated from platelet concentrates (PC) and the levels of complement activation products contained in PC. *A: schematic timeline of production process of Apheresis-PC stored in plasma or Platelets additive solution (PAS)-E and buffy coat (BC)-PC from morning and afternoon whole blood (WB) donation stored in PAS-E. Apheresis-PC are produced in plasma or PAS-E and stored overnight until sampling for quality control (QC). BC-PC are produced from morning or afternoon WB donation. Subsequently WB of morning donations is separated producing BC and stored overnight until further processing and sampling. WB of afternoon donations is kept overnight and separated and processed to PC the following morning. B: Washed platelets from apheresis-PC stored in PAS-E and BC-PC stored in PAS-E were incubated with storage buffer or Thrombin Receptor Activating Peptide (TRAP) (1uM). n=6 ***p<0.0005. The levels of C: C3b/c; and D: C4b/c were analyzed in apheresis PC stored in plasma or PAS-E. The levels of E: C3b/c; and F: C4b/c were analyzed in apheresis and PC from pooled Buffy coat (BC). PC in plasma n=38, PC stored in PAS-E n=50. *p<0.05; ****p<0.0001.*

Statical analysis

Mann-Whitney test was performed to compare apheresis PC stored in plasma to apheresis PC stored in PAS-E. Kruskall-Wallis test with additional Dunn's multiple comparisons test was performed to analyze statistical differences between PC stored in PAS-E (apheresis PC and BC PC from morning and afternoon).

RESULTS

More platelet activation in apheresis-PC than BC-PC after TRAP stimulation

We first assessed platelet activation by analyzing expression of platelet activation marker CD62p (P-selectin) during rest and after incubation with TRAP. After stimulation with a suboptimal concentration of TRAP we observed a higher percentage of CD62p+ platelets in apheresis-PC stored in PAS-E than in pooled BC-PC stored in PAS-E (morning and afternoon donations)(Figure 1B). Furthermore, platelets from BC-PC from morning donations seem to become activated more than platelets from BC-PC from afternoon donations. No significant differences were observed without TRAP stimulation. No analysis were done on apheresis-PC stored in plasma, because these were no longer produced at the time of these experiments.

Complement activation

To analyze complement activation in PC, we analyzed levels of complement activation products of C3 and C4. The levels of complement activation products C3b/c and C4b/c are affected by the amount of plasma that is contained in the product, as evidenced by the reduction observed between apheresis-PC stored in plasma and apheresis-PC stored in PAS-E (Figure 1C and 1D). No significant differences in the levels of C3b/c were measured between the different types of PC (Figure 3E). Surprisingly, we observed significantly elevated levels of C4b/c in BC-PC from morning blood collections compared to apheresis-PC in PAS-E and BC-PC from afternoon collection (Figure 3F).

DAMPs are present in apheresis-PC and BC-PC

In a next step we analyzed DAMPs, specifically mtDNA, nucleosomes and heme, in the PCs. No significant differences in mtDNA concentrations were observed (Figure 2A and 2E). We observed that the nucleosome levels of most apheresis-PC stored in plasma and BC-PC stored in PAS-E are above the concentrations found in plasma of healthy controls (Figure 2B and 2F). In addition, apheresis-PC stored in PAS-E have significantly lower levels of nucleosomes compared to apheresis-PC stored in plasma, but also compared to BC-PC stored in PAS-E (Figure 2B and 2F). The levels of heme in the supernatant of apheresis-PC and BC-PC from afternoon donations seem to be slightly higher than in BC-PC from morning donations (Figure 2D). In addition, we observed a statistical lower heme levels in apheresis-PC stored in PAS-E as compared to BC-PC stored in PAS-E of the lysed pellet (Figure 2H). This suggests that there are more red blood cells present in BC-PC than in apheresis-PC. No measurements were done on apheresis-PC stored in plasma. Apheresis-PC stored in PAS-E have significantly lower levels of EA compared apheresis-PC stored in plasma (Figure 2C). Strikingly, BC-PC from morning collection had increased EA concentrations compared to other PC stored in PAS-E (Figure 2G).

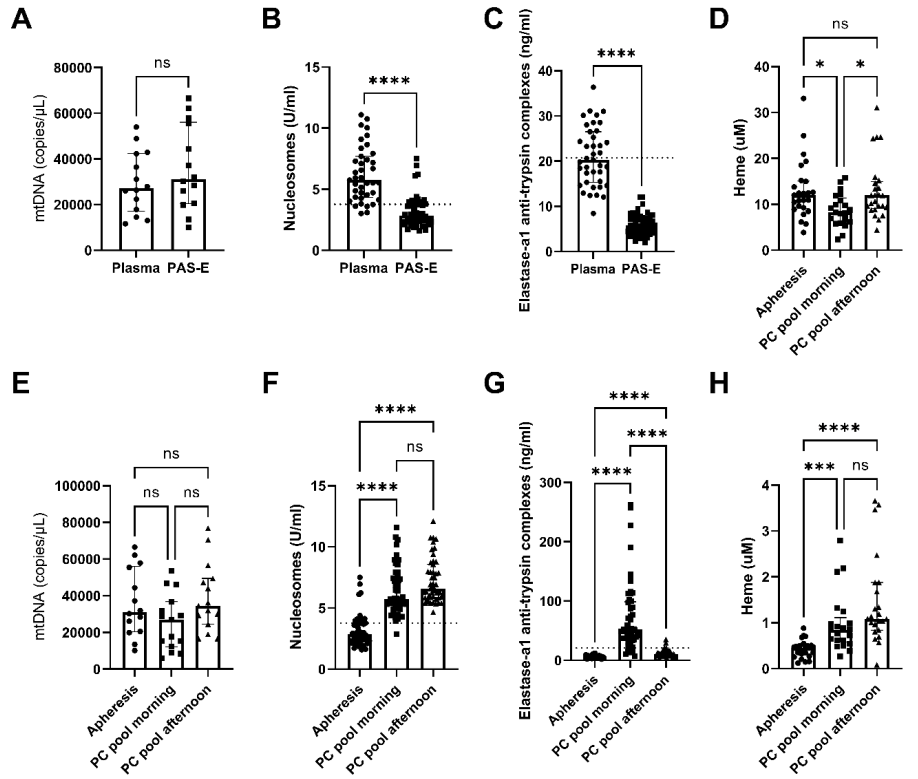

Figure 2 The levels of Damage-associated molecular patterns (DAMPs) contained in Platelets concentrates (PC). *The levels of A: mtDNA; B:nucleosomes; and C: elastase-α1-antitrypsin complexes (EA) were analyzed in apheresis platelets stored in plasma or Platelet additive solution (PAS)-E. D: the levels of heme were measured in the supernatant of PC. The levels of E: mtDNA; F: nucleosomes; and G: EA were analyzed in apheresis and PC from pooled BC. H: the levels of heme were measured in the lysed pellet of PC. PC in plasma n=38, PC stored in PAS-E n=50. *p<0.05; ***p<0.0005; ****p<0.0001.*

DISCUSSION

PC can be prepared from whole blood donation or by apheresis. In the last decade a global shift from PRP-derived PC toward BC-derived PC has occurred and nowadays PC preparation by BC pooling is standard practice in Europe, US, South America and in some countries in Asia[3]. This means that were previously PC were produced from PRP for both whole blood donation and apheresis procedures, now PC are produced from BC and apheresis, respectively. This alternative production method may lead to differences between BC-PC and apheresis-PC. In this study we observed variation in platelet,-complement,-and neutrophil activation and concentrations of DAMPs between types of PC.

Here we demonstrated that during rest there is no difference in platelet activation between apheresis-PC and BC-PC. However, after stimulation with a suboptimal concentration of TRAP we observed higher activation by apheresis-PC. A previous study indicated that CD62p was not differently expressed on apheresis-PC or BC-PC[8]. However, exposure of phosphatidylserine (PS), a marker for apoptosis, was found to upregulated by a higher percentage of platelets on day 5 of storage in apheresis-PC as compared to BC-PCs[8]. Moreover, an association was observed between PS exposure and the formation of micro vesicles (MV)[8]. Due to pooling, there is an increased risk for viral and bacterial contamination. Viral infections became very rare because of improved donor screening techniques. However, in non-pathogen-inactivated products bacterial contamination remains an issue. In a large retrospective study all cases in which platelets that tested positive in the BacT/ALERT® bacterial screening, that had already been transfused, during 2013–2019 were examined and this identified a higher percentage of positive BC-PC than apheresis-PC[9].

Additionally, in this study we observed higher levels of nucleosomes and EA, which is a marker for neutrophil activation, in BC-PC than in apheresis-PC. This suggests that neutrophils contained in the BC are activated and/or die between blood collection and processing in BC-PC resulting in a higher DAMP content as compared to apheresis-PC where neutrophils and platelets are separated during collection. In addition, we observed increased activation of complement and neutrophils (EA complexes) in BC-PC from morning collections as compared to BC-PC from afternoon collections. This indicates that storing platelets in whole blood or BC overnight until further processing affects neutrophils. Most literature has focused on quality of platelets such as storage lesion while comparing apheresis-PC and BC-PC. We previously, observed an association between AR and complement activation products and DAMPs[6]. In this study we aimed to link different PC production procedures to DAMPs and activation of neutrophils and complement with regard to their role in adverse reactions after transfusion[10–12].

This study demonstrate that the apheresis and whole blood collection techniques have an effect on PC. Apheresis-PC are easier activated by stimulation and have lower levels of DAMPs and complement activation than BC-PC. Furthermore, the different processes of BC-PC regarding morning and afternoon blood collections, also seems to have an effect on platelets and complement and neutrophil activation and subsequent DAMPs contained in the product.

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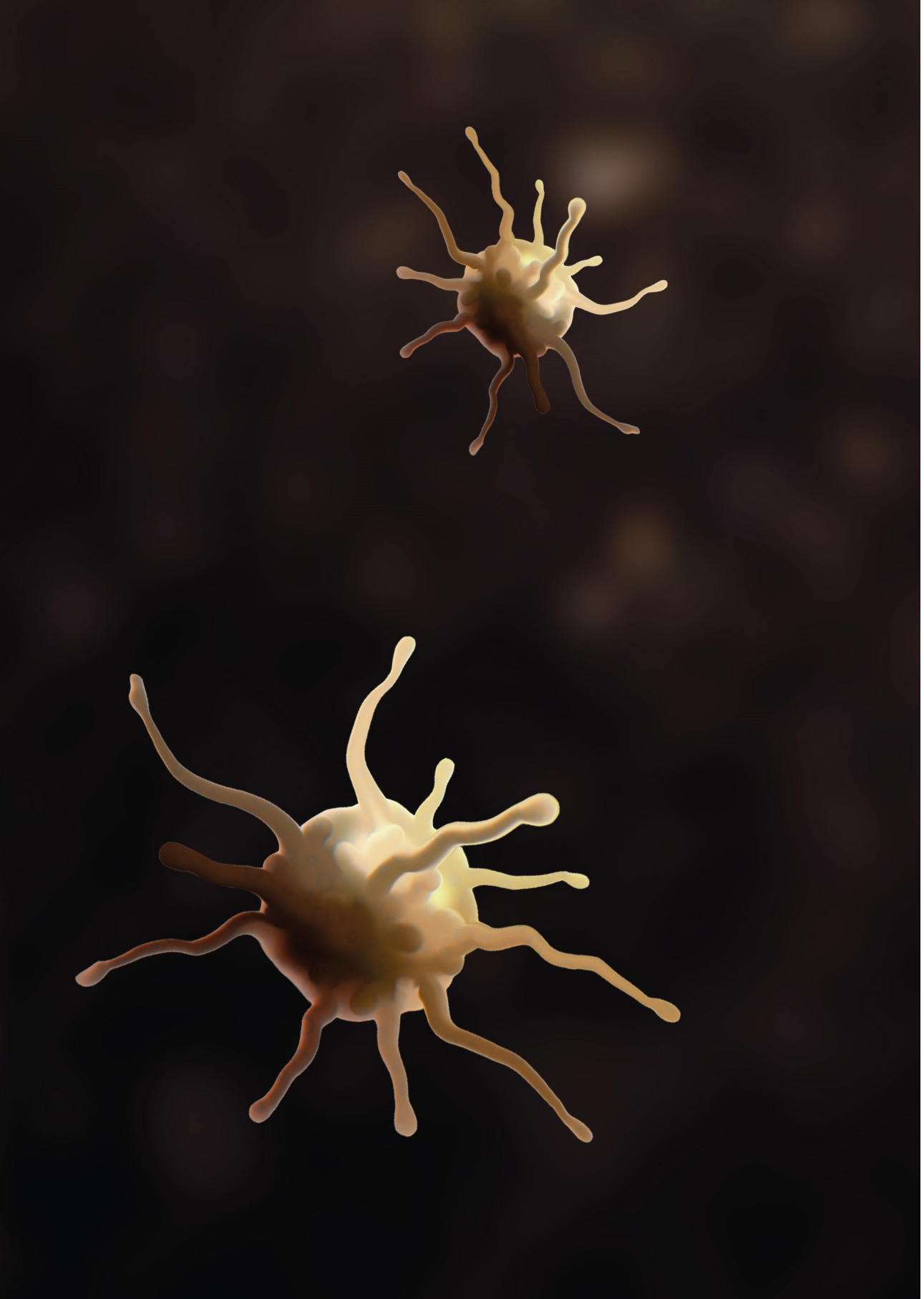

CHAPTER 6

DAMPS and complement activation in platelet concentrates that induce adverse reactions in patients

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Transfusion, 2022

ABSTRACT

Background
Patients with severe thrombocytopenia due to bone marrow failure and after chemotherapy are still treated with platelet transfusions. Platelet concentrates (PC) are associated with a high incidence of adverse reactions (AR). Platelet-derived damage-associated molecular patterns (DAMPS) and complement were proposed to play a role in the pathology of AR.

Study design and methods
Single donor apheresis platelet concentrates (SDA PCs) were produced in a regional setting of the French Blood Establishment. After transfusion samples were collected from PC and possible AR in patients were recorded. Platelet activation markers, High mobility group box 1 (HMGB1) and complement activation products (CAP) were measured. The correlation between platelet activation, and HMGB1 and complement activation was analyzed.

Results
A total of 56 PC were included in the study. 30 PC induced no AR, and 26 induced AR (Febrile non-hemolytic transfusion reaction n=16; Atypical Allergic Transfusion Reactions n=11; hemodynamic instability n=5) in the patients. The levels of P-selectin, sCD40L, HMGB1, C3b/c and C4b/c were all significantly increased in PC that induced AR following transfusion in patients.

Additionally, HMGB1, C3b/c and C4b/c were positively correlated with P-selectin and sCD40L.

Conclusion
In this study we observed an association between HMGB1 and CAP and the incidence of AR. Furthermore, we demonstrated that both HMGB1 and complement activation were correlated to platelet activation.

INTRODUCTION

Therapeutic platelet transfusions are important measures to treat bleeding in severe thrombocytopenic patients. Prophylactic platelet transfusion is most commonly applied to patients suffering from hematologic malignancies and receive myeloablative chemotherapy. In comparison with other blood products, platelet concentrates (PC) are associated with the highest incidence of transfusion related adverse reactions (AR)[1]. According to the Dutch hemovigilance report of 2020 this has remained unchanged (0,44AR /1000 PC versus 0,26AR/1000 RBC and 0,04AR/1000 SD-treated plasma). Among these reactions, febrile non-hemolytic transfusion reactions (FNHTR), as well as allergic and anaphylactic reactions are the most frequently reported. By replacing plasma as storage solution for platelets by platelet additive solution (PAS), the rate of allergic and anaphylactic reactions has decreased. However, the incidence of FNHTR remained unchanged[2].

It has previously been shown that platelet-derived damage-associated molecular patterns (DAMPs), such as mtDNA and High mobility group box 1 (HMGB1) are associated with AR[3,4]. Inflammatory mediators, such as complement anaphylatoxins increase during PC storage and also have been suggested to play a role in AR[5,6]. However, so far, the role of complement activation in AR has not been studied.

In this study we measured DAMPs and complement activation products (CAP) in SDA-PC that induced or did not induce AR in patients. Additionally, we analyzed whether the levels of DAMPs and CAP contained in the PC correlated to the levels of platelet activation markers and to each other.

MATERIAL AND METHODS

Study design
SDA-PC samples from a previous cohort were used[7]. SDA-PCs were produced in a regional setting of the French Blood Establishment (EFS) before using 100% pathogen reduction of platelets. All SDA-PCs were leukoreduced (<10^6 white blood cells/bag), suspended in 35% native plasma/65% PLT additive solution (AS; InterSol, Fenwal, la Châtre, France; or SSP+TM, MacoPharma, Mouveaux, France), and stored at 22°C ± 2°C under gentle rotation and shaking (60 rpm) until being issued for transfusion. The maximum storage time was 5 days. Within 3h after transfusion samples were collected from PC and possible AR in patients were recorded according to the ISBT scaling system. If transfusion took place overnight, the processing time never exceeded 12h. The previously reported study included 2850 PC samples (issued to patients in the 2

university hospitals), of which a total of 140 ARs were reported[7]. Platelets incriminated in a AR were immediately shipped back the Blood Bank for investigation. All samples obtained from PCs not associated with AR were used as controls. All considered cases were scored as 3 (severe) according to the ISBT scaling system, i.e., necessitating medical assistance, with no grade 4 (lethal) cases observed in this survey. The cases with accountability grades of 3 ("probable") and 4 ("certain")—in terms of accountability according to this international scale—were retained for the survey, and the "unlikely" and "possible" cases were discarded. After having excluded hazards obviously linked to the causal pathology in the transfused patients as well as the infectious (TTBI) causes, the diagnosis of inflammatory-type AR was made on the immediate observation/report of 1) FNHTR, generally associated with fever, rigors, and/or chills; 2) Atypical Allergic Transfusion Reactions (AATR), which commonly involves erythematous rash, urticaria, and/or pruritus or more severe reactions with angioedema, which are combined with the further discharge of typical allergic biology, such as elevated serum tryptase, histamine, or IgE; and 3) in rare occasions, Hemodynamic instability (HT) with tachy-/bradycardia and/or hyper-/hypotension resembling non-septic shock. We also excluded all cases of ATR with a known Ag/Ab conflict such as allo-immunization (against the donor's HLA and/or HPA), post-transfusion purpura, or refractoriness (and bleeding). This was in accordance with previous work (PMID: 28720587 and PMID: 24830754). Supernatants were collected after centrifugation (402 g: 10 min) and aliquots frozen at −80°C within 12h after transfusion. PCs were collected from non-remunerated blood donors who signed an informed consent form, approved by the ethical committees of "Etablissement Français du Sang"

Table 1 Demographics of patients that received transfusion.

		Control (n=30)	Transfusion related adverse reactions (n=26)
Sex (%)	Male	60	62
	Female	40	38
Age (Years)	Median	46.4	51.0
	SD	11.6	9.2

Quantification of soluble factors in PC

P-selectin, and soluble CD40 ligand (sCD40L) were quantified in PC supernatants using Luminex technology as described previously[8]. HMGB1 and nucleosome levels were measured by ELISA, as previously described[4,6]. Tumor necrosis factor α (TNFα) and interleukin-8 (IL-8) were determined by ELISA (Peli-Kine-compact, Sanquin Reagents, Amsterdam, the Netherlands), according to the manufacturer's instructions. Neutrophil activation was analyzed by measuring elastase-α1-anti trypsin (EA) complexes by ELISA[6]. Complement activation was determined by measuring CAP C3b/c and C4b/c by ELISA[6].

Since repetitive thawing and freezing of plasma samples induces complement activation, CAP have only been measured in non-thawed samples. Groups: no AR (n=7); AR (n=7).

Statistical analysis

Results are represented as median with interquartile range. Groups were compared using non-parametric tests (Mann-Whitney test). Correlation between groups were calculated using spearman rank correlation. For correlations we only used non-thawed samples (n=15). Bonferroni post-hoc analysis was used for multiple testing. Statistical analyses and figures were computed with GraphPad Prism software v 9.1.1 (GraphPad Software, La Jolla, CA, USA). Results were considered significant at p<0.05

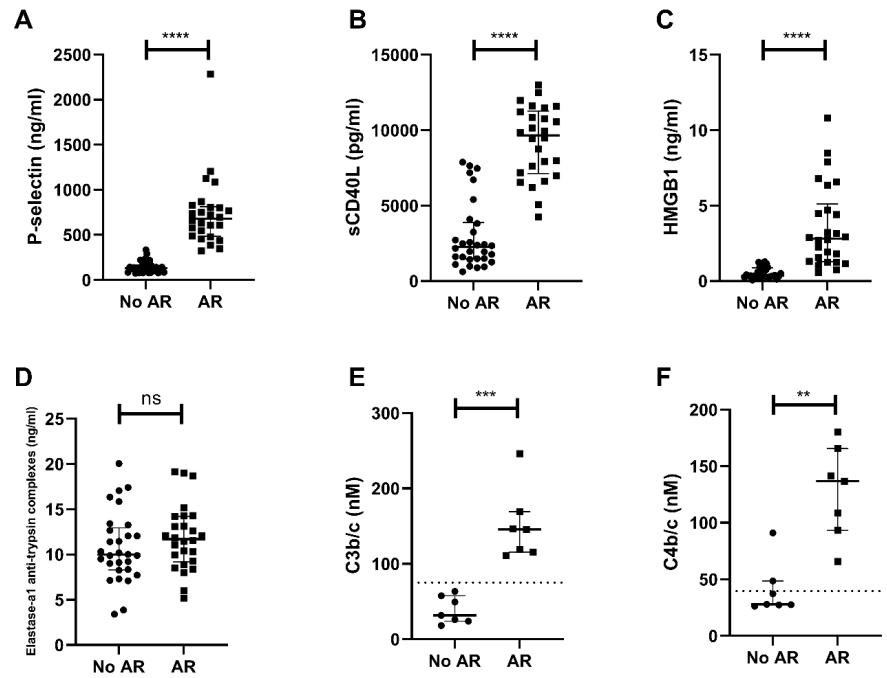

Figure 1 Markers of platelet, -and complement activation and HMGB1 are increased in PC that induce AR in patients. *A: P-selectin levels (no AR median: 130.7ng/mL IQ: 84.35-166.7; AR median:677.3ng/mL IQ: 484.8-812.8); B: sCD40L levels (no AR median: 2254pg/mL IQ: 1472-3887; AR median: 9658pg/mL IQ: 7136-11272); C: HMGB1 levels (no AR median: 0.35ng/mL IQ: 0.275-0.8775; AR median: 2,81ng/mL IQ: 1.298-5.115); D: Elastase-a1-anti-trypsin complex levels (no AR median: 10.02ng/mL IQ: 8.298-12.95; AR median: 11.7ng/mL IQ: 9.186-14.21); E: C3b/c levels (no AR median: 31.7nM IQ: 23.95-57.55; AR median 145.5nM IQ: 115.5-169); F: C4b/c levels (no AR median: 27.95nM IQ: 27.4-48.55; AR median: 136.7nM IQ: 93.3-165.5). The dotted line represents normal value in plasma. Data represented as median + IQ range. **p<0,005; ***p<0,001; ****p<0,0001.*

RESULTS

Groups

A total of 56 PC from 56 non-consecutive transfusions from a previous cohort were included in this study[7]. 30 PC induced no AR, and 26 induced AR in patients. Observed AR (n=26) were FNHTR (n=16), AATRs (n=11), HT (n=5). The 5 patients with hemodynamic instability developed more than one AR. Hemodynamic instability was observed in combination with FNHTR (n=3), AATR (n=1), and FNHTR and AATRs (n=1). Recipients in the control and AR group were of similar age and had a similar distribution of males and females (Table 1).

Higher platelet activation in products that induce AR

We first assessed platelet activation by analyzing platelet activation markers P-selectin and soluble CD40 ligand (sCD40L) in the supernatants of the products. Increased platelet activation was observed in PC that induce AR, as P-selectin and sCD40L levels were significantly higher in products that caused AR in patients compared to products that did not cause AR (Figure 1 A and B).

DAMPs are present in PC that cause AR

In a next step we analyzed DAMPs, specifically HMGB1 and nucleosomes, in the PCs. Significantly higher HMGB1 levels were measured in PC that induced AR in patients as compared to PC that induced no reaction (Figure 1C). In contrast, there was no difference in nucleosome levels between the 2 groups. Nucleosome levels were below detection limit (<1 U/ml). In addition, based on low EA levels there was no evidence of neutrophil activation in any product included in this study (Figure 1D).

Complement activation

To evaluate complement activation in these products, we analyzed CAP levels of C3 and C4. As mentioned above, since repetitive freeze-thawing cycles induced complement activation, complement activation in the form of C4b/c and C3b/c was only measured in unthawed samples. We observed increased complement activation in PC that induced AR in patients, as levels of C3b/c and C4b/c were significantly higher in PC that induced AR (Figure 1E and F).

Cytokines

To exclude the influence of inflammatory factors produced by leukocytes during the apheresis - and production process, levels of inflammatory cytokines tumor necrosis factor-alpha (TNF-α) and Interleukin 8 (IL-8) were measured in the PCs. TNF-α and IL-8 levels were below the respective detection limit in all tested PC (data not shown).

Correlation between platelet activation, HMGB1 and complement activation

Figure 2 shows representative correlations of the whole data set. Increased platelet activation was significantly correlated with CAP levels (Figure 2A-D). In addition, increased levels of P-selectin and sCD40L were also associated with increased levels of HMGB1 (Figure 2E and F). On the contrary, there was no significant correlation between HMGB1 and CAP (Figure 2 G and H). Interestingly, the correlation figures clearly indicated a separation of the groups (Figure 2). Although the whole data set results in a significant correlation, we did not observe a correlation within the groups AR and no AR (data not shown).

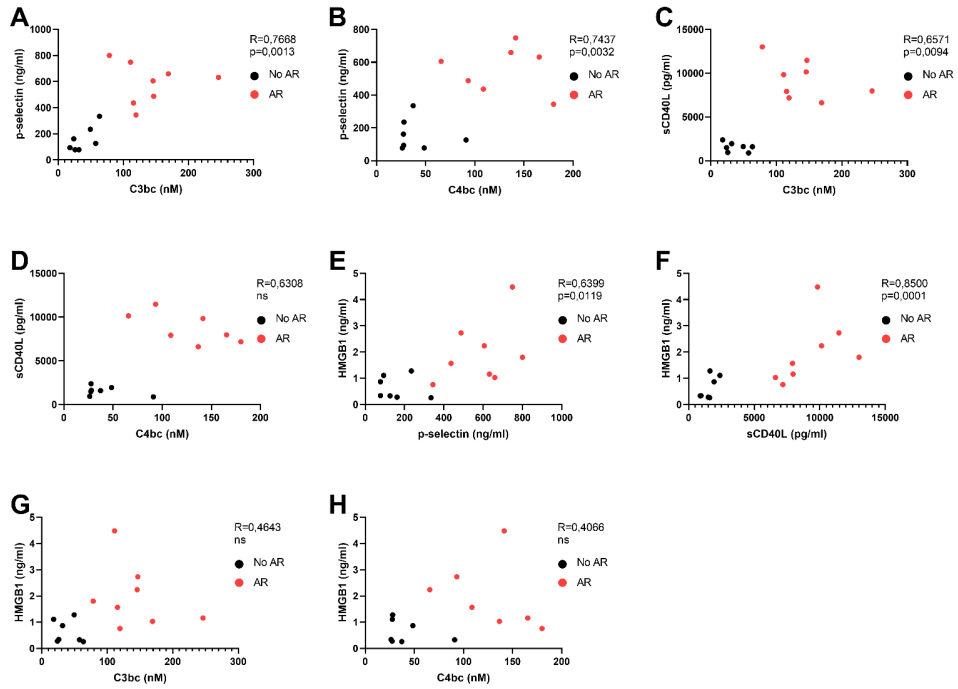

Figure 2 Increased levels of platelet activation markers correlate with complement activation. *Spearman correlations between P-selectin, sCD40L, C4b/c, C3b/c, and HMGB1. R denotes correlation coefficient; p<0.05 is considered to be statistically significant, Bonferroni correction was applied for multiple testing.*

DISCUSSION

AR following PC transfusion are unwanted as they negatively affect the outcome of transfusion and could increase morbidity and mortality in patients. Although most AR are usually non-life-threatening, they cause patient discomfort, hospital admission or prolong hospital stay. Moreover, AR may entail laborious and costly clinical and laboratory investigation to exclude infection or another cause of the clinical symptomatology.

In this study we observed elevated levels of platelet activation markers, HMGB1, and CAP in SDA-PCs that induced AR. Moreover, we observed that HMGB1 and CAP levels correlated with platelet activation. This suggests that the activation state of platelets in PC is linked to the occurrence of AR and may affect the outcome in recipients. This is in line with a previous study that demonstrated an association of sCD40L with adverse reactions to platelet transfusions[9]. Therefore, improving PC storage, in order to reduce platelet activation, is an important goal.

We confirm that HMGB1 levels seem to be associated with AR. DAMPs have been proposed to be involved in the pathology of FNHTR and allergic reactions, which are the most common type of AR seen in patients undergoing platelet transfusion. We have previously shown that platelet-derived DAMPs, such as HMGB1 and mtDNA, are present in PC that induced AR in patients[4,10].

Furthermore, we observed an increase in CAP in PC that induced AR. Previously, we and others have shown that complement activation occurs in platelet products during storage[5,6]. Furthermore, it was suggested that complement activation may play a role in AR[5]. Nonetheless, we have previously shown that CAP levels are decreased in PC stored in PAS[6].

A poor storage condition induces storage lesion, which is related to AR and negatively impacts the transfusion outcome[11,12]. Longer storage leads to activation and deterioration of platelets, which is associated with AR[13,14]. The nature of storage solutions has a significant impact on platelet activation and soluble factors, such as, cytokines, complement and DAMPs, contained in the product. Even though these factors increase during storage, the use of a PAS supplemented with magnesium and potassium results in reduced platelet activation and cytokines compared to PAS without[15]. Furthermore, replacement of plasma by PAS supplemented with magnesium and potassium results in lower CAP as compared to storage in plasma only[6]. This study demonstrates a correlation between platelet activation markers and HMGB1. Platelet, -and complement activation was also correlated, whereas HMGB1 and complement activation were not. Accordingly, this may suggest that platelet activation and/or deterioration of platelet may be the common cause of HMGB1 release and complement activation. This hypothesis is further supported by evidence that CAP, platelet activation and the risk for AR all increase during storage[5,16]. Moreover, upon activation platelets upregulate and release HMGB1 in various inflammatory conditions[17].

This study demonstrates that despite the use of PAS, platelet become activated causing release of HMGB1 and complement activation in PC, which finally cause AR. This further amplifies the need to improve storage by using the most optimal storage solution. However, another important point is the impact of the donor selection, which may impact the activation status of the collected platelets. Future studies can investigate donor and recipients related factors (age, gender, ABO type, previous transfusion history [reactions and others] and underlying disease) for the transfusion associated with AR. Recently, it has been demonstrated that platelet from older and/or diabetic donors showed big changes in the proteome and were of poor in-vitro quality, which may induce AR[18,19]. This suggests that a combination of the most optimal storage solution and specific donor selection is necessary to reduce the incidence of AR. Future studies could increase the sample size and may potentially determine a cut-off value for HMGB1 or complement activation products that could be used as quality control marker to remove potentially harmful PC from being transfused. One limitation of this study was that we were unable to retrieve ABO types for recipient and donor. Another limitation of the study is that in the analysis we could not correlate the data to storage time, because information on the storage time was not recorded and could later not be retrieved.

In summary, in this study we observed an association between HMGB1 and CAP and the risk for AR. Furthermore, we demonstrated that both HMGB1 and complement activation were correlated to platelet activation.

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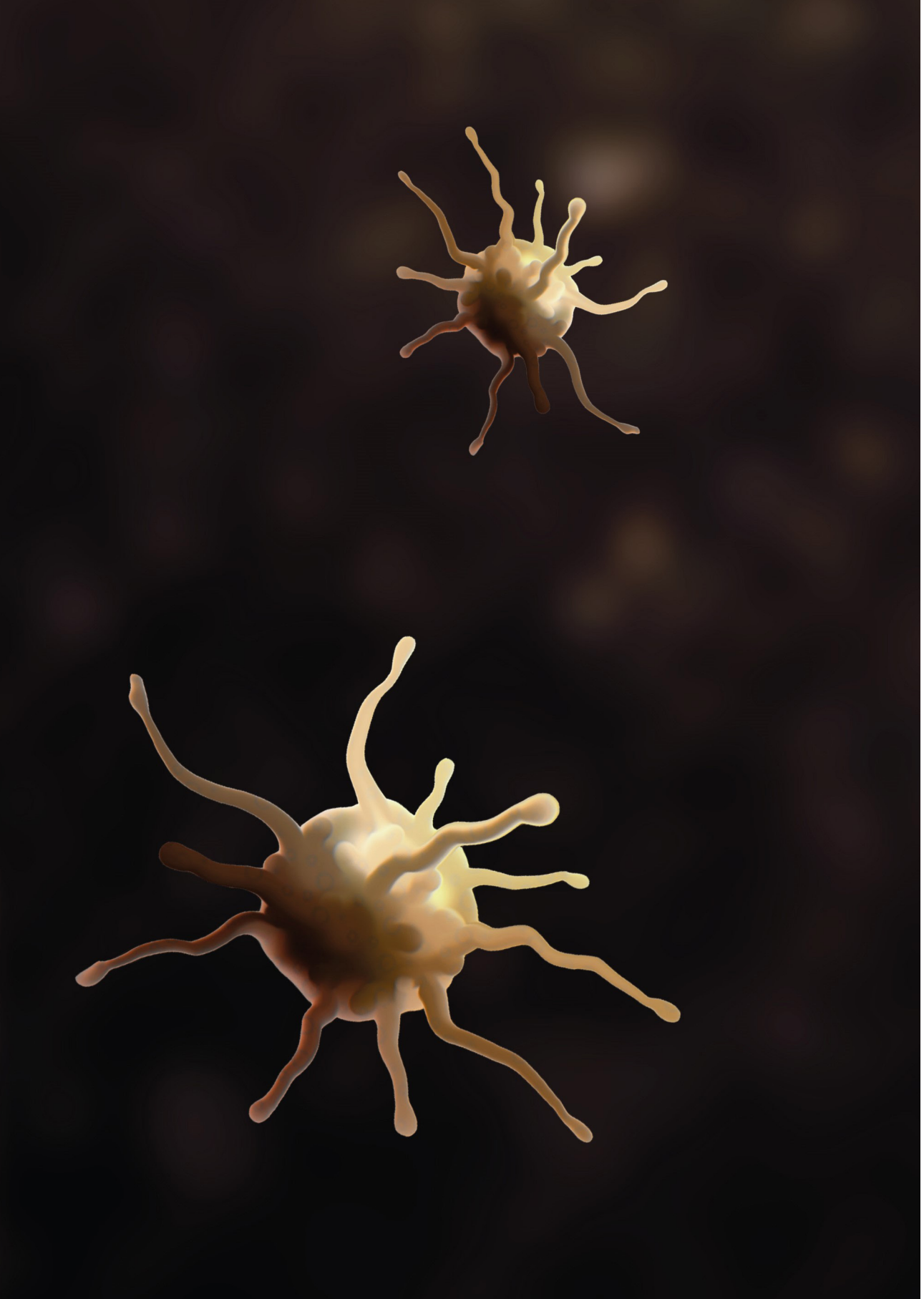

CHAPTER 7

Plasma exchange therapy using Solvent Detergent-treated plasma: an observational pilot study on complement-, neutrophil and endothelial cell activation in a case series of patients suffering from atypical Hemolytic Uremic Syndrome

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ABSTRACT

Introduction
Plasma exchange therapy (PEX) was standard treatment for Thrombotic microangiopathy (TMA) before eculizumab was available and is still widely applied. However, most PEX patients still ultimately progress to end-stage renal disease (ESRD). It has been suggested that infusion of plasma that contains active complement may induce additional complement activation with subsequent activation of neutrophils and endothelial cells, leading to exacerbation of organ damage and deterioration of renal function.

Objective
This observational pilot study, examines the effect of hemodialysis, eculizumab and PEX before and after treatment in plasma of aHUS patients on complement-, neutrophil and endothelial cell activation.

Methods
Eleven patients were included in this pilot study. Six patients were treated with hemodialysis, two patients received regular infusions of eculizumab, and three patients were on a regular schedule for PEX. Patients were followed during three consecutive treatments. Blood samples were taken before and after patients received their treatment.

Results
Complement activation products increased in plasma of patients after PEX, as opposed to patients treated with hemodialysis or eculizumab. Increased levels of complement activation products were detected in omniplasma used for PEX. Additionally, activation of neutrophils and endothelial cells was observed in patients after hemodialysis and PEX, but not in patients receiving eculizumab treatment.

Conclusion
In this pilot study we observed that PEX induced complement and neutrophil activation, and that omniplasma contains significant amounts of complement activation products. Additionally, we demonstrate that hemodialysis induces activation of neutrophils and endothelial cells. Complement activation with subsequent neutrophil activation may contribute to the deterioration of organ function and may result in ESRD. Further randomized controlled studies are warranted to investigate the effect of PEX on complement- and neutrophil activation in patients with thrombotic microangiopathy.

INTRODUCTION

Thrombotic microangiopathy (TMA) is characterized by hemolytic anemia, the presence of schistocytes in the peripheral blood smear, and thrombocytopenia. Furthermore, TMA is often complicated by organ damage due to microvascular thrombosis, resulting among others in acute kidney failure and cerebral ischemia. Activation and injury of endothelial cells is the central pathological event that results in the clinical picture of TMA[1]. However, the etiologies of endothelial cell activation and damage are very broad, ranging from infection, pregnancy, tumors and drugs to organ- and hematopoietic stem cell transplantation[2]. Genetic mutations and/or autoimmune responses may render individuals susceptible to develop TMA as a response to the mentioned triggers[1]. On one end of the spectrum, genetic mutations leading to a complete lack of ADAMTS13 or autoantibodies decreasing ADAMTS13 activity result in an inadequate proteolytic degradation of ultra large von Willebrand factor (vWF) molecules. This leads to subsequent activation of endothelial cells and platelets, as seen in classical thrombotic thrombocytopenic purpura (TTP)[2]. Atypical hemolytic uremic syndrome (aHUS) at the other end of the spectrum is caused by an acquired and/or congenital defect affecting the function of complement proteins, such as complement regulatory proteins (e.g. factor H, CD46) or complement factors (e.g. gain of function mutation C3)[3]. This results in the activation of, and occasionally damage to endothelial cells with subsequent platelet activation and aggregation, a process finally culminating in microvascular thrombosis[1]. As matter of fact, the etiology of aHUS lies in an overwhelming complement activation caused by inadequate control. However, activation or injury of endothelial cells regardless of the cause as seen in TMA will lead to secondary complement activation, thereby perpetuating endothelial cell damage[4].

Given the high morbidity and mortality, plasma exchange (PEX), also known as therapeutic plasma exchange (TPE), and –in case of acute renal insufficiency- hemodialysis are considered the emergency treatment of patients presenting with acute TMA in order to prevent fatality[4,5]. Nowadays, complement inhibition using anti-C5 (eculizumab) is considered the long-term standard treatment for aHUS, since it efficiently halts complement-induced renal damage and prevents end stage renal disease (ESRD)[6–11]. However, PEX has been the standard treatment for aHUS before the availability of complement inhibitors and is still applied as emergency treatment upon presentation and in case complement inhibitors are not available[4,8]. Although approximately two-third of the aHUS patients initially show a positive response to PEX with preservation of renal function for multiple years, most patients still finally progress to ESRD[12,13].

The main aim of PEX is to substitute defective or deficient plasma proteins, such as ADAMTS13 in case of TTP or factor H in aHUS[1,2]. However, plasma used for PEX contains

a wide range of activatory and inhibitory complement proteins[2]. Although potentially beneficial, the safety of PEX in patients with an activated complement system is debated, as infusion of plasma that contains active complement may induce additional complement activation. This may result in the activation of neutrophils, platelets and endothelial cells with subsequent exacerbation of organ damage, in particular deterioration of renal function[14,15]. Indeed, neutrophil activation in the form of NETs has been reported to play a role in the pathogenesis of TMA[16,17]. In this observational pilot study, we examined complement-, neutrophil and endothelial cell activation products in plasma of aHUS patients before and after treatment with hemodialysis, anti-C5 infusion (eculizumab) or PEX.

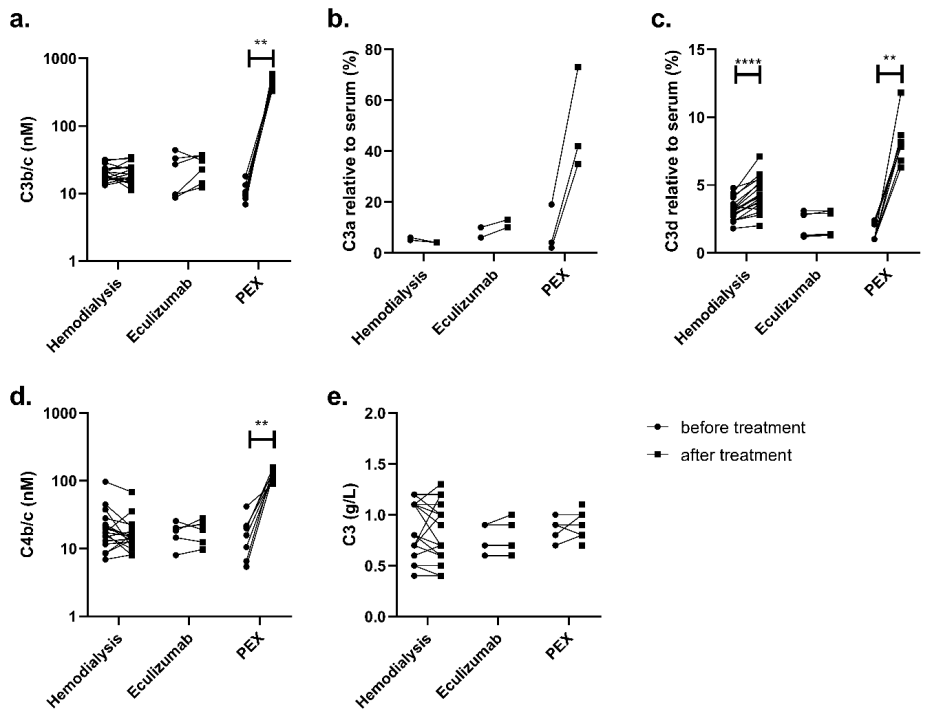

Fig. 1. Complement activation is elevated in patients after PEX. *a. C3b/c; b. C3a; c. C3d; d. C4b/c; e. C3 concentrations were measured in patients before and after they received treatment. n=18 hemodialysis (3 consecutive treatments of 6 patients), n=6 eculizumab (3 consecutive treatments of 2 patients), n=8 PEX (3 consecutive treatments of 2 patients and 2 consecutive treatments of 1 patient). *p<0,05; **p<0,01; ****p<0,0001.*

MATERIALS AND METHODS

Patients

Patients with a very high suspicion of the diagnosis of aHUS (based on clinical and/or histopathological presentation with hemolytic anemia, thrombocytopenia and acute kidney injury) at an age of 12 years or older treated on a regular schedule with hemodialysis (in case of renal failure), eculizumab, or PEX at the department of Nephrology at Amsterdam University Medical Centre, location Academic Medical Center (AMC) Amsterdam were enrolled in the current study. Identification of gene mutations of complement proteins or complement autoantibodies was not required for inclusion. The only exclusion criterion was pregnancy. Informed consent was obtained from all patients or their legal representative. The study was approved by the ethical medical committee of the Amsterdam University Medical Centre location AMC, Amsterdam, the Netherlands.

Study design

Patients were followed during three consecutive treatments. Patients with ESRD were on regular hemodialysis therapy, which meant a hemodialysis session of 4 hours three times a week. They were dialyzed using standard filter (F80) and received prophylactic dose of low molecular weight heparin as anticoagulant prior to dialysis. Plasma exchange was performed once or twice a week using centrifugation technique (Spectra Optia, Terumo BCT©). During each treatment, plasma was exchanged for 1.5-fold plasma volume against Solvent detergent (SD)-treated plasma (omniplasma, OctaplasLG®). Patients on eculizumab (Soliris®) therapy received infusions every other week. All patients were in the maintenance phase including a fixed dose of 1200 mg every 14 days. Blood samples using EDTA and Citrate as anticoagulant were taken 30 minutes before and after patients received their treatment. From citrate and EDTA tubes, platelet rich plasma was collected by centrifugation (1500g, 10min) and platelets were removed by an additional centrifugation step (1500g, 10min). The plasma was aliquoted and stored at -80°C. Serum was collected by allowing the blood to clot at room temperature for 60-90 minutes with subsequent removal of the clot by centrifuging at 1500g for 10min. Serum was aliquoted and stored at-80°C.

Complement assays

C3 antigen levels have been determined by nephelometric measurement in the routine diagnostic lab. The levels of C3 and C4 activation products (C3b/c and C4b/c) were determined by ELISA [18]. For determination of C3d levels, plasma was treated with 22% Polyethylene glycol (PEG) (to remove macromolecules such as C3) for 2 hours at 4 °C. Afterwards, the samples were centrifuged at 3800 RPM for 20 min at 4 °C. The supernatant, which now only contained low molecular weight proteins or protein

degradation products, was used in the ELISA as previously described[18]. Serum pool was used as standard and arbitrarily set as 100%. Levels of C3a were determined by ELISA. In brief, microtiter plates were coated with 1µg/ml monoclonal anti-C3a (#HM1072, Hycult, USA) diluted in 0.1 M Carbonate – bicarbonate buffer (pH 9.6). After 5 washes with Phosphate Buffered Saline 0.02% Tween-20 (PBST), samples diluted in PBS Tween 0.1% Gelatin 0.2% (PTG)-Ethyl-Diamino-Tetra-Acetic acid (EDTA) (10mM) were added. After 5 washes, biotinylated rabbit polyclonal anti-C3a (1gG fraction: Behring, Marbug) diluted in PTG (0.5 µg/ml) was added to the wells. After 5 washes, streptavidin polymerized horseradish peroxidase diluted 10,000 times in High Performance Elisa buffer (HPE) was added to the wells. After another 5 washes, plates were developed by adding 100 µg/ml Tetra Methyl Benzidine (TMB) and 0.003% (v/v) hydrogen peroxide in 0.11M sodium acetate buffer (pH 5.5). The reaction was stopped by adding 2M H2SO4. Absorbance was measured at 405 nm. Serum pool was used as standard and arbitrarily set as 100%.

Endothelial cell- and neutrophil activation markers

Human neutrophil elastase-α1-antitrypsin complex (EA) and human lactoferrin levels were determined by ELISA[19]. Cell-free DNA levels in the form of nucleosomes were measured by ELISA[20]. vWF antigen and vWF propeptide levels were determined as previously described[21].

Quantification of mitochondrial (mt)DNA

MtDNA was purified from patient plasma samples or omniplasma using the QIAamp DSP Virus kit (#60704, Qiagen). Patient DNA samples were subsequently diluted (1:5) in DNase-free water. A digital droplet PCR was performed according to manufactures instructions: in this study, the digital droplet (dd)PCR system included automated droplet generator and reader from Biorad, (QX200 Droplet Digital PCR, Bio-rad, Hercules, California, USA) and a T100 thermal cycler (Biorad). For mtDNA quantification, a primer and probe targeting the mitochondrial encoded NADH dehydrogenase 1 (MT-ND1) (NADH dehydrogenase 1 (ND1), Human (FAM), Biorad, unique assay ID dHsaCNS669425578) was used. Results were analyzed using the Quantasoft software, and absolute values of mtDNA (ND1) (copies/µl) were calculated for each DNA sample.

Statistical analysis

Measurements of patient's samples of three consecutive treatments and of plasma products are individually shown. All results are indicated as median and interquartile range. Statistical analyses were performed using a paired Wilcoxon test in GraphPad Prism 8 software. P<0.05 was considered significantly different from the null hypothesis.

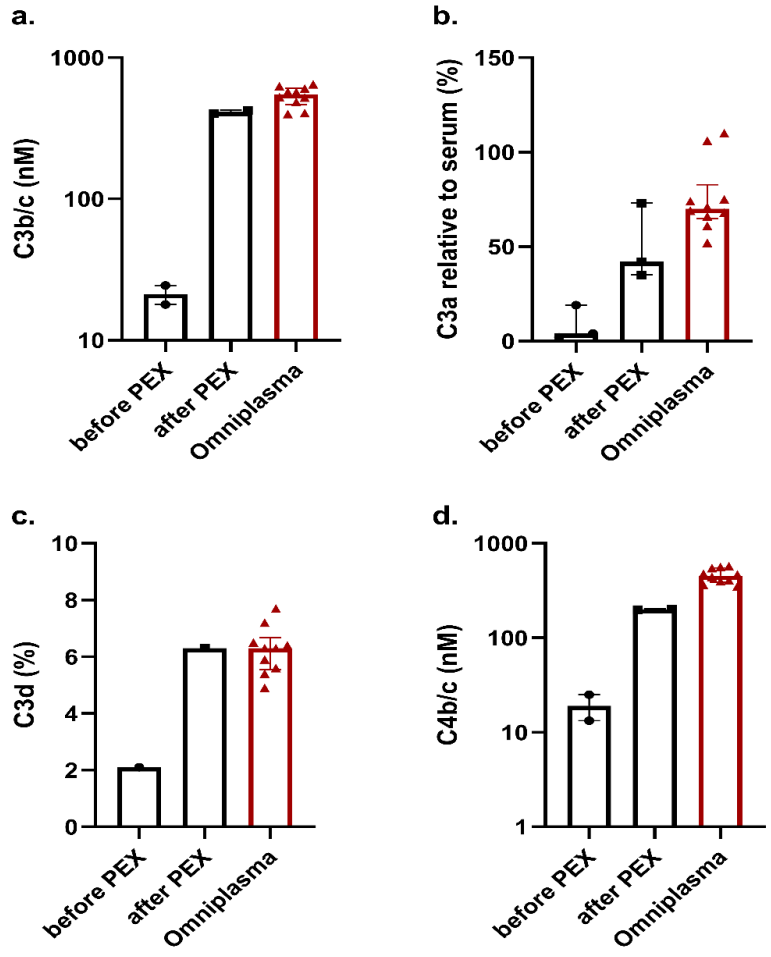

Figure 2. Omniplasma contains high levels of activated complement products. *a. C3b/c; b. C3a; c. C3d; d. C4b/c concentrations were measured in plasma before and after treatment of two patients and in 10 omniplasma units administered to these two patients subjected to PEX.*

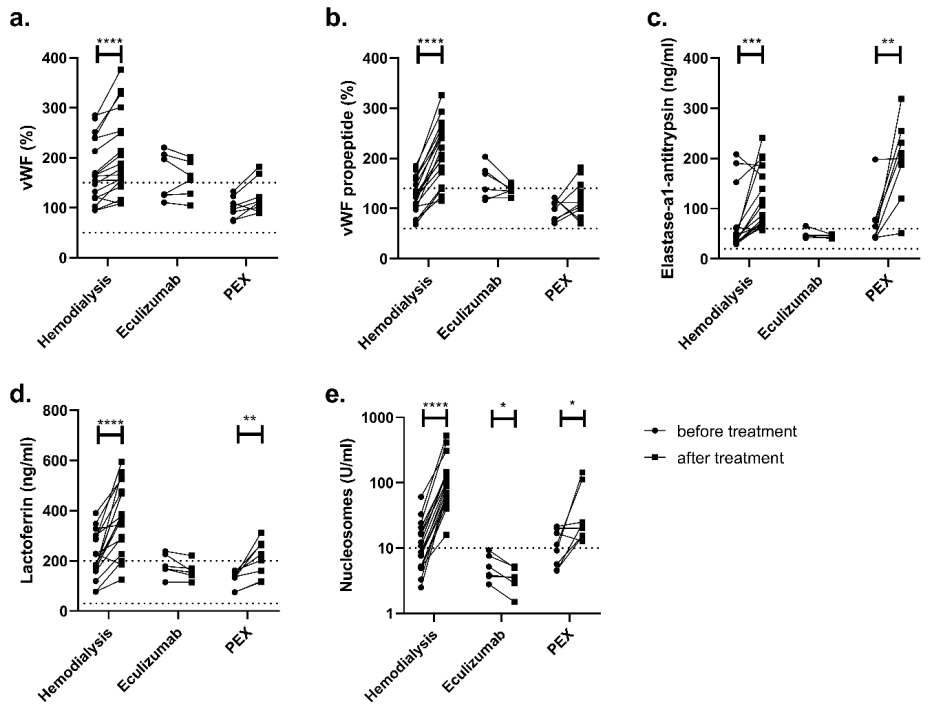

Figure 3. Endothelial, -and Neutrophil activation were observed after hemodialysis and PEX. *a. von Willebrand factor antigen; b. von Willebrand factor propeptide; c. elastase-α1-antitrypsin complexes; d. lactoferrin; e. nucleosomes were measured in patients before and after they received treatment. n=18 hemodialysis (3 consecutive treatments of 6 patients), n=6 eculizumab (3 consecutive treatments of 2 patients), n=8 PEX (3 consecutive treatments of 2 patients and 2 consecutive treatments of 1 patient). The dotted line represents the normal value or range in plasma. *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001.*

RESULTS

Patient characteristics

In total, 11 patients were included in this pilot study. Six patients were treated with hemodialysis, 2 patients received regular infusions of eculizumab, and 3 patients were on a regular schedule for PEX. Table 1 presents the basic characteristics of the 11 patients who completed the study according to the protocol. Based on the clinical and/or histopathological presentation with microangiopathic hemolytic anemia, thrombocytopenia and acute kidney injury all patients were diagnosed with aHUS. In 8 patients genetic analysis for mutations in complement factors associated with aHUS revealed a mutation. The majority (n=5) had factor H mutations, 1 patient had a mutation in factor I, 1 patient had a mutation in factor B, and 1 patient had a deletion in factor H related proteins 1 and 3. Patients within the hemodialysis group were older (median 50, range 45-57) compared to patients treated with eculizumab (median 26, range 22-30) or PEX (median 18, range 16-18). Three patients received a kidney transplant and three patients suffered from arterial hypertension.

Complement activation products increased after PEX

First, we measured complement activation in all patients before and after treatment. Complement C3 activation products C3b/c, C3a, and C3d, were significantly increased in the circulation of the patients after PEX, whereas levels of these markers did not change when comparing before and after eculizumab treatment (shown in Fig. 1a-c). After hemodialysis treatment we observed a significant increase of C3d, but not of C3b/c or C3a (shown in Fig. 1a-c). In addition, classical complement activation as evidenced by C4 activation products (C4b/c) could be detected after PEX (shown in Fig. 1d), whereas no difference could be detected before and after treatment with either eculizumab or hemodialysis. There was no difference in complement regulators factor H and B levels before and after treatment in all groups (data not shown). C3 antigen levels remained comparable before and after treatment in all three groups indicating that measurements of total complement protein levels are not reflecting activation (shown in Fig. 1e).

In a next step, we investigated whether the increase of complement activation products after PEX could be caused by complement activation products present in the plasma products used for PEX. Therefore, we assessed complement activation products (C3b/c C3a, C3d and C4b/c) in 10 omniplasma units that were administered to two patients. Interestingly, C3b/c, C3a, C3d, and C4b/c levels were considerably increased in 10 omniplasma units compared to patients before PEX treatment (shown in Fig. 2a-d). These results identified the complement activation products in omniplasma as a potential source of the complement activation products observed in patients after PEX.

Activation of endothelial cells and neutrophils after hemodialysis and PEX

Subsequently, we investigated the effect of the three treatment modalities on endothelium by measurement of the direct markers for endothelial cell activation von Willebrand factor antigen (vWF:Ag) and vWF-Propeptide (vWFpp). Hemodialysis resulted in a significant increase of vWF:Ag and vWFpp (shown in Fig. 3a-b), whereas the levels remained comparable before and after treatment with PEX and eculizumab, respectively.

Neutrophil activation was determined using plasma levels of elastase-alpha1 antitrypsin complexes (EA), lactoferrin, and cell-free DNA in the form of nucleosomes (shown in Fig. 3c-e). Interestingly, all three markers of neutrophil activation were significantly increased in the circulation of patients after PEX and hemodialysis, whereas there was no

change observed for EA- and lactoferrin-levels after eculizumab treatment. In contrast, nucleosome levels significantly decreased upon treatment with eculizumab (shown in Fig. 3e). Additionally, we could detect neutrophil activation products, such as EA and lactoferrin, in the omniplasma products (EA: median 619.0 ng/ml, range 591.3-644.0; lactoferrin median 1160.0 ng/ml, range 1103.0-1220.0), whereas nucleosome levels in omniplasma products were not increased as compared to control plasma (median 2.3 U/ml, range 2.3-2.7; control plasma <10 U/ml) (data not shown).

We also measured mtDNA, which is released from platelets upon activation. We observed no differences in levels of mtDNA before or after treatment in the plasma of hemodialysis, PEX or eculizumab patients (shown in Fig. 4a), and the level of mtDNA in omniplasma was low (median 132 copies/ul, range 114-182, Shown in Fig. 4b). Finally, we assessed plasma levels of markers for hemolysis before and after treatment. There were no significant changes in plasma levels of haptoglobin, hemopexin and LDH in hemodialysis, PEX or eculizumab patients, ruling out significant hemolysis (data not shown).

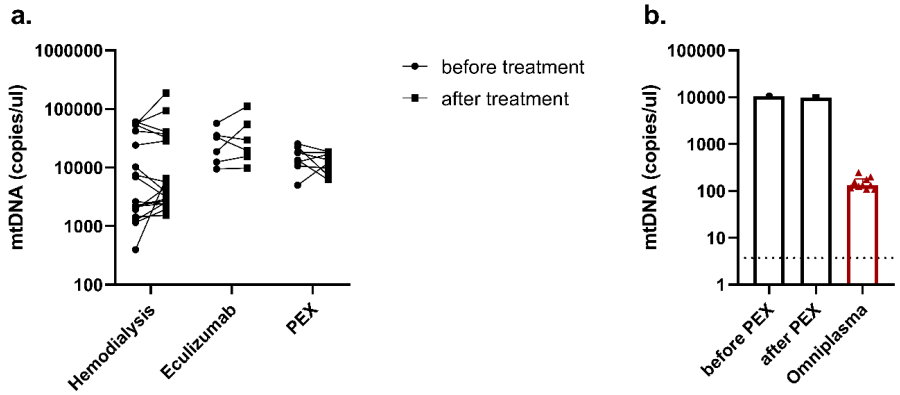

Fig. 4. mtDNA is not affected by treatment in aHUS patients. *a. mtDNA concentrations were measured in patients before and after they received treatment. n=18 hemodialysis (3 consecutive treatments of 6 patients), n=6 eculizumab (3 consecutive treatments of 2 patients), n=8 PEX (3 consecutive treatments of 2 patients and 2 consecutive treatments of 1 patient). b. mtDNA concentrations were measured in plasma before and after treatment of two patients and in 10 omniplasma units administered to these two patients subjected to PEX.*

DISCUSSION/CONCLUSION

Since recently, complement inhibition using eculizumab is now an established and standard treatment of atypical HUS in most high-income countries. However, in many countries, PEX is still widely applied and in case of renal insufficiency, patients are on hemodialysis. In fact, aHUS treatment using PEX may prevent acute complication, ESRD finally occurs in many of these patients. In the present observational pilot study, we noticed that complement is activated in patients after PEX and that omniplasma used for PEX contains high amounts of complement activation products. No complement activation was observed in patients treated with eculizumab. In addition, we observed activation of neutrophils and endothelial cells in patients after hemodialysis and PEX, which was not observed in patients receiving eculizumab treatment.

The observation of complement activation products after PEX is in line with previous studies reporting complement activation after plasma exchange and apheresis[22,24,27-29]. Most probably, the etiology of complement activation observed in our study after PEX is multifactorial. It has been suggested that the plasma separator and filtration steps may induce in complement activation[22,24,25]. PEX using centrifugation technique also results in complement activation, but to a lower degree compared to combined techniques including centrifugation and filtration, respectively[26]. Centrifugation is the physical technique used to perform PEX in our study, which may account for at least a part of complement activation detected after PEX. In addition, we also detected complement activation products in omniplasma products. Indeed, the presence of complement activation products in quarantine plasma as well as in Solvent Detergent (S/D) plasma has been described[25,30,31]. Plasma apheresis using centrifugation technique to collect plasma from donors may in part be responsible for the complement activation detected in the plasma products[26]. In addition, next to the apheresis procedure, the S/D process step may also induce significant complement activation in the product[30]. Unfortunately, it remains difficult to establish to what degree PEX itself contributes to the measured complement activation in our patients, as the exchanged apherisate for complement measurement has not been collected. Therefore, omniplasma administered to the patient itself is not only a source of complement proteins but also of complement activation products. S/D plasma, especially omniplasma, has an excellent safety profile, which is reflected by the very low incidence of allergic reactions and TRALI[32]. The most common adverse effect in patients of plasma exchange therapy, hypotension, was suggested to be caused by the complement activation products C3a and C5a[28]. It has been suggested that high plasma concentration of complement activation products in the treated patients may influence the clinical outcome of the treatment[24]. Patients with aHUS are characterized by ongoing complement activation, that results in damage to endothelial cells with subsequent microvascular thrombosis resulting in organ failure[4].

Especially the kidneys, where endothelial and epithelial integrity is strictly dependent on the availability of functional fluid-phase complement regulators such as, FH, are prone to complement-mediated attack[4]. By introducing complement activation products in the patient's circulation, PEX may theoretically not cease but rather maintain the ongoing complement activation. Administration of plasma that already contains complement activation products may therefore cause more harm than benefit. This is supported by the fact that- although PEX delays deterioration of renal function- a large proportion of aHUS patients finally still progress to ESRD[12,13].

In the current study, we report that C3d levels were significantly upregulated in patients after hemodialysis. However, although significant, the levels did not increase to biological relevant concentrations. Moreover, there were no other signs of relevant ongoing complement activation as reflected by low C3bc values in patients after hemodialysis. In the past, using cellulose membranes for hemodialysis, inflammatory activation products of complement components C3 and C5 were demonstrable after dialysis[22]. However, this issue was resolved with the use of advanced filter technology (e.g. application of hydrophilic polysulfone hemodialysis membrane), which is in accordance to our results not demonstrating relevant ongoing complement activation[23].

Additionally, we observed neutrophil-and endothelial cells activation after hemodialysis and PEX, while no neutrophil and endothelial cell activation was observed in patients treated with eculizumab. In a recent study in Paroxysmal Nocturnal Hemoglobinuria (PNH) patients we demonstrated neutrophil activation to be dependent on complement activation and that treatment with eculizumab completely abrogated neutrophil activation in these patients[33]. Our results are in line with these earlier findings, since no complement- and neutrophil activation were observed in aHUS patients treated with eculizumab. Therefore, although neutrophil activation observed after PEX is most probably a multifactorial process, complement activation in this activation process might play an important role[37,38]. For the last decades hemodialysis membranes have been extensively studied and were found to activate the alternative pathway of the complement system; activate platelets; induce cytokine release; induce neutrophil degranulation; and activate monocytes[34–36]. Complement as the main inducer of neutrophil activation in our hemodialysis patients can be ruled out since there was no relevant complement activation observed after hemodialysis, apart from a limited increase in C3 activation product C3d, suggesting no ongoing activation. Interestingly, vWF and vWFpro concentrations were increased after hemodialysis and to a lesser extent after PEX. Increased levels of markers of endothelial damage have been demonstrated in patients with ESRD on hemodialysis[39,40]. Therefore, hemodialysis and probably PEX may affect endothelial cell homeostasis resulting in activation of endothelial cells.

The clinical consequences of neutrophilic mediators released during PEX and hemodialysis are not entirely clear. There is evidence that Neutrophil Extracellular Trap (NET) formation plays an important role in the pathogenesis of thrombotic microangiopathy[16,41]. Neutrophil activation in the form of NETs may result in the release of pro inflammatory and cytotoxic molecules, such as histones, and induce a procoagulant state[38,42]. Neutrophils have been reported to release NETs upon storage of red blood cell concentrates[43]. Therefore, it cannot be ruled out that neutrophils may release NETs during the production process of blood products. The S/D pool plasma production process includes a filtration step using a membrane with pores of 0.2 µm in diameter which at least will remove DNA-fragments, such as (poly)nucleosomes[44]. However, neutrophilic products such as elastase and lactoferrin will not be removed by this filtration step. The present observational pilot study, however, is too small to answer the question on whether these identified markers correlate with adverse events that occur during hemodialysis and PEX. Interestingly, we observed no difference in mtDNA levels before or after treatment, whereas we did observe a difference in mtDNA levels between the different treatments. Given the large range of these values, an interpretation of these findings remains difficult, especially when considering the limited number of measurements.

Here we have to point out, that in the patient population in this observational pilot study is very heterogeneous regarding the underlying forms as well as the treatment of aHUS, which impedes to draw firm conclusions on the definite therapeutically consequences of our findings.

In summary, we detected activation products of complement and neutrophils in patients after PEX and in S/D plasma products in this small cohorts of aHUS patients. In addition, we observed neutrophil activation in hemodialysis patients. It remains surprising that in this observational pilot study PEX, a therapy used to treat complement-mediated diseases, in fact seems to increase the amount of complement activation products in patients. Moreover, it seems remarkable that the products used for this procedure contain complement activation products. Since this pilot study contained only a very small and heterogeneous number of patients future studies will have to determine the relevance of the activation of complement.

Acknowledgement

We would like to acknowledge all the patients that participated in this study.

Statement of Ethics

This study protocol was reviewed and approved by METC AMC, Amsterdam UMC, approval number 2012_255, NL nr 41994.018.12. Informed consent was obtained from

all patients or their legal representative.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Y.W., A.R., G.M., and F.B. substantially contributed to the conception of the work, data acquisition, analysis, and interpretation, and drafted the manuscript. D.W., A.B., and S.S. contributed to the analysis and interpretation of the data and revised the work critically for important intellectual content. All authors approved the final version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author. The data that support the findings of this study not publicly available due to information that could compromise the privacy of research participants but are available from the corresponding author upon reasonable request.

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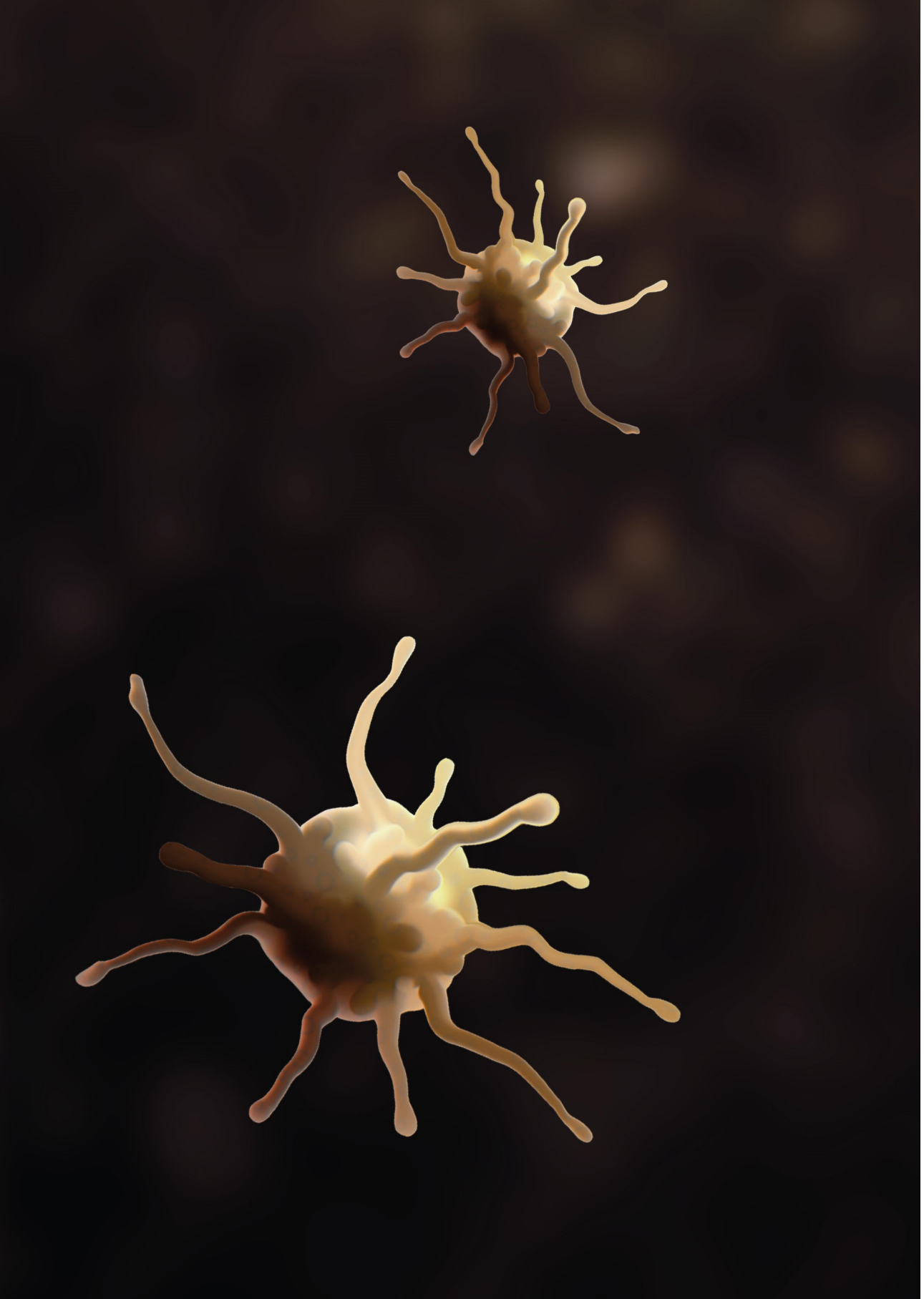

CHAPTER 8

Summary and Discussion

Systemic inflammation is a (chronic) immune response throughout the whole body against harmful stimuli[1]. Whereas inflammation takes place during an infection, inflammation does not necessarily mean that there is an infection[2,3]. An infection is the invasion of a foreign pathogen that results in tissue injury[2]. While the purpose of inflammation is to mediate a response following an injury to remove injurious stimuli, and initiate regeneration of damaged tissue[4]. Numerous noninfectious diseases feature inflammation, such as atherosclerosis, ischemic heart disease, diabetes, and aHUS. Inflammation is also a frequently observed characteristic of adverse reactions (AR) following transfusions of blood products. Inflammation is an interplay between immune cells, endothelial cells and soluble mediators, that consists of three phases: recruitment, proliferation and resolution[5]. In the acute phase complement, platelets, coagulation, and leukocytes respond and recruit to the site off injury. Subsequent proliferation is induced by release of cytokines and chemokines. Finally, neutrophils are the most abundant leukocyte present in the circulation and contribute to the resolution of inflammation[6]. Upon activation, neutrophils release pro-resolution mediators (such as lipoxins, annexin A1, and protectins) that form negative feedback for degranulation and promote tissue reparative programs. Neutrophil activation products are a robust marker for disease severity[7]. More abundant than neutrophils are platelets. Nowadays it becomes more and more acknowledged that platelets play a major role during the initiation and progression of inflammation[1]. The acute-phase response (APR) is the first response to infection or injury, and it has been indicated that platelets induce the APR[8]. Elevated levels of activated complement are induced in APR and are a potent marker for systemic inflammation. Platelets act, together with complement and coagulation factors, as scavengers of danger signals, activate immune cells, and locally confine the inflamed area to prevent spreading[9]. As a consequence, elevated activation of platelets, complement and neutrophils can result in uncontrolled thrombosis and inflammation[10].

Thrombotic complications are often observed during progression of systemic inflammation. During infection, sepsis may result in the development of disseminated intravascular coagulation (DIC) resulting in microvascular complications [11]. Coagulation and difficulties to form a stable cloth, due to fibrin dissolution/fibrinolysis, may occur causing a vicious cycle. Microthrombi can develop throughout the circulation and mainly block the micro capillaries of tissues, thereby limiting blood flow in many parts of the body, including limbs and organs. This can ultimately lead to multi organ failure. On the other side of the spectrum, DIC can increase bleeding due to consumption of coagulation factors. Microvascular thromboembolic complications are frequently observed in COVID-19 patients because of a process called immunothrombosis and mainly affect the capillaries of the lungs. A role of immunothrombosis is also demonstrated in pathogenesis of aHUS, which is characterized by thrombotic microangiopathy due to activation of the alternative pathway of complement as a result of improper control. The common pathogenetic

features in aHUS are concurrent damage to endothelial cells, hemolysis, and activation of platelets. This causes a procoagulative state with subsequent formation of microthrombi and tissue damage, most profoundly in the kidneys.

NETWORK OF PLATELET, COMPLEMENT AND NEUTROPHILS ORCHESTRATE IMMUNOTHROMBOSIS IN COVID-19

Strong activation of neutrophils can induce NETosis in severely ill patients. Even though NETs contribute to pathogen clearance, excessive NET formation is unwanted as it induces additional inflammation and tissue damage[12]. Because it is still debated what the source of cell-free DNA in systemic inflammatory conditions could be, we focused on our studies only on evidence for systemic activation of neutrophils (e.g., nucleosomes and human neutrophil elastase (HNE)). It has been argued that, during infection, cell-free DNA and histones are mainly released by neutrophils during NETosis[13,14]. However, some studies proposed that, in sepsis, cell-free DNA in the form of nucleosomes is actively released from other cell types and that widespread cell death also contributes to extracellular release of DNA and histones[7,15–17]. At the side of inflammation, tissue injury and activation of platelets and neutrophils takes place, which results in a meshwork of DNA decorated with microbicidal and cytotoxic enzymes, tissue factor (TF), citrullinated histone H3 (ci-H3), high mobility group box 1 (HMGB1), and aggregated platelets. The assays to measure NETs are generally based on the detection of non-covalent complexes of neutrophilic enzymes, e.g., MPO and DNA, or the presence of complexes of DNA with citrullinated histone 3. It remains difficult to determine whether these complexes have been formed during the process of NETosis or whether they are a result of neutrophilic proteases binding to DNA released by parenchymal cells. A previous study of our group demonstrated that initially the levels of nucleosomes and MPO increased and correlated well, but over time the ratio of nucleosomes and MPO increased[7]. Therefor it was proposed that there is a biphasic pattern of cell-free DNA release in systemic inflammation, initially by hematopoietic cells and later additionally by nonhematopoietic cells[7]. Several studies have concluded that neutrophils form procoagulant NETs in COVID-19 patients, as evidenced by the presence of co-localized myeloperoxidase (MPO) and cell-free DNA in lung autopsies[18,19]. In light of the recent COVID-19 pandemic, **Chapter 2** summarizes the latest publications on immunothrombosis in COVID-19. Currently, it is believed that platelets mediate infiltration of neutrophils and subsequent NETosis in the lung and that this contributes to acute respiratory distress syndrome (ARDS)[20]. More specifically, procoagulant NETs are presumed to mediate diseases, thrombosis, mucous secretions in the airways and cytokine production in pulmonary disease such as COVID-19[21]. Platelet-mediated NET-driven thrombogenicity has been proposed to be complement dependent, as blockade of the C5a receptor attenuated thrombotic activity[18]. The concurrent activation of

platelets and neutrophils and the complement- and coagulation system strongly point towards thrombotic complications as a consequence of immunothrombosis. In addition to inflammation caused by SARS-CoV-2 itself, overwhelming inflammation is also caused by direct endothelial cell damage by the virus. This could explain why in severe COVID-19 there is very little regulation of inflammation, which results in the thrombotic complications in mainly the lungs as reviewed in **Chapter 2**. In **Chapter 3** we could demonstrate in COVID-19 patients that neutrophil activation was related to increased levels of D-dimers (a clinical parameter for coagulation) and cell-free DNA. Our data contributes to the current knowledge on how inflammation drives the deterioration of COVID-19.

During sepsis, the complement system is activated and proinflammatory cytokines such as IL-6 and IL-8 are released[22,23]. IL-8 and the complement activation products C3a and C5a are potent inducers of activation of neutrophils[23,24]. It is increasingly recognized that complement-mediated neutrophil activation plays an ambivalent role in the pathophysiology of sepsis and COVID-19[25]. Our study (**Chapter 3**) furthermore contributes to increasing evidence that inflammation in COVID-19 is induced via the complement-platelet-neutrophil axis. Previous data from a sepsis cohort demonstrates that increased plasma levels of IL-8 and C3a associated with elevated elastase-α1-antitrypsin complexes (EA) levels[26]. This suggests that neutrophil activation as shown by elevated levels of EA are the result of a strong inflammatory response that includes complement activation and IL-8 release [26]. One could argue that in such a strong inflammatory response it is likely that neutrophils release NETs. Therefore, we suggest that EA may be a good marker for the severity of an inflammatory response during COVID-19. Furthermore, we hypothesize that by inhibition of complement components, such as C5, neutrophil activation by C5a could be decreased. C5 may therefore be a potential therapeutic target during the treatment of systemic inflammation.

HYPERACTIVATION OF PLATELETS, COMPLEMENT AND NEUTROPHILS IS DETRIMENTAL FOR OUTCOME IN COVID-19 PATIENTS

In Chapter 3 we observed a point of remission in the activation of neutrophils and complement in mild and moderate COVID-19, whereas in severe disease the activation of neutrophils and complement progresses (**Fig. 1**). This pattern is also frequently observed in sepsis patients[27]. This is also reflected in the circulating levels of DAMPs, as determined by nucleosome and mtDNA levels in these patients. While the circulatory levels of DAMPs in mild COVID-19 patients do not increase compared to healthy individuals, the levels in severe patients significantly increase and do not restore to normal levels. One could argue that there may be a tipping point beyond which inflammation creates more damage than you can clear and regenerate, leading to a vicious cycle of cell damage and inflammation.

We provide evidence that, based on markers of inflammation (e.g., complement and neutrophil activation markers), we can determine which patient falls within which severity category at the moment of admission, before the disease progresses. This may provide new possible diagnostics tools to determine the right treatment plan for a patient. Breaking the amplification loop of the complement pathway, using complement inhibitors (e.g., Compstatin) may prevent crossing the tipping point beyond which damage by inflammation is greater than clearance and tissue regeneration.

Although we did not measure platelet activation in COVID-19 patients, we can conclude from the early increase in the levels of complement activation products and D-dimers that the complement- and coagulation system, and potentially also platelets, are likely activated. Hyperactivation of platelets is frequently described as an important characteristic of COVID-19[28–30]. This is not a surprising event, due to the fact that platelets maintain a close interaction with the endothelium. When endothelial cells become infected and damaged by SARS-CoV-2, platelets may immediately respond by interacting with the immune- and coagulation system. Therefore, platelet inhibition by aspirin may be of great therapeutic potential, as aspirin is well-known for its anti-inflammatory, analgesic, and antithrombotic properties. Both complement and platelet inhibition could decrease neutrophil activation and therefore may improve disease outcome in COVID-19 patients.

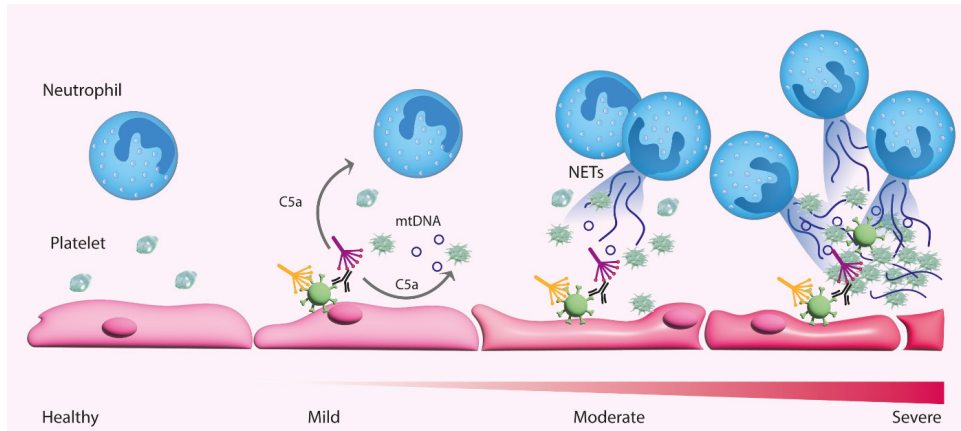

Figure 1. Severe COVID-19 results in hyperactivation of complement, platelets, and neutrophils. *A mild form of COVID-19 results in activation of complement, platelets, and neutrophils. The levels of complement activation products, mitochondrial DNA (mtDNA) and neutrophil activation increase with disease severity. Ultimately, in severe COVID-19 this results in hyperactivation of complement, platelets, and neutrophils, with excessive release of Neutrophil Extracellular Traps (NETs).*

PLATELET, -AND COMPLEMENT ACTIVATION GO HAND IN HAND IN PLATELET CONCENTRATES (PC)

Although platelet transfusion have proven to effectively reduce the incidence of bleeding complications in thrombocytopenic patients, they are associated with transfusion related adverse reactions (AR)[31–33]. According to the Dutch hemovigilance report of 2020, febrile non-hemolytic transfusion reactions are the most frequently recorded transfusion reaction following platelet transfusion. Symptoms or signs include, fever, chills (rigors), painful muscles (myalgia) and or nausea and can therefore be classified as inflammation without an infectious cause. In order to prevent AR, it is essential to minimize platelet activation in the product. In **Chapter 4** we described how platelets become activated during storage and how the levels of DAMPs, such as HMGB1, increased with it. This is both disadvantageous for the platelet increment after transfusion and for AR. In addition, we demonstrated how complement activation products are generated in PC during storage. HMGB1, which is potent DAMP or alarmin, and the complement system are two powerful soluble mediators of host defense that closely interact and provide immune surveillance. Both DAMPs and anaphylatoxins are potent inducers of systemic inflammation that together with activated platelets promote neutrophil activation. Increasing knowledge about crosstalk between platelets and complement demonstrates a tightly regulated network of pathways. Several studies have demonstrated a feedback loop involving complement and HMGB1 towards inflammation resolution and immune balance[34,35]. Therefore, it sounds plausible that an increase in these mediators during storage can negatively impact the transfusion efficiency. In addition, one could argue that this indicates that these platelets may undergo a phenotypic change, which makes them more proinflammatory. PAS as a storage solution reduces the amount of complement activation products compared to storage in plasma due to the dilution of the plasma content to ~30%. However, PAS does not prevent complement activation. With this chapter we gained more insights into the effect of storage solution for PC on proinflammatory mediators present in the PC. Despite the fact that plasma and PAS are both suitable storage solution for platelets, there is still room for improvement. Considering that platelet activation markers follow a similar trend in platelets stored in plasma versus platelets stored in PAS-E, future studies could attempt to further decrease platelet activation during storage.

It is well established that AR increase with storage duration and sCD40L, a platelet activation marker for which it has been shown to be linked to AR[36–39]. **Chapter 6** sheds more light on why it is important to decrease platelet activation in PC. In line with the previous studies stated above, an association between platelet activation and AR was also demonstrated in our study[36–39]. Moreover, platelet activation was linked to elevated amounts of HMGB1 and mtDNA in PC that induced AR in patients receiving platelet transfusion.

Taking the data from **Chapter 5 and 6** together we can paint a picture how PC production and storage can ultimately result in activated complement and DAMP content that may induce AR in the recipient. Not only do we demonstrate that these inflammatory mediators increase during storage, but in **Chapter 5** we also showed that the product type (apheresis versus PC from pooled buffy coats) and production process (differences between morning and afternoon blood donations) can have a significant effect on the content of the product. In addition, whereas we could only speculate in **Chapter 4 and 5** that these proinflammatory mediators may influence the recipient, we are able to support this hypothesis with the data presented in **Chapter 6**. In this chapter we provide compelling evidence that complement activation products and DAMPs are associated with AR and that they are linked to platelet activation. Based on this I advise to further investigate the possibility of testing the PC on complement activation products and DAMPs prior to the administration to the patient, next to ongoing optimization of PAS.

COMPLEMENT AND DAMPs AS QUALITY/SAFETY CONTROL MARKER IN PCs

Currently, PCs are evaluated for basic quality characteristics including platelet count, leukocyte count and swirl. In addition, products are assessed for bacterial contamination. We demonstrate in **Chapter 6** that there is a significant difference in the levels of soluble platelet activation markers, complement activation products and DAMPs between products that induced AR and those that did not (**Fig. 2**). Therefore, I would propose that with additional research we a threshold for the amount of soluble platelet activation markers, complement activation products and DAMPs that is allowed in the PC for it to be released for transfusion can be established and that DAMPs and complement activation products can serve as an additional quality measure to decrease the incidence of AR.

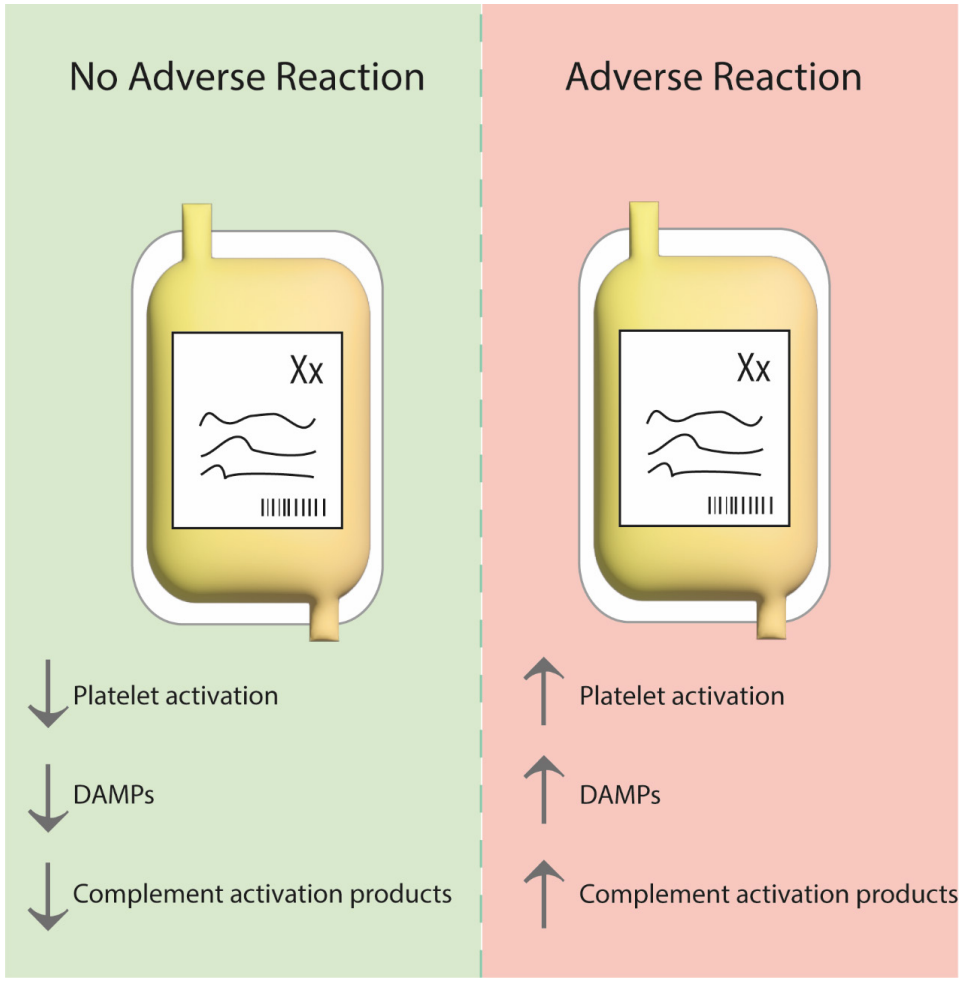

Figure 2. Increased levels of platelet activation, complement activation products, and Damage-Associated Molecular Patterns (DAMPs) in Platelet concentrates (PC) are associated with adverse reactions after platelet transfusion.

DONOR CHARACTERISTICS POTENTIALLY DETERMINE QUALITY PC

In addition to storage and production, we recommend to also take donor characteristics into account when producing PC. In **Chapter 2** we reviewed how the phenotype of a platelet can change from procoagulant to proinflammatory depending on the environment of megakaryocytes. In addition, platelets of patients with diabetes have also shown to have a more proinflammatory phenotype. Therefore, we advocate that if a donor is diagnosed with a proinflammatory disease (e.g., type 2 diabetes) their blood donation

should be averted for the production of PC. In addition to classical proinflammatory diseases platelets are also being considered to provide a connection between mental diseases and an inflammatory response[40,41]. A recent study demonstrated alterations in platelet parameters in patients with affective disorders[42]. This study showed unique platelet parameter variation patterns among patients with first-episode major depression disorder (MDD), recurrent MDD, bipolar disorder (BD) manic episodes and BD depressive episodes. This suggests that different affective disorders have specific characteristics of platelet-associated parameters. Based on this we advise to include mental disorders on the intake form in addition to chronic diseases such as diabetes. Future studies could be performed on PC products of donors with an increased risk of an altered platelet phenotype to see whether certain platelet parameters can be used to determine a threshold to divert proinflammatory platelets from PC production.

EFFECT OF COMPLEMENT IN POOLED PLASMA IN SEVERELY ILL PATIENTS

Solvent detergent (SD)-treated plasma is used to correct coagulopathy in liver transplantation patients and in patients with severe trauma. In addition, SD-plasma is used to substitute ADAMTS13 in patients with thrombotic thrombocytopenic purpura (TTP). In patients with aHUS, targeted therapy to block complement is nowadays standard of care. However, before complement inhibitory therapy was available, therapeutic plasma exchange therapy using SD-treated plasma was applied to replace dysfunctional complement components with functional components to inhibit ongoing complement activation. In countries where expensive treatment with complement inhibitors is not available, therapeutic plasma exchange is still applied in aHUS patients. It was surprising to find elevated levels of complement activation products in SD-treated plasma, especially since our pilot study in aHUS patients demonstrated that complement activation products in the circulation of aHUS patients increased after therapeutic plasma exchange therapy (PEX). However, we could not identify whether this is due to the complement content in the SD-treated plasma or due to the PEX procedure. It would be interesting to investigate if such an effect is also observed after transfusion of SD-treated plasma in a different setting such as trauma or liver transplantation. Overall, SD-treated plasma is a very safe and effective blood product[43]. Compared to its predecessor, fresh frozen plasma, SD-treated plasma is associated with fewer allergic transfusion reactions and a low frequency of TRALI[44]. According to the Dutch hemovigilance report of 2019, there are very few adverse reactions reported for SD-treated plasma, particularly compared to red blood cell and platelet transfusions. The incidence of AR after red blood cells, platelets or fresh frozen plasma are 16, 11 and 5 times higher than after SD-treated plasma, respectively. However, patients that are on a regular schedule for PEX receive

SD-treated plasma every other day, which exposes them to an accumulating risk that is ultimately far greater than that of a single blood transfusion.

HOLD YOUR PLATELETS! PLATELET PHENOTYPE POTENTIAL INDICATOR FOR INFLAMMATION AND THROMBOEMBOLISM

The role of platelets as scavengers of danger signals in the circulation and tissues makes them one of the first sensors and responders to a change in hemostasis. This suggests that a change in platelet activation status could already predict a detrimental outcome in the progression of inflammation. Previously, it was suggested that the aggregation rate of platelets and acquired platelet GPVI dysfunction could serve as a possible predictor for early sepsis diagnosis and poor outcome[45,46]. Platelet factor 4 (PF4) has shown to predict risk of fatal cerebral malaria[47]. A decline in platelet counts is an early predictive hematologic marker of human immunodeficiency virus (HIV)-induced central nervous system (CNS) disease[48].

Furthermore, sCD40L, soluble P-selectin and increased expression levels of inflammatory adhesion receptors (intracellular adhesion molecule 1 (ICAM1); glycoprotein Ibα (GPIbα); GPαIIbβ3, GPαIIbβ3) appear to have predictive value in the development of an atherosclerotic plaque even at very early stages of atherogenesis[49]. Platelet aggregation also plays a key role in acute myocardial infarction (AMI). Elevated levels of GPIIb-IIIa complexes on platelets and an increased mean platelet volume (MPV) are observed in AMI patients[50]. This study proposed that since platelet size and protein content are determined during thrombopoiesis, the majority of these platelets must have been circulating prior to AMI. It is therefore presumed that larger platelets, with more GPIIb-IIIa, may be causally related to AMI and could have a predictive value.

Overall, results presented in this thesis further substantiate that the phenotype of platelets could potentially be a good predictor of inflammation.

CONCLUDING REMARK

The combined results presented in this thesis provide new insights into the role of platelets in inflammation.

We discussed the role of platelets in inflammation and immunothrombosis during a SARS-CoV-2 infection. Platelet dysfunction as well as platelets with a proinflammatory or procoagulant phenotype were associated with severe COVID-19 and immunothrombosis.

Additionally, we provide evidence that during the early phase of infection, markers of inflammation such as DAMPS, neutrophil activation markers and complement activation products can specify disease severity in COVID-19. Together, we suggest that both platelet phenotype and markers of inflammation could be used as a diagnostic tool to determine the severity of COVID-19 during early onset of the disease. This could potentially also apply to systemic inflammation in other types of infections.

Furthermore, we present new insights that provide a better understanding of adverse reactions that take place after platelet transfusion. PC that induce an adverse reaction in recipients have shown to contain increased amounts of platelet activation markers, DAMPs and complement activation products. We hypothesize that this may (partly) trace back to the platelet characteristics of the donor. Platelets may have a phenotype that results in poor storage, these include a proinflammatory phenotype as is observed in diabetic donors. Additional research might elucidate which other conditions might result in PC with inferior quality. If future studies suggest that donor characteristics and platelets parameters can anticipate poor storage quality, then we propose that post donation screening of platelets and additional questions on intake forms for donors of platelets may divert platelets with poor storage quality.

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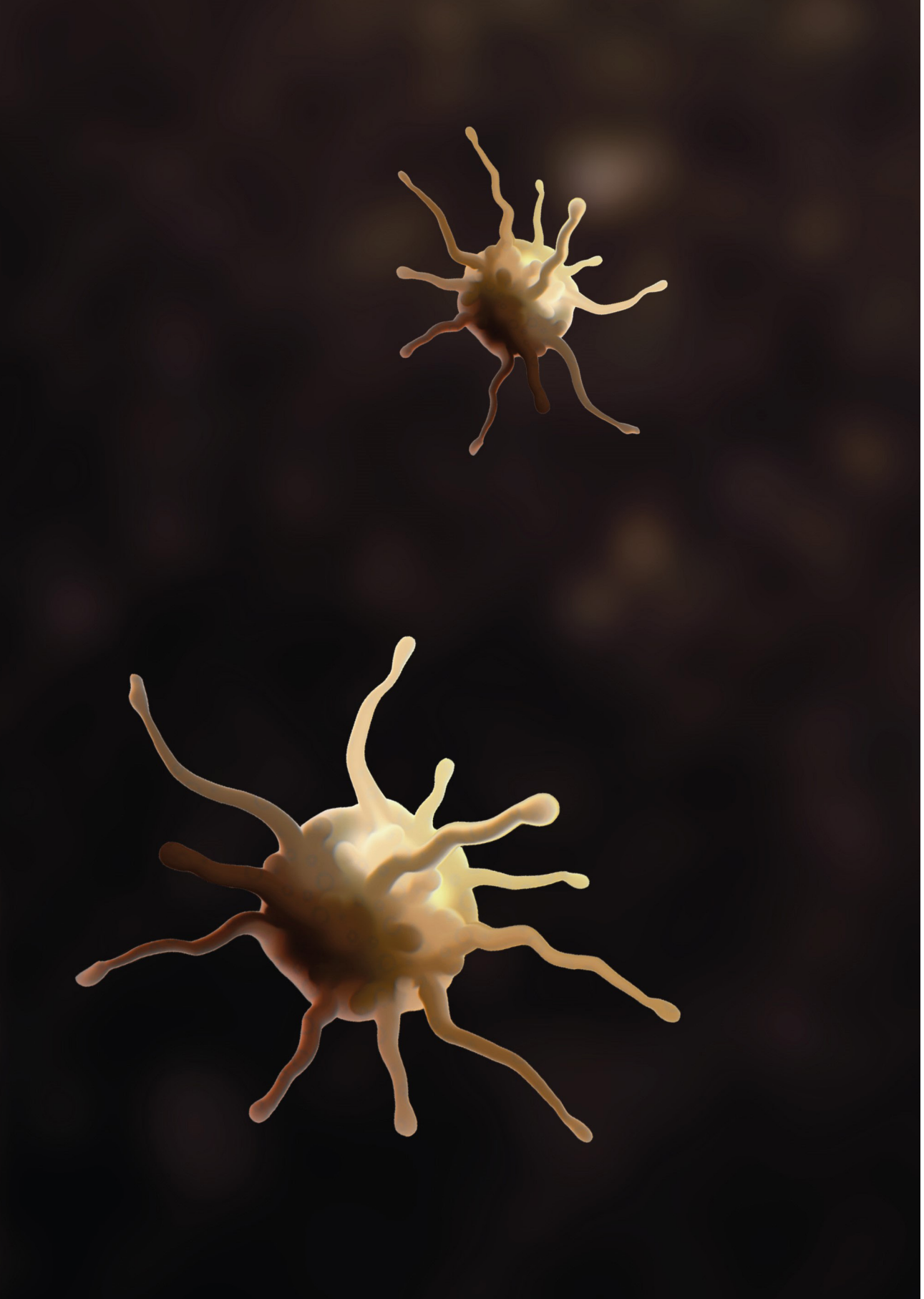

CHAPTER 9

APPENDIX

English summary

Nederlandse samenvatting

List of publications

List of co-authors and their contribution to the manuscript

ENGLISH SUMMARY

Hold your platelets: The role of complement and neutrophils in infection, inflammation, and immunity

Platelets are the second most abundant blood cell type in the circulation and are key players in hemostasis and thrombosis. Activation of platelets by soluble ligands or subendothelial exposure of collagen, von Willebrand factor (vWF) or tissue factor (TF) results in upregulation of membrane-bound glycoproteins/integrins and granule release. Integrins or soluble glycoproteins from α-granules are essential for platelets to bind coagulation factors and agglutinate, but also to adhere to the vessel wall. Additionally, platelets secrete dense (δ) granules, which contain vasoconstrictors and reenforce platelet activation and aggregation.

To maintain hemostasis, platelets are involved in vascular repair, danger sensing and closely interact with endothelial cells and leukocytes. However, platelets can also detect and respond to local danger signs, such as infectious pathogens. Platelets use Toll-like receptors (TLR) and adhesion receptors to mechanically probe the microenvironment. Recent studies demonstrate how platelets closely interact with neutrophils and the complement system during the innate immune response. Platelets express numerous complement components and receptors and a large spectrum of pro-inflammatory/modulatory chemokines and cytokines.

As described in **Chapter 1** the role of platelets in immune defense is nowadays increasingly being recognized. In circulation there is an intensive crosstalk between complement, platelets, leukocytes, coagulation, and fibrinolysis. Recent studies link coagulation to innate immunity and this indicates a key role for platelets in the first-line immune defense. In this thesis we discused the role of platelets, complement and neutrophils in immunothrombosis, infection and inflammation. We performed observational studies to gain insight into the dynamics of platelets, neutrophils, complement and DAMPs in multiple different clinical settings. We specifically focused on the role of platelets, complement and neutrophils during Coronavirus Disease 2019 (COVID-19) and in adverse reactions after platelet transfusion. Finally, we deliberate about the role of complement and neutrophil activation in atypical hemolytic uremic syndrome (aHUS) during therapeutic plasma exchange therapy and in solvent/detergent treated plasma.

The role of platelets, complement and neutrophils in Coronavirus Disease 2019 (COVID-19)

In addition to recruiting leukocytes to the area of inflammation, platelets promote coagulation and the formation of Neutrophil extracellular traps (NETs) to locally confine the infection in a process called immunothrombosis. This process requires a tight interaction between platelets, complement, neutrophils and the coagulation system. If immunothrombosis is dysregulated, it may result in thrombotic complications. In **Chapter 2** we elaborate on the collaboration of platelets with complement and neutrophils in immunothrombosis, with a specific focus on COVID-19. Microvascular thrombotic complications are frequently observed in COVID-19 and are considerably associated to mortality. Severe COVID-19 is characterized by an overwhelming innate immune response with activation of innate immune cells, complement and coagulation resulting in vascular complications. Hyperactivated platelets with an “pro-inflammatory” signature are key in this process- catalyzing NETosis and promoting the procoagulant state in the microvasculature. Furthermore, hyperactivated platelets offer a template for coagulation- as well as complement activation and release procoagulant mediators and inhibitors of DNAse1, thereby preventing NET degradation. This menage a trois between complement, platelets and neutrophils results in an orchestrated interplay of neutrophil activation in the form of NETs, hyperactivated platelets, complement activation and coagulation activation paralleled by the neutralization of anticoagulants.

There is evidence that the complement - and neutrophil activation in the form of neutrophil extracellular traps are key drivers to develop microvascular complications in COVID-19. In **Chapter 3** we studied cell-free DNA, neutrophil activation, complement activation, and D-dimers in longitudinal samples of COVID-19 patients with differing severity of disease (ranging from mild to severe). We observed that all the above markers increased with disease severity. Moreover, we provide evidence that in severe disease there is continued neutrophil and complement activation, as well as D-dimer formation and nucleosome release, whereas in mild and moderate disease all these variables decrease over time. These findings support other studies that have suggested that neutrophil and complement activation are important drivers of microvascular complications in COVID-19 patients.

Activation of platelets and complement and accumulation of Damage-associated molecular patterns (DAMPs) are associated with Adverse reactions (AR) after platelet transfusion

Prophylactic therapeutic platelet transfusions are often administered to patients with low platelet counts. Although, these transfusions are effective they can be associated with adverse reactions (AR), such as febrile nonhemolytic transfusion reaction (FNHTR). Damage-associated molecular patterns (DAMP) and complement have been suggested to play a role in FNHTR. In **Chapter 4** we investigated the nature of DAMPs and complement activation products contained in platelet concentrates (PC) during storage with a specific focus on different platelet storage solutions. During storage equal levels of free nucleosomes and increasing concentrations of HMGB1 were present in all groups. Complement was activated during storage of platelets in plasma and in Platelet

additive solution (PAS)-E and PAS-C. However, the use of PAS reduced C3b/c levels by ~90% and C4b/c levels by ~65% at day 8. These proinflammatory mediators in PC might induce FNHTR.

PCs available for transfusion are prepared either from whole blood donations or by platelet apheresis procedures. The predominant methods of PC production are apheresis-PC and Buffy coat (BC)-PC derived from whole blood. In **Chapter 5** we analyzed platelet activation markers, DAMPs and complement activation products in single donor apheresis-PC and BC-PC. A comparison was made between apheresis-PC and BC-PC. Furthermore, we divided the BC-PC in groups of morning and afternoon blood donations, as this effects the production timeline. Apheresis-PC can be better activated than BC-PC. The levels of nucleosomes, and C4b/c were significantly increased in BC-PC as compared to apheresis-PC. In addition, the levels of elastase-α1-antitrypsin complexes (EA) were higher in BC-PC from morning blood collections than in apheresis-PC or BC-PC from afternoon blood collections, which indicates increased neutrophil activation. BC-PC contain higher levels of nucleosomes and EA, most likely due to the presence of neutrophils in the BC. In addition, higher levels of complement activation products were observed in BC-PC.

After we established the presence of complement activation products and DAMPs in PC, we assessed the association of these markers with AR. In **Chapter 6** we collected samples from PC and possible AR in patients were recorded. Platelet activation markers, High mobility group box 1 (HMGB1) and complement activation products were measured. Subsequently, the correlation between platelet activation, and HMGB1 and complement activation was analyzed. The levels of P-selectin (CD62p), sCD40L, HMGB1, C3b/c and C4b/c were all significantly increased in PC that induced AR following transfusion in patients. Additionally, HMGB1, C3b/c and C4b/c were positively correlated with P-selectin and sCD40L. This suggests an association between HMGB1 and complement activation products and the incidence of AR. Furthermore, we demonstrated that both HMGB1 and complement activation were correlated to platelet activation, which may indicate that platelet activation is the mutual factor involved in AR.

Plasma exchange therapy (PEX) results in increased complement activation products in patient

Plasma exchange therapy (PEX) was standard treatment for Thrombotic microangiopathy (TMA) before eculizumab was available and is still widely applied. However, most PEX patients still ultimately progress to end-stage renal disease (ESRD). It has been suggested that infusion of plasma that contains active complement may induce additional complement activation with subsequent activation of neutrophils and endothelial cells, leading to exacerbation of organ damage and deterioration of renal

function. In **Chapter 7** we performed an observational pilot study that examines plasma of aHUS patients before and after treatment (hemodialysis, eculizumab or PEX) on complement-, neutrophil and endothelial cell activation. We observed that PEX induced complement and neutrophil activation, and that omniplasma used for PEX contains significant amounts of complement activation products. Additionally, we demonstrate that hemodialysis induces activation of neutrophils and endothelial cells. Complement activation with subsequent neutrophil activation may contribute to the deterioration of organ function and may result in ESRD.

In **Chapter 8** the general results of this thesis are summarized and discussed. Based on this discussion, we have come up with some future recommendations. Platelet phenotype and markers of inflammation could be used as a diagnostic tool to determine the severity of COVID-19 during early onset of the disease. This could potentially also apply to systemic inflammation in other types of infections. Furthermore, we present new insights that provide a better understanding of adverse reactions that take place after platelet transfusion. PC that induces an adverse reaction in recipients have shown to contain increased amounts of platelet activation markers, DAMPs and complement activation products. We hypothesize that this may (partly) trace back to the platelet characteristics of the donor.

NEDERLANDSE SAMENVATTING

Rustig aan met je plaatjes: De rol van complement en neutrofielen bij infectie, ontsteking en immuniteit

Bloedplaatjes zijn het op een na meest voorkomende type bloedcellen in de bloedsomloop en spelen een sleutelrol bij hemostase en trombose. Activering van bloedplaatjes door liganden of subendothele blootstelling van collageen, von Willebrand-factor (vWF) of weefselfactor (TF) resulteert in opregulatie van membraangebonden glycoproteïnen/integrinen en afgifte van granulen. Integrinen of oplosbare glycoproteïnen uit α-granulen zijn essentieel voor bloedplaatjes om stollingsfactoren te binden en te agglutineren, maar ook om zich aan de vaatwand te hechten. Bovendien scheiden bloedplaatjes dense (δ)-granulen af, die vasoconstrictoren bevatten en de activering en aggregatie van bloedplaatjes versterken.

Om hemostase te behouden, zijn bloedplaatjes betrokken bij vasculair herstel, gevaardetectie en hebben ze een nauwe wisselwerking met endotheelcellen en leukocyten. Bloedplaatjes kunnen echter ook tekenen van lokaal gevaar, zoals infectieuze ziekteverwekkers, detecteren en erop reageren. Bloedplaatjes gebruiken Toll-like receptoren (TLR) en adhesiereceptoren om de micro-omgeving mechanisch te onderzoeken. Recente studies tonen aan hoe bloedplaatjes nauw samenwerken met neutrofielen en het complementsysteem tijdens de aangeboren immuunrespons. Bloedplaatjes brengen talloze complementcomponenten en receptoren tot expressie en een groot spectrum van pro-inflammatoire/modulerende chemokines en cytokines. Zoals beschreven in **Hoofdstuk 1** wordt de rol van bloedplaatjes in de immuun afweer tegenwoordig steeds meer erkend. In de circulatie is er een intensieve wisselwerking tussen complement, bloedplaatjes, leukocyten, coagulatie en fibrinolyse. Recente studies koppelen coagulatie aan aangeboren immuniteit en dit wijst op een sleutelrol voor bloedplaatjes in de eerste linie van de immuun afweer. In dit proefschrift bespreken we de rol van bloedplaatjes, complement en neutrofielen bij immunotrombose, infectie en ontsteking. We hebben observationele studies uitgevoerd om inzicht te krijgen in de dynamiek van bloedplaatjes, neutrofielen, complement en DAMP's in meerdere verschillende klinische settingen. We richten ons specifiek op de rol van bloedplaatjes, complement en neutrofielen tijdens Coronavirus Disease 2019 (COVID-19) en bij bijwerkingen na bloedplaatjestransfusie. Ten slotte discussiëren we over de rol van complement- en neutrofielactivatie bij atypisch hemolytisch-uremisch syndroom (aHUS) tijdens therapeutische plasma-uitwisselingstherapie en in met oplosmiddel/detergens behandeld plasma.

De rol van bloedplaatjes, complement en neutrofielen in Coronavirus Disease 2019 (COVID-19)

Naast het rekruteren van leukocyten naar het ontstekingsgebied, bevorderen bloedplaatjes de stolling en de vorming van neutrofielen extracellulaire vallen (NET's) om de infectie lokaal te beperken in een proces dat immunotrombose wordt genoemd. Dit proces vereist een nauwe interactie tussen bloedplaatjes, complement, neutrofielen en het stollingssysteem. Als immunotrombose ontregeld is, kan dit leiden tot trombotische complicaties. In **Hoofdstuk 2** gaan we dieper in op de samenwerking van bloedplaatjes met complement en neutrofielen bij immunotrombose, met een specifieke focus op COVID-19. Microvasculaire trombotische complicaties worden vaak waargenomen bij COVID-19 en zijn aanzienlijk geassocieerd met mortaliteit. Ernstige COVID-19 wordt gekenmerkt door een overweldigende aangeboren immuunrespons met activering van aangeboren immuuncellen, complement en coagulatie resulterend in vasculaire complicaties. Hypergeactiveerde bloedplaatjes met een "pro-inflammatoire" fenotype zijn de sleutel in dit proces - het bevorderen van NETosis en van de procoagulante toestand in de microvasculatuur. Bovendien bieden hypergeactiveerde bloedplaatjes een opstap voor coagulatie, evenals complementactivering en afgifte van procoagulante mediatoren en remmers van DNAse1, waardoor NET-degradatie wordt voorkomen. Deze menage a trois tussen complement, bloedplaatjes en neutrofielen resulteert in een georkestreerd samenspel van neutrofielactivering in de vorm van NET's, hypergeactiveerde bloedplaatjes, complementactivering en stollingsactivering parallel met de neutralisatie van anticoagulanten.

Er zijn aanwijzingen dat de complement- en neutrofielactivering in de vorm van NETs de belangrijkste drijfveren zijn voor het ontwikkelen van microvasculaire complicaties bij COVID-19. In **Hoofdstuk 3** bestudeerden we celvrij DNA, neutrofielactivatie, complementactivatie en D-dimeren in longitudinale monsters van COVID-19-patiënten met verschillende ernst van de ziekte (variërend van mild tot ernstig). We hebben waargenomen dat alle bovenstaande markers toenamen met de ernst van de ziekte. Bovendien leveren we bewijs dat er bij ernstige ziekte sprake is van voortdurende activering van neutrofielen en complement, evenals D-dimeervorming en nucleosoom afgifte, terwijl bij milde en matige ziekte al deze variabelen in de loop van de tijd afnemen. Deze bevindingen ondersteunen andere onderzoeken die hebben gesuggereerd dat activering van neutrofielen en complement belangrijke oorzaken zijn van microvasculaire complicaties bij COVID-19-patiënten.

Activering van bloedplaatjes en complement en accumulatie van Damage-associated molecular patterns (DAMP's) worden in verband gebracht met bijwerkingen na bloedplaatjestransfusie

Profylactische therapeutische bloedplaatjestransfusies worden vaak toegediend

aan patiënten met een laag aantal bloedplaatjes. Hoewel deze transfusies effectief zijn, kunnen ze gepaard gaan met bijwerkingen, zoals febriele niet-hemolytische transfusiereactie (FNHTR). Er is gesuggereerd dat Damage-associated molecular patterns (DAMP) en complement een rol spelen bij FNHTR. In **Hoofdstuk 4** onderzochten we de aard van DAMP's en complementactiveringsproducten in bloedplaatjesconcentraten (PC) tijdens opslag met een specifieke focus op verschillende bewaarvloeistoffen voor bloedplaatjesopslag. Tijdens opslag waren in alle groepen gelijke niveaus van vrije nucleosomen en toenemende concentraties van HMGB1 aanwezig. Complement werd geactiveerd tijdens opslag van bloedplaatjes in plasma en in Platelet Additive Solution (PAS)-E en PAS-C. Het gebruik van PAS verlaagde de C3b/c-waarden echter met ~90% en de C4b/c-waarden met ~65% op dag 8. Deze pro-inflammatoire mediatoren bij PC kunnen FNHTR induceren.

PC's die beschikbaar zijn voor transfusie worden bereid uit volbloeddonaties of door bloedplaatjesafereseprocedures. De voornaamste methoden voor PC-productie zijn aferese-PC en buffy coat (BC)-PC bereid uit volbloed. In **Hoofdstuk 5** analyseerden we markers voor bloedplaatjesactivatie, DAMP's en complementactiveringsproducten in single donor aferese-PC en BC-PC. Er werd een vergelijking gemaakt tussen aferese-PC en BC-PC. Verder hebben we de BC-PC opgedeeld in groepen van ochtend- en middagbloeddonaties, omdat dit de productietijdlijn beïnvloedt. Aferese-PC kan beter geactiveerd worden dan BC-PC. De niveaus van nucleosomen en C4b/c waren significant verhoogd in BC-PC in vergelijking met aferese-PC. Bovendien waren de niveaus van elastase-α1-antitrypsinecomplexen (EA) hoger in BC-PC van bloedafnames in de ochtend dan in aferese-PC of BC-PC van bloedafnames in de middag, wat wijst op een verhoogde activering van neutrofielen. BC-PC bevatten hogere niveaus van nucleosomen en EA, hoogstwaarschijnlijk vanwege de aanwezigheid van neutrofielen in de BC. Bovendien werden hogere niveaus van complementactiveringsproducten waargenomen in BC-PC.
Nadat we de aanwezigheid van complementactiveringsproducten en DAMP's in PC hadden vastgesteld, beoordeelden we de associatie van deze markers met transfusiereacties. In **Hoofdstuk 6** verzamelden we monsters van PC en werden mogelijke transfusiereacties bij patiënten geregistreerd. Bloedplaatjesactiveringsmarkers, High mobility group box 1 (HMGB1) en complementactiveringsproducten werden gemeten. Vervolgens werd de correlatie tussen activering van bloedplaatjes en HMGB1 en activering van complement geanalyseerd. De niveaus van P-selectine (CD62p), sCD40L, HMGB1, C3b/c en C4b/c waren allemaal significant verhoogd in PC die transfusiereacties induceerde na transfusie bij patiënten. Bovendien waren HMGB1, C3b/c en C4b/c positief gecorreleerd met P-selectine en sCD40L. Dit suggereert een verband tussen HMGB1 en complementactiveringsproducten en de incidentie van transfusiereacties. Verder hebben we aangetoond dat zowel HMGB1 als complementactivatie gecorreleerd waren met bloedplaatjesactivatie, wat erop kan wijzen dat bloedplaatjesactivering de wederzijdse factor is die betrokken is bij transfusiereacties.

Plasma-uitwisselingstherapie (PEX) resulteert in verhoogde complement-activeringsproducten bij de patiënt
Plasma-uitwisselingstherapie (PEX) was de standaardbehandeling voor trombotische microangiopathie (TMA) voordat eculizumab beschikbaar was en wordt nog steeds veel toegepast. De meeste PEX-patiënten ontwikkelen uiteindelijk echter nog steeds eindstadium nierziekte (ESRD). Er is gesuggereerd dat infusie van plasma dat actief complement bevat, aanvullende complementactivering kan induceren met daaropvolgende activering van neutrofielen en endotheelcellen, wat leidt tot verergering van orgaanbeschadiging en verslechtering van de nierfunctie. In **Hoofdstuk 7** hebben we een observationele pilotstudie uitgevoerd die plasma van aHUS-patiënten voor en na behandeling (hemodialyse, eculizumab of PEX) onderzoekt op complement-, neutrofielen- en endotheelcelactivatie. We hebben waargenomen dat PEX complement- en neutrofielactivering induceerde en dat omniplasma dat voor PEX wordt gebruikt, aanzienlijke hoeveelheden complementactiveringsproducten bevat. Bovendien tonen we aan dat hemodialyse activering van neutrofielen en endotheelcellen induceert. Complementactivering met daaropvolgende activering van neutrofielen kan bijdragen aan de verslechtering van de orgaanfunctie en kan leiden tot ESRD.

In **Hoofdstuk 8** worden de algemene resultaten van dit proefschrift samengevat en besproken. Op basis van deze discussie zijn we tot enkele aanbevelingen voor de toekomst gekomen. Het fenotype van bloedplaatjes en ontstekingsmarkers kunnen worden gebruikt als diagnostisch hulpmiddel om de ernst van COVID-19 tijdens het beginstadium van de ziekte te bepalen. Dit zou mogelijk ook van toepassing kunnen zijn op systemische ontsteking bij andere soorten infecties. Verder presenteren we nieuwe inzichten die een beter inzicht geven van bijwerkingen die optreden na trombocytentransfusie. Van PC's die een bijwerking veroorzaken bij ontvangers is aangetoond dat ze verhoogde hoeveelheden markers voor bloedplaatjesactivatie, DAMP's en complementactiveringsproducten bevatten. Onze hypothese is dat dit (deels) terug te voeren is op de bloedplaatjeskenmerken van de donor.

LIST OF PUBLICATIONS

Yasmin E.S. de Wit; Richard Vlaar; Eric Gouwerok; Hind Hamzeh-Cognasse; Gerard van Mierlo; Ingrid Bulder; Johan W.M. Lagerberg; Dirk de Korte; Fabrice Cognasse; Anja ten Brinke; Sacha S. Zeerleder. Platelet concentrates in Platelet additive solutions generate less complement activation products during storage than platelets stored in plasma. Blood Transfus, 2022; doi: 10.2450/2022.0323-21

Yasmin E.S. de Wit; Hind Hamzeh-Cognasse; Fabrice Cognasse; Anja ten Brinke; Sacha S. Zeerleder. DAMPS and complement activation in platelet concentrates that induce adverse reactions in patients. Transfusion, 2022; 62(9):1721-1726. doi: 10.1111/trf.17061.

Yasmin E.S. de Wit; Arne Rethans; Gerard van Mierlo, Diana Wouters, Anja ten Brinke; Frederike Bemelman, Sacha Zeerleder. Plasma exchange therapy using Solvent Detergent-treated plasma: an observational pilot study on complement-, neutrophil and endothelial cell activation in a case series of patients suffering from atypical Hemolytic Uremic Syndrome. Transfus Med Hemother, 2022; 49:288–296 https://doi.org/10.1159/000522137

LIST OF CO-AUTHORS AND THEIR CONTRIBUTION TO THE MANUSCRIPT

Menage a trois in COVID-19: Platelets, Complement and NETs in immunothrombosis
Authors: Yasmin E.S. de Wit; Anja ten Brinke; Sacha S. Zeerleder
Concept: YW, SZ
Writing of the manuscript: YW, AB, SZ
Final approval of the manuscript: YW, AB, SZ

Devils Dance: Complement, NETs, and Thrombosis in COVID-19
Authors: Tiphaine Ruggeri, Yasmin De Wit, Noëlia Schärz, Gerard van Mierlo, Anne Angelillo-Scherrer, Justine Brodard, Joerg C. Schefold, Cédric Hirzel, Ilse Jongerius, and Sacha Zeerleder
Study concept: CH, SZ
Study design: TR, CH, SZ
Acquisition of data: TR, YW, NS, GM, AS, JB, JS
Analysis and interpretation of the results: TR, YW, CH, SZ
Writing of the manuscript: TR, YW, SZ
Critical reviewing of the manuscript: TR, YW, CH, IJ, SZ
Final approval of the manuscript: all named authors

Platelet concentrates in Platelet additive solutions generate less complement activation products during storage than platelets stored in plasma
Authors: Yasmin E.S. de Wit; Richard Vlaar; Eric Gouwerok; Hind Hamzeh-Cognasse; Gerard van Mierlo; Ingrid Bulder; Johan W.M. Lagerberg; Dirk de Korte; Fabrice Cognasse; Anja ten Brinke; Sacha S. Zeerleder
Study concept: JL, DK, FC, AB, SZ
Study design: YW, JL, DK, FC, AB, SZ
Acquisition of data: YW, RV, EG, HH, GM, IB
Analysis and interpretation of the results: YW, RV, EG, HH, JL, DK, FC, AB, SZ
Writing of the manuscript: YW, FC, AB, SZ
Critical reviewing of the manuscript: JL, DK, FC, AB, SZ
Final approval of the manuscript: all named authors

Blood collection technique and processing impacts the contents of damage-associated molecular pattern (DAMP) contents of platelet products
Authors: Yasmin E.S. de Wit; Rick van Andel; Ido J. Bontekoe; Thomas R.l. Klei; Anja ten Brinke; Sacha S. Zeerleder
Study concept: YW, AB, SZ
Study design: YW, AB, SZ

Acquisition of data: YW
Analysis and interpretation of the results: YW, TK, AB, SZ
Provided critical samples: RA
Writing of the manuscript: YW, AB, SZ
Critical reviewing of the manuscript: YW, IB, TK, AB, SZ
Final approval of the manuscript: all named authors

DAMPS and complement activation in platelet concentrates that induce adverse reactions in patients
Authors: Yasmin E.S. de Wit; Hind Hamzeh-Cognasse; Fabrice Cognasse; Anja ten Brinke; Sacha S. Zeerleder
Study concept: FC, SZ
Study design: YW, FC, SZ
Acquisition of data: YW, HH
Analysis and interpretation of the results: all authors
Writing of the manuscript: YW, FC, SZ
Critical reviewing of the manuscript: YW, FC, AB, SZ
Final approval of the manuscript: all named authors

Plasma exchange therapy using Solvent Detergent-treated plasma: an observational pilot study on complement-, neutrophil and endothelial cell activation in a case series of patients suffering from atypical Hemolytic Uremic Syndrome
Authors: Yasmin de Wit; Arne Rethans; Gerard van Mierlo, Diana Wouters, Anja ten Brinke; Frederike Bemelman, Sacha Zeerleder
Study concept: DW, FB, SZ
Study design: DW, FB, SZ
Acquisition of data: YW, AR, GM
Analysis and interpretation of the results: YW, AR, DW, AB, SZ
Provided critical samples: FB
Writing of the manuscript: YW, AB, FB, SZ
Critical reviewing of the manuscript: YW, DW, AB, FB, SZ
Final approval of the manuscript: all named authors

PHD PORTFOLIO

PhD student: Yasmin E.S. de Wit
PhD period: May 2018 – May 2022
Promotor: Prof. Dr. Sacha Zeerleder
Co-promotor: Dr. Anja ten Brinke

PhD TRAINING

Courses	**Year**	**ECTS**
Communication program, Anneke van der Kuip Training & Coaching	2021	2
Personal Development program, Mennen Training & Consultancy	2019-2022	2
Advanced Immunology, Vumc and Sanquin Research	2019	2.5
Mouse Morphology, Function & Genetics	2019	1.5
European Network of Immunology Institutes (ENII)	2019	2.5
Advanced Immunology Summer School, Porto Cervo, Italy		
Laboratory Animal Science Course (Article 9)	2018	3.9
Sanquin science course	2018	0.5

Seminars and workshops	**Year**	**ECTS**
UTG reference meeting Trombotische MicroAngiopathie Sanquin	2021	0.5
Sanquin Spring seminar	2019	0.25
Weekly department meeting	2018-2022	5
Weekly Sanquin Research Seminar	2018-2022	5
Landsteiner Lectures	2018-2022	2
Monthly IP Seminars	2018-2020	2
Journal Club	2018-2022	4
Sanquin Science Day (2x poster presentation)	2018-2022	0.75

(Inter)national conferences	**Year**	**ECTS**
13th and 14th Dutch Hematology Congress (DHC), Papendal, The Netherlands	2019-2020	2
5th European Congress of Immunology, Amsterdam, The Netherlands	2018	1
European Symposium on Platelet and Granulocyte Immunobiology	2018	0.5

Teaching	**Year**	**ECTS**
Course assistant Immunology research and clinic (4x)	2018-2022	1.5

Grants
EFIS-IL Short-term Fellowship

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