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Faculty of Health Sciences

Department of Clinical Medicine

The role of complement and Toll-like receptors in Thromboinflammation

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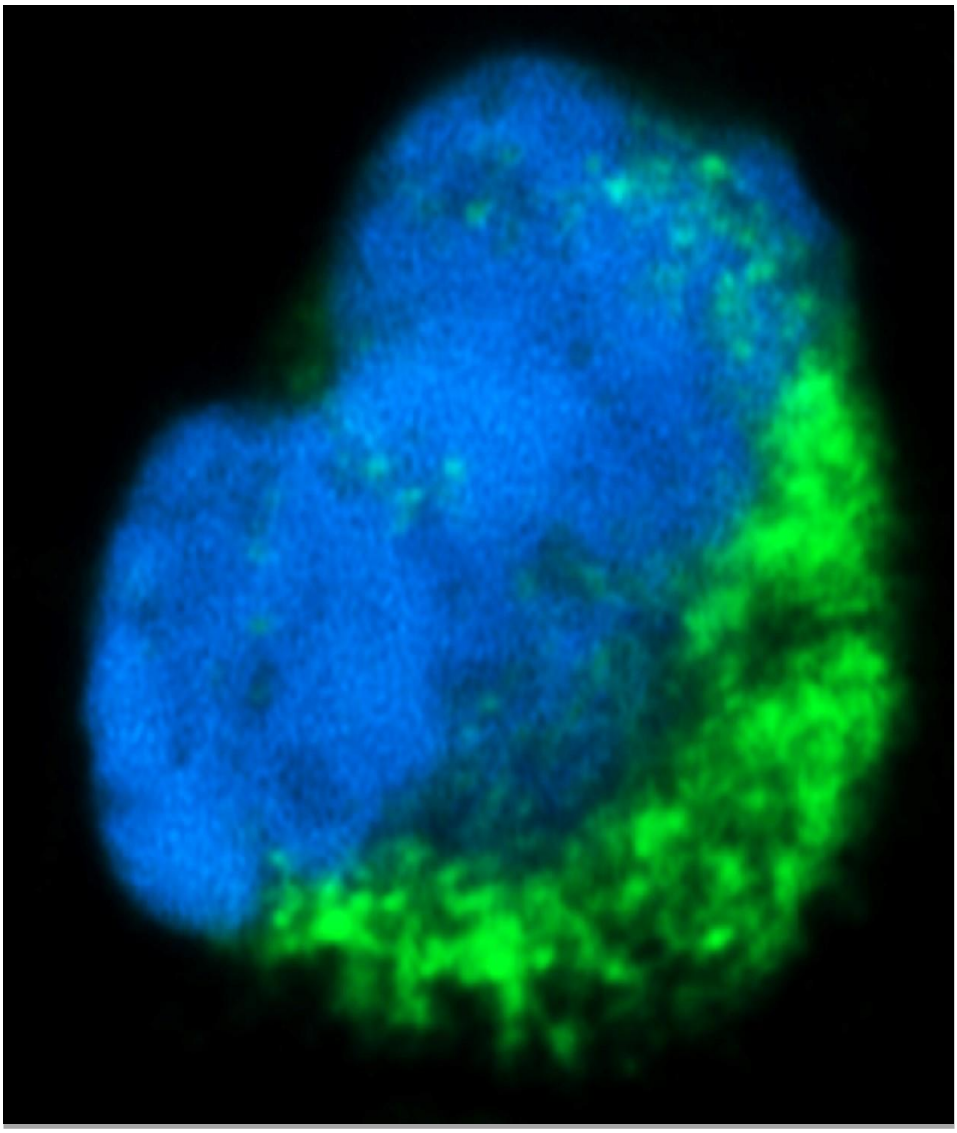

UNIVERSITY OF TROMSØ

The role of complement and Toll-like receptors in thromboinflammation

PhD Thesis
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Cover: Monocyte with tissue factor (TF) expression in blood smear after 2 hours stimulation of human whole blood with *Escherichia coli*, confocal microscopy (630x). Tissue factor in green was stained using a FITC labeled anti-TF antibody and DNA was stained using DAPI in blue. The picture was obtained by post. doc. Bård Ove Karlsen.

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Selected abbreviations

C	Complement component
C5aR	C5a receptor
C5D	C5 deficient
C1-INH	C1-inhibitor
CD	Cluster of differentiation
CT	Clotting time
DAMP	Damage-associated molecular pattern
DIC	Disseminated intravascular coagulation
E. coli	*Escherichia coli*
EXTEM	Extrinsic thromboelastometry
ELISA	Enzyme-linked immunosorbent assay
FVII	Factor VII
HAE	Hereditary angioedema
IL	Interleukin
INTEM	Intrinsic thromboelastometry
IRF	Interferon regulatory transcription factor
kDa	Kilo Dalton
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
MASP	MBL associated serine protease
MBL	Mannose-binding lectin
MCF	Maximum clot firmness
MD2	Myeloid differentiation protein 2
MFI	Median fluorescence intensity
mL	Milliliter
MP	Microparticle
mRNA	Messenger RNA
NATEM	Non-activated thromboelastometry
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
PAMP	Pathogen-associated molecular pattern
PAR	Protease-activated receptor
PBS	Phosphate-buffered saline
PRR	Pattern recognition receptor
PS	Phosphatdylserine
PTF1.2	Prothrombin fragment F 1 + 2
PTX3	Long-pentraxin 3
ROTEM®	Rotational thromboelastometry
qPCR	Quantitative polymerase chain reaction
S. aureus	*Staphylococcus aureus*
TCC	Terminal complement complex
TF	Tissue factor
TF-MP	Tissue factor function in plasma microparticles
TLR	Toll-like receptor
TNF	Tumor necrosis factor
vWF	von Willebrand factor

List of papers

Paper I

***Landsem A**, Fure H, Christiansen D, Nielsen EW, Østerud B, Mollnes TE, Brekke OL* The key roles of complement and tissue factor in *Escherichia coli*-induced coagulation in human whole blood. Clin Exp Immunol. 2015 Oct; 182 (1):81-9.

Paper II

***Landsem A**, Fure H, Mollnes TE, Nielsen EW, Brekke OL.* C1-inhibitor efficiently delays clot development in normal human whole blood and inhibits *Escherichia coli*-induced coagulation measured by thromboelastometry. Thrombosis Res. 2016 Apr 28; 143:63-70

Paper III

***Landsem A**, Fure H, Ludviksen JK, Christiansen D, Lau C, Mathisen M, Bergseth G, Nymo S, Lappegård KT, Woodruff TM, Espevik T, Mollnes TE, Brekke OL.* Complement component 5 does not interfere with physiologic hemostasis but is essential for *Escherichia coli*-induced coagulation accompanied by Toll-like receptor 4. Clin Exp Immunol. 2018 Nov 16

Paper IV

Gustavsen A, Nymo S, ***Landsem A***, Christiansen D, Ryan L, Husebye H, Lau C, Pischke SE, Lambris JD, Espevik T, Mollnes TE. Combined Inhibition of Complement and CD14 Attenuates Bacteria-Induced Inflammation in Human Whole Blood More Efficiently Than Antagonizing the Toll-like Receptor 4-MD2 Complex. J Infect Dis. 2016 Jul 1;214 (1):140-50.

Abstract

The interaction between the complement system as a part of the innate immune system and the coagulation system is close. Increased knowledge about the interaction between these systems is important to enable development of more efficient medications. Sepsis is still a disease with a high mortality despite years of research [1]. Globally, sepsis is the cause of death for more than five million individuals annually and the mortality is 26% [2]. In Norway the incidence of sepsis is 140 per 100.000 inhabitants per year and the overall mortality was 13.5% [3]. Mollnes et al. hypothesized that combined inhibition of complement and cluster of differentiation 14 (CD14) blocks the sepsis-induced immune response [4]. The complement system and the Toll-like receptor (TLR) interact and work synergically and a combined upstream inhibition may reduce the immune responses [5, 6]. Thus, the focus in this thesis is the effect of the selective and combined complement and CD14/TLR inhibition on bacteria-induced coagulation. The human whole blood model of inflammation [7] was used to study some of the key components, including complement component 3 (C3) and 5 (C5), C1-inhibitor (C1-INH), CD14, TLR4 and tissue factor (TF). In paper I, we used the C3 specific inhibitor compstatin alone or combined with an anti-CD14 antibody (anti-CD14), an important cofactor for several TLRs including TLR4. The combined inhibition significantly reduced the *Escherichia coli (E. coli)*-induced coagulation which was TF dependent. In paper II, we studied the effects of increasing amounts of C1-INH on coagulation kinetic and on *E. coli*-induced coagulation as C1-INH was postulated to be procoagulant [8]. High supraphysiological doses of C1-INH abolished the *E. coli*-induced coagulation analyzed using rotational thromboelastometry. In paper III we showed that C5 had no effect on the normal physiological hemostasis *in vitro*. In contrast, eculizumab reduced the *E. coli*-induced TF messenger RNA (mRNA) and TF function in microparticles (TF-MP). The combined inhibition with eculizumab and anti-CD14 or eritoran most efficiently reduced the *E. coli*-induced coagulation. The effect of the combined inhibition on inflammation, phagocytosis and leukocyte markers CD11b and CD35 was studied in paper IV. Anti-CD14 was a better inhibitor than eritoran on the *E. coli*-induced TF and the combined inhibition was even more effective. In conclusion, this thesis discloses new knowledge on treatment of sepsis-induced coagulation.

1 Background

1.1 Introduction

Traditionally, the coagulation and immune systems have been considered two different cascade systems with separate functions. However, in the last years crosstalk and multiple interactions between the two systems have been found [9]. Some molecules seem to have an especially important role and complement C5, TLRs and TF are all involved in these interactions. The process involving interaction of both coagulation and immune systems in diseases such as sepsis is now called immunothrombosis as a part of the innate immunity [10]. This is probably a beneficial local process since it may limit the spread of bacteria into the blood stream from a local bacterial infection [10]. Thromboinflammation is another term used about the intravascular activation of immune and endothelial cells caused by activation of the cascades [11]. However, in a "systemic whole-body inflammation" such as severe sepsis with disseminated intravascular coagulation (DIC) the immunothrombosis is disadvantageous and does more harm than good [12]. There are also strong interactions between the complement system and TLRs [6]. Several studies have shown that complement system and TLR act in synergy during inflammation [6]. Combined upstream inhibition of both complement and TLRs may thus be necessary to inhibit systemic bacteria-induced thromboinflammation [6].

The term acute inflammation was introduced by Celsus about 2000 years ago. The Latin word *inflammare* means "to set on fire". The five cardinal symptoms of acute inflammation are *rubor* (redness), *calor* (heat), *tumor* (swelling), *dolor* (pain) and *function laesa* (loss of function), illustrated in Fig. 1. The heat and redness is due to vasodilation and tumor is due to increased vascular permeability. Pain is due to activation of nociceptors. Loss of function is due to pain, reflex muscle inhibition and disruption of tissue structure. In more recent definitions of inflammation, clinical signs and enhanced levels of inflammatory mediators are often included. Inflammation is now considered to be due to activation of complex cascades and repair mechanisms.

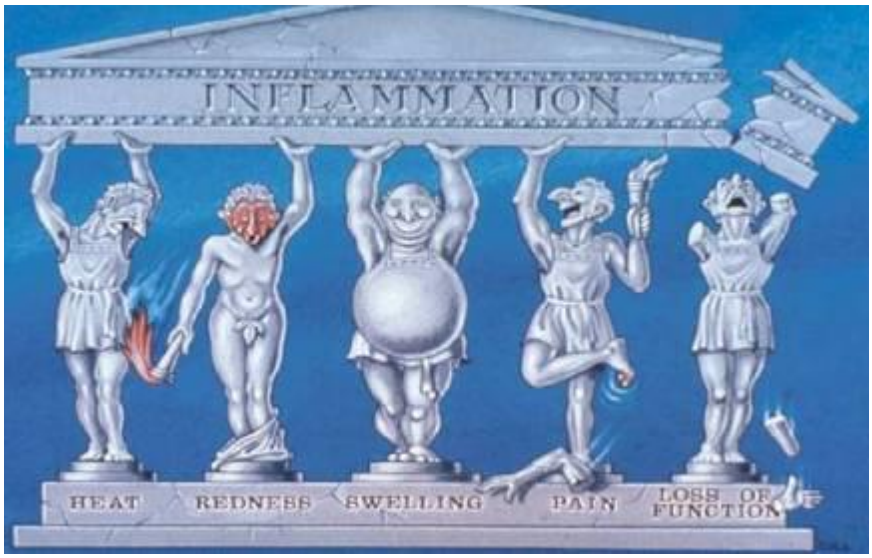

Fig. 1. Inflammation characterized by five signs: heat, redness, swelling, pain and loss of function.

Sepsis is a typical example of acute systemic inflammation from the clinical situation with interaction between the complement and coagulation systems [1, 13]. The whole blood model developed at Nordland Hospital is an *in vitro* model of inflammation constructed by adding bacteria to fresh human whole blood [7]. The main advantage of this model is that the anticoagulant used, namely lepirudin, does not affect complement activation, measured as terminal complement complex (TCC) [7]. This thesis brings forward new knowledge about the role of innate immune systems in bacteria-induced thromboinflammation, focusing on complement, TLRs, coagulation, and the crosstalk between them.

1.2 Sepsis

What is sepsis? The answer is complex and has changed over time. Homer used the term sepsis around 2700 years ago. The term comes from *sepo* meaning, "I rot" [14]. Hippocrates described sepsis as an undesirable putrefaction that could occur, for example, in the colon and induce "dangerous principles" and, furthermore, "auto-intoxication". First in 1991, "a consensus conference group" agreed upon a diagnosis of sepsis, systemic inflammatory response syndrome (SIRS), severe sepsis and septic shock [15]. The International sepsis definitions conference in 2001 extended the description of signs and symptoms [16]. In 2016, the Third International Consensus Definitions for Sepsis and

Septic Shock (Sepsis-3) defined sepsis as "a life-threatening organ dysfunction caused by a dysregulated host response to infection" [1]. They included a sequential (sepsis-related) organ failure assessment (SOFA) score to identify the organ dysfunction. Septic shock is a severe situation with hypotension and increased mortality. In septic shock patients vasopressors are needed to maintain the mean arterial pressure ≥ 65 mm Hg and the lactate concentration in plasma below 2 mmol/L despite fluid resuscitation [1].

Sepsis is a heterogeneous condition, but in general the immune response overreacts against pathogens and several systems are disturbed. After some time, the immune system is suppressed, and the body has problems maintaining a normal homeostasis [13]. The inflammatory response thus varies over time. In fact, the mortality has been shown to be higher in the immunosuppressive later phase [17]. Activated immune cells induce coagulation activation. The coagulation activation observed during infections is thought to be an attempt to inhibit pathogen spreading [18]. However, in sepsis the infection is spread via the bloodstream and the microthrombi in the microvasculature is harmful and induce organ failure and even death [19].

Due to the huge coagulation activation with increased consumption of coagulation factors and platelets, the production of coagulation factors and inhibitors is reduced and the degeneration is increased. That induce an increased risk of bleeding [20]. The anticoagulation system and the fibrinolytic system are also reduced during sepsis. Thus, the complications of sepsis can be both thrombosis and bleeding [21]. Disseminated intravascular coagulation (DIC) is a state with widespread generation of microthrombosis [10]. The reduced blood flow can induce organ failure e.g. in the kidneys and brain. [20]. The general treatment for septic shock is antibiotics, fluid resuscitation, corticosteroids and oxygen administration. This treatment is given to inhibit bacterial growth and maintain fluid homeostasis [22]. *Escherichia coli (E. coli)* and *Staphylococcus aureus* (*S. aureus)* bacteria are the most common causes of for sepsis [2].

1.3 Innate immunity

The immune system is divided into innate and adaptive immunity. This thesis will focus on innate immunity and the complement system. The host has several defenses against invading pathogens. Examples are physical, chemical and biological barriers like skin,

sweat, saliva, gastric acid and gut flora. However, a cellular defense is needed when the pathogens cross these barriers. Microbes have molecular patterns named pathogen-associated molecular patterns (PAMPs) [23]. These foreign molecules are detected by receptors on human cells called pattern recognition receptors (PRRs). The danger-associated molecular patterns (DAMPs) refer to molecules expressed in response to a cell injury or cell death [23]. Bacteria and other particles opsonized by antibodies (IgG or IgM) are recognized by Fcγ receptors, while complement opsonins like iC3b, C3b or C3d are recognized by complement receptor (CR) 3 [24]. CR3 consists of CD11b/CD18, and the main ligand is iC3b. Monocytes express CR3 and the expression is increased by activation and during differentiation to macrophages [25]. Resting neutrophils hardly express CR3, but the expression is increased by cell activation [25]. CR1, also called CD35 is another complement receptor that recognize C3b and C4b opsonized particles and induces phagocytosis [26]. Macrophages, monocytes and neutrophils ingest pathogens by phagocytosis. The first step in this process is recognition of a target particle larger than 0.5 µm, followed by activation of the internalization machinery through signaling. The generated phagosomes fuse with lysosomes and form phagolysomes. The ingested particle is then broken down by enzymes in the phagolysomes [24]. *S. aureus*-induced TLR response occurs after phagocytosis [27]. The phagosome maturation induces an acid environment which activates important enzymes. These enzymes release ligands from the bacteria required for a full immune response [27]. Inhibition of *E. coli* phagocytosis did not inhibit the TLR4 dependent response [27]. Ip et al. conclude that *E. coli* can induce cytokine production from the cell surface in contrast to *S. aureus*, which required degradation in the endosome [27].

1.3.1 Cytokines

Cytokines are small proteins that are released from different cells. They are important molecules involved in the communication and interaction between different cells. Cytokines can act in several ways, and may have autocrine actions on the cell itself, paracrine effects on cells close by or endocrine effects on cells at a distance [28]. Tumor necrosis factor (TNF), Interleukin (IL)-1β and IL-6 are mainly proinflammatory cytokines [28]. Some cytokines are chemokines that activate and induce migration of leukocytes. The chemokine IL-8 also called chemokine (C-X-C motif) ligand 8 (CXCL8)

acts on neutrophils and T-cells [28]. IL-10 is one of the anti-inflammatory cytokines [28]. Many cytokines are released after binding of PAMPs such as lipopolysaccaharide (LPS) to TLR4, leading to activation of intracellular signaling and the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [29]. The activation of NF-κB leads to upregulation of several cytokine mRNAs [6]. TLR4 can also induce signals through another pathway. Translocation of TLR4 to endosomes or phagosomes activates a TRAM-TRIF dependent pathway that results in interferon regulatory transcription factor (IRF) 3 phosphorylation and IFN-β production [6]. Some cytokines like IL-6 induce synthesis of acute phase proteins such as CRP in the liver [30].

1.4 The complement system

1.4.1 Overview

The complement system (Fig. 2) is an important part of the innate immunity system. Everything, i.e. cells or substances, including debris, microorganisms and artificial materials, that do not express factor H (FH) is attacked by a complement component. Self cells are protected by expressing FH due to the competition between FB and FH, which inhibits the further activation of the complement [6]. In addition, complement molecules recognize antibodies bound to pathogens or conserved patterns from pathogens. The complement system is a cascade system, i.e. the activation of one component activates the next and so on [25]. The complement system consists of more than 50 proteins [31] and constitutes about 3 g protein per liter plasma [32]. Complement activation leads to opsonization, increased phagocytosis and recruitment of inflammatory cells to the site of infection [33]. The terminal product, TCC (C5b-9) also induces lysis of certain complement-sensitive bacteria or activation of immune competent cells. Soluble TCC in plasma is commonly used as a marker of complement activation. Anaphylatoxins, including C3a, C4a and C5a are released during the complement activation. They have several effects for example activation of myeloid cells, platelets and inducing chemotaxis to enhance the immune response [33, 34]. Previously, the complement system was thought to be present only extracellularly. However, intracellular stores of both C3 and C5 have been detected in human T-cells [35]. Complement inhibitors and regulators in cells prevent damage from complement activation [35]. Complement regulators are both in the fluid phase and membrane-bound on cell surfaces [36]. An increasing number of

diseases are associated with disorders in the complement system, caused by both local and systemic activation [37]. Examples of diseases with local complement activation are adult macular degeneration, stroke, myocardial infarction and periodontitis [37]. While examples of diseases with systemic complement activation are sepsis, trauma, paroxysmal nocturnal hemoglobinuria (PNH), atypical hemolytic uremic syndrome (aHUS) and systemic lupus erythematosus [37].

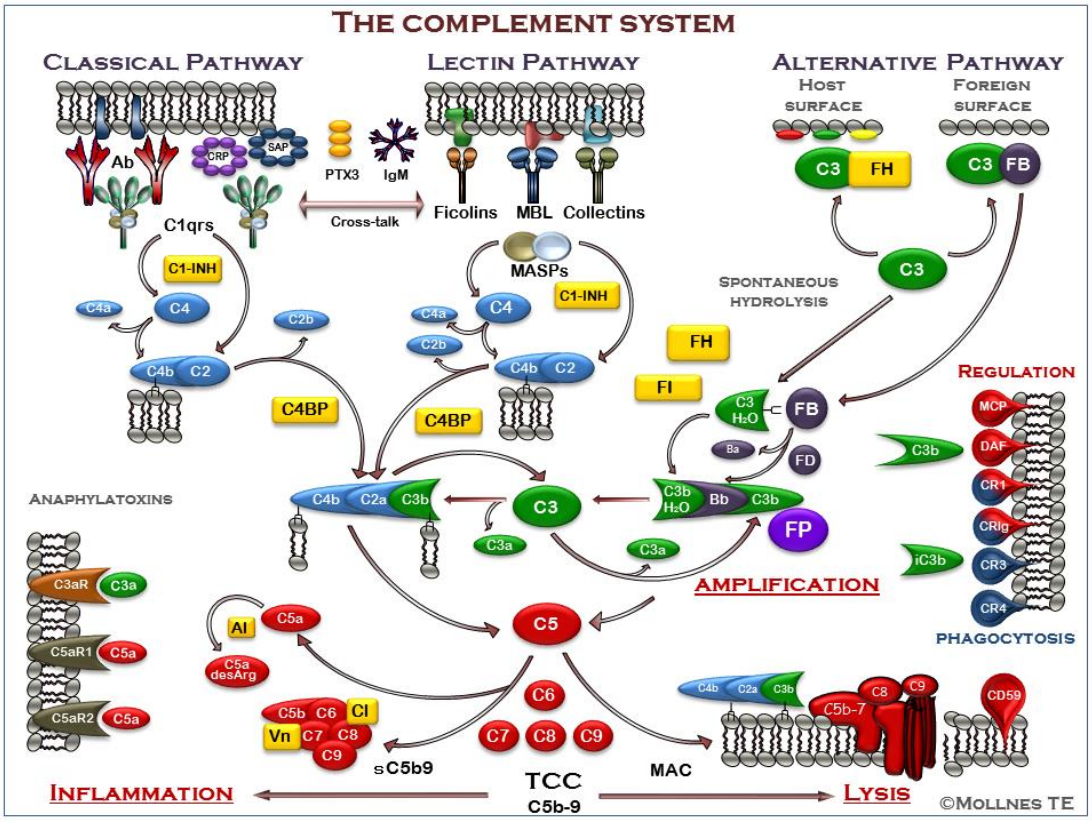

Fig. 2. The complement system. *The complement system is activated via three pathways the classical, lectin and alternative pathway. Surfaces covered by antibodies and pentraxins activate the classical pathway. Ficolins, MBL or collectins bound to carbohydrates activate the lectin pathway. Foreign or damaged cells activate the alternative pathway through spontaneous hydrolysis of C3. The C3 convertases is formed (C4bC2a for classical and lectin pathway and C3bBb for the alternative pathway). C3 is cleaved to the anaphylatoxin C3a and C3b which activates the cascade further. Then the C5 convertase is formed and C5 is cleaved to the anaphylatoxin C5a and C5b which together with C6, C7, C8 and C9 form the MAC or a soluble form sC5b9. Inhibitors are yellow in the figure.*

Abbreviations: a = activated, Ab = antibody, Al = anaphylatoxin inhibitor, C = complement component, C1-INH = C1-inhibitor, Cl = clustrin, CRP = C-reactive protein, DAF = decay accelerating factor, F = factor, I = inactivated, IgM = immunoglobulin M, MAC = membrane

attack complex, MASP = MB associated serine protease, MBL = mannose-binding lectin, MCP = membrane cofactor protein, PTX3 = long pentraxin 3, R = receptor, SAP = serum amyloid P component, TCC = terminal complement complex, Vn = vitronectin. Reprinted with permission from T.E. Mollnes.

1.4.2 Activation pathways

The complement system has three main activation pathways (Fig. 2). The classical pathway (CP) is activated after contact between immunoglobulin G or M complexes and C1q. The binding between antigen and antibody induces a slight configuration change in the Fc-domain of the antibody that permits C1q binding [38]. C1q has also other ligands such as phosphatidylserine (PS) [39], pentraxins like C-reactive protein (CRP) [40] and long-pentraxin 3 (PTX3) [41]. C1q bound to a ligand proteolytically activates C1r and C1s, which provide a further activation of the complement cascade [38]. In the lectin pathway (LP), conserved pathogen-specific structures in microorganisms and altered self-antigens are detected by soluble mannose-binding lectin (MBL), collectins (CL-10 and CL-11) or ficolins (Ficolin-1, -2 or -3) [42]. CL recognizes sugar patterns [33]. The MBL, which is a part of the CL family, has up to six trimeric subunits, is similar to C1q [32] and recognizes glucose, mannose and N-acetyl-glucosamine [33]. These molecules are expressed in viruses, bacteria and dying cells [33]. Ficolins recognize and bind to acetyl groups on bacteria [33]. The binding between receptors in LP and their ligands leads to binding and activation of the MBL-associated serine proteases (MASP)-1, and -2 [33]. The activation of CP or LP leads to cleavage of C4 and C2. The complex of C4b and C2a, C4bC2a formed in CP and LP, is called C3 convertase and cleaves C3 to C3a and C3b [33]. The third pathway is the alternative pathway (AP). In contrast to the other pathways, this pathway's activation is spontaneous hydrolysis of C3 to C3 (H_2O) [43]. The changed C3 structure facilitates binding to factor B (FB). FB is cleaved by factor D to Ba and Bb. Bb and C3 (H_2O) forms a complex. C3bBb is the alternative pathway C3 convertase [44].

The C3 convertases can cleave C5 only to a small extent. If a C3b molecule binds to the C3 convertase, the cleavage of the C5 molecules to C5a and C5b by the CP C5 convertase is increased a thousand-fold compared to CP C3 convertase [33]. The CP C5 convertase is six to nine time faster than the AP C5 convertase [45]. Properdin stabilizes the AP C5

convertases and increases the halftime [33]. Properdin and the AP amplification loop gives many C3b molecules making AP C5 convertase to the main contributor of C5b-9 [33]. Properdin as a pattern recognization molecule in the alternative pathway is controversial and the study by Harboe et al. showed that properdin did not have this function [46]. C5b interacts with C6, C7, C8 and several C9 molecules to form the C5b-9 complex, the membrane-attack complex. The MAC induces a pore in the membrane, causing calcium flux and lysis of certain complement sensitive bacteria [33]. In addition to lysis, the membrane-attack complex can induce both cellular activation [47] and tissue damage [48].

The terminal pathway with lysis is the major defense against infections with *Neisseria meningitidis*. These bacteria can survive intracellularly, and extracellular lysis is necessary to kill them. People that have deficiencies of the terminal complement components, such as C5 deficiency, have 7000-10 000-fold higher risk for disease caused by meningococcal infections [49]. However, in most cases the disease is limited to recurrent meningitis with milder symptoms than in healthy subjects [50].

1.4.3 Anaphylatoxins and their receptors

The C3 gene is localized to chromosome 19 and glycoprotein is 185 kilodalton (kDa) and the matured protein has 1641 amino acids [51, 52]. C3 is cleaved by C3 convertases to a small fragment, C3a, and a larger fragment, C3b. C3a is an anaphylatoxin, i.e., an inflammatory mediator and C3b is an opsonin [33]. Carboxypeptidase N inactivates C3a by removing the C-terminal arginine group and this des-Arginated molecule is called C3a des-Arg [53]. C3a, in contrast to C3a des-Arg binds to the C3a receptor (C3aR) [54, 55]. Enzyme release from leukocytes is dependent on C3a [56]. C3a des-Arg has only one known receptor, which is C5L2 [57]. However, whether this binding is direct is still unclear [58].

C5 is located at chromosome 9 q32-q34 [59]. The protein has 1676 amino acids and its molecular weight is 188 kDa. C5 is produced by hepatic cells, but also locally by for example macrophages [60]. C5 convertase cleaves C5 into C5a and C5b. C5a is an approximately 12–14.5 kDa glycoprotein with 74 amino acids [61]. There are three

disulfide linkages that stabilize the protein [61]. Carboxypeptidases remove the C-terminal arginine from C5a, and C5a des-Arg is formed [53]. C5a des-Arg has 10–100-fold lower affinity to C5a receptor 1 (C5aR1) [62]. However, the C5L2 receptor has 10-fold higher affinity to C5a des-Arg [35]. The human C5aR was cloned in 1991 [63, 64], and C5L2 was detected in 2000 [65]. C5aR is expressed in different cell types, including myeloid cells, T-cells and cells from different organs such as the kidney, liver, brain and lung [66]. C5a induces synthesis and release of proinflammatory cytokines, including TNF, IL-1β and IL-6 in human monocytes [67]. C5a is a strong chemoattractant for neutrophils [68]. In addition, C5a enhances phagocytosis and induces oxidative burst in neutrophils [7]. TF expression in endothelial cells is mediated by C5a [69]. Other effects of C5a are increased vascular permeability, vasodilation [70] and histamine release from mast cells [71].

C4a from CP and LP is an anaphylatoxin produced by cleavage of C4 molecules after activation of the CP or LP. Tsuruta et al. showed in 1993 that C4a has an inhibitory effect on monocyte chemotaxis [72]. The role of C4a in humans has been debated; some observe effects, while others find few or no effects [33, 34]. Contamination with C3a and C5a has disrupted several studies and the interpretation of these results is therefore difficult [34]. Recombinant C4a has shown to inhibit C5a-induced neointima formation [73]. C4a has protective effects in arterial injuries [73]. A specific receptor for C4a, like C3aR or C5aR1, has not yet been detected. However, C4a has antimicrobial effects [34]. Recently, Wang et al. published a study indicating that C4a is probably a ligand for protease-activated receptor (PAR) 1 and PAR4 [74].

1.4.4 Complement regulators

The complement system is tightly regulated by both membrane-bound CR1, decay-accelerating factor (DAF) and membrane cofactor protein (MCP), in addition to plasmatic inhibitory proteins [36]. Factor H (FH) is an important regulator for the alternative pathway as well as a membrane-bound and plasmatic inhibitor [36]. Host cells have sialic acid-capped glycans and proteoglycans with glycosaminoglycan (GAG) chains as markers of self. FH binds to these surfaces and inhibits complement activation on host cells [75]. The interaction between FH and “self marker” increases the affinity for C3b

binding in competition with FB [75]. The stability of the alternative pathway C3 convertase (C3bBb) is reduced by FH by the increased dissociation of C3b and Bb. FH is also one of the cofactors for factor I (FI). FI inactivates C3b to iC3b and C3f by proteolysis; however, FI needs a cofactor [76, 77]. iC3b does not bind to FB [78]. iC3b is further cleaved to C3c and C3dg by FI, and to C3g and C3d by other proteases [77]. iC3b, C3b and C3d opsonize microbes. They are ligands for complement receptors, including CR1 and CR3, involved in phagocytosis [77]. If C4b-binding protein (C4BP) acts as a cofactor for FI, FI is able to cleave C4b from C4bC2a, which is a C3-convertase [76]. C4BP increase the natural decay of the C3 convertase. C4BP also has a role in the alternative pathway acting as cofactor for FI and cleaving the C3b molecules in the fluid phase [79]. C1-INH is described in more detail below. CR1, also named CD35, is a membrane bound regulator. CR1 on erythrocytes remove C3b and C4b-opsonized immune complexes and pathogens from the circulation by delivering them to the liver and spleen [76]. CR1 also inactivates C3b and C4b by acting as cofactor for FI [33]. CD55, also called DAF, acts on the AP C3 convertase and the CP and LP C3 convertase. CD55 decreases the stability of all C3 convertases [76]. MCP, also known as CD46, is a cofactor for FI and inactivates both C3b and C4b [33].

1.4.5 C1-inhibitor (C1-INH)

C1-INH was first described by Ratnoff in 1957 [80]. C1-INH was earlier called C1-esterase inhibitor. It belongs to the serpin family which inhibits serine proteases. The gene for C1-INH is located at chromosome 11, p11.2-q13 [81]. The protein is 105 kDa and consists of a protease recognition domain and a glycosylated amino terminal domain. In human plasma, the concentration is approximately 0.25 g/L [82]. Citrated plasma from a healthy person contains 1 unit (U)/ milliliter (mL) of C1-INH. Hepatocytes, monocytes, macrophages, fibroblasts, endothelial cells, microglia cells and amnionic epithelial cells produce C1-INH [83]. The production is increased by cytokines like IFN-γ, TNF and IL-6 [84]. Alpha granula in platelets contain C1-INH, and activation of platelets can locally increase the C1-INH concentration several times [85, 86].

1.4.6 C1-INH is a serpin

Serpins inhibit serine proteases by a two-step process. The serpin mimics the target for the protease and the binding between protease and serpin induces a cleavage, for C1-INH of Arg444-Thr445 [87]. The following molecular rearrangement induces an irreversible covalent binding between the active seat in the protease and the serpin. The protease is now inactivated [88, 89]. Some of the serpins are affected by the glycosaminoglycan (GAG). GAG increases the inhibitory effect C1-INH has on C1s and FXIa [83]. However, the inhibition on kallikrein and FXIIa is not affected by GAG [83]. The complex of C1-INH and protease binds to serpin-enzyme receptors on monocytes and hepatocytes and is then removed from the circulation [90]. C1-INH inactivates several proteases (Fig. 3), in the complement, coagulation and fibrinolytic systems [91]. C1-INH is also an important protease inhibitor in the kallikrein-kinin system, see below [91].

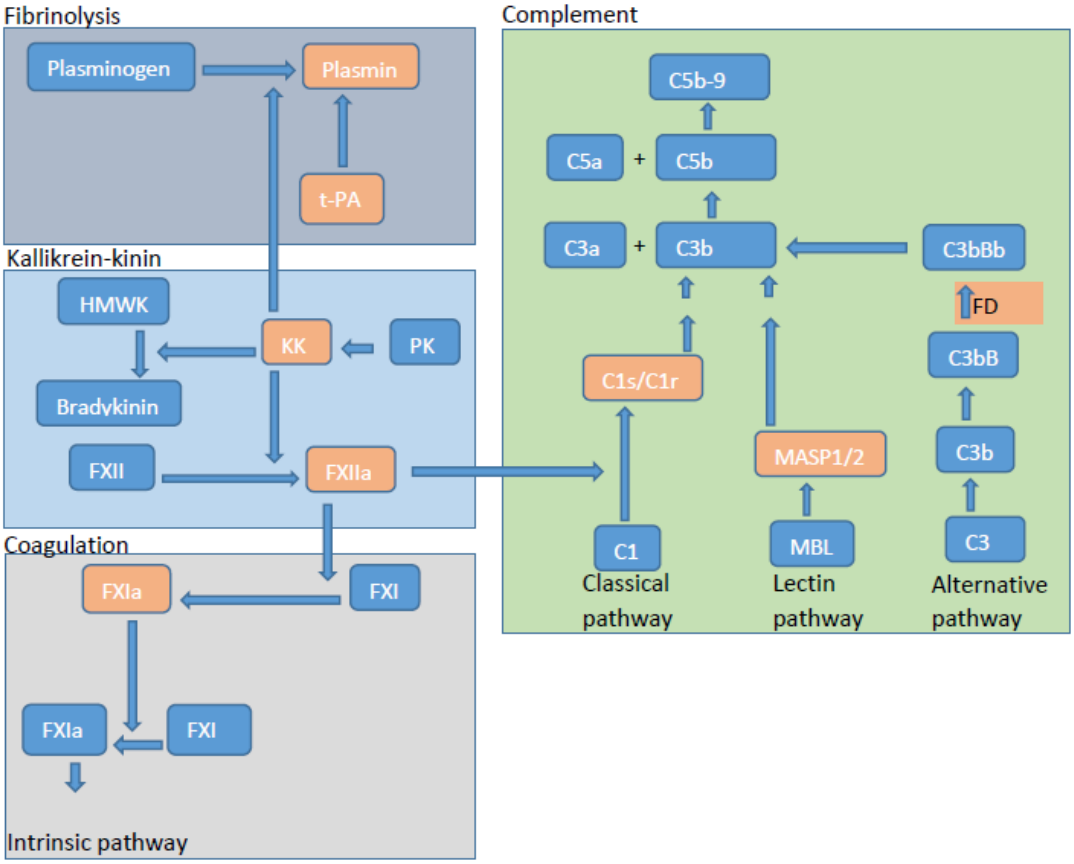

Fig. 3. An overview of the effects of C1-inhibitor on the fibrinolysis, kallikrein-kinin, coagulation and complement system. *The orange boxes indicate that C1-inhibitor inhibits these*

proteases. The effect of Factor VII activating protease and the direct activation of FVII via FXIIa are not included in the figure. Abbreviations: a = activated, C = complement component, F = factor, HMWK = high-molecular-weight kininogen, KK = kallikrein, MASP = MB associated serine protease, MBL = mannose-binding lectin, PK = prekallikrein, t-PA = tissue plasminogen activator.

1.4.7 C1-INH and the complement system

In the CP, C1-INH binds and inactivates C1r and C1s, and they form the complex C1rC1s $(C1\text{-}INH)_2$ [92]. The inactivation by C1-INH inhibits the autoactivation of C4 and C2. C1-INH stabilizes the C1 complex without covalent binding [93]. In the LP, C1-INH forms stabile complexes with both MASP-1 and MASP-2 [94]. C1-INH inhibits the alternative pathway by a non-covalent binding to C3b and consequently inhibiting factor B binding to C3b [95]. A comparison between the effects of C1-INH on the three complement activation pathway showed that C1-INH in supraphysiological doses inhibited both CP and LP, however the inhibition in LP was more pronounced in low doses. Only the fluid AP activation was inhibited by C1-INH [96].

1.4.8 C1-INH and hemostasis

C1-INH is the main inhibitor of the FVII activating protease [83]. C1-INH also inhibits FXI [97, 98], plasmin [99] and tissue plasminogen activator (t-PA) [91]. In addition, C1-INH inhibits FXII that activate FVII. HAE patients have a increased level of FVIIa compare to their siblings [100]. Horstick et al. reported that C1-INH in high doses induced a procoagulant situation [101].

1.4.9 Hereditary angioedema (HAE) and the kallikrein-kinin system

Landerman et al. were the first to show that plasma from patients with hereditary angioedema (HAE) lacked an inhibitor of kallikrein and FXII [102]. Donaldson et al. reported that HAE patients had reduced activity of C1-INH [103]. Several years later C1-INH was shown to be the major inhibitor of kallikrein [104] and FXII [97]. There are several types of HAE: Both type I and type II have one mutated gene from one of the

parents. In type I HAE, the mutated gene produces no C1-INH, or a type which is not detected by antigenic measurements. In type II HAE, the mutated allele produce a C1-INH without function, but it can be measured antigenically. In type I and II HAE the one normal gene makes a protein with normal function and normal antigenic-value. It will be consumed rapidly, however, because 50% production (only from one gene) is not sufficient to hinder autoactivation of all proteases C1-INH should have kept in check. This explains why the C1-INH function in both HAE type I and II patients is approximately 10–20% of the normal level [105]. In addition, Haslund et al. have shown that normal C1-INH and the mutated C1-INH generate aggregates that also can explain why the functional activity is lower than 50% [106]. HAE type II patients have normal to supranormal antigenic values since the abnormal C1-INH-product from the mutated gene in type II is not consumed. The third HAE type includes several subtypes with normal C1-INH concentration and function. In a Danish review the four subgroups are caused by mostly gain of function mutations in the FXII gene, plasminogen gene, angiopoitin or an unknown mutation group [107]. HAE-patients suffer from edema in the skin and in mucosa of airways, gastrointestinal tract and genitourinary region [108]. Low activity of C1-INH allows for increased activity of the contact system and increased concentration of bradykinin [105]. Bradykinin increase vascular permeability and edema follows [105]. During HAE attacks, coagulation is very modestly activated as shown by increased FVIIa and thrombin levels [109, 110]. Nielsen et al. also found increased TCC levels during HAE attacks, indicating a very small complement activation beyond C4 and C2 [109]. The first-line treatment of HAE attacks is plasma-derived or recombinant C1-INH concentrate, inhibition of kallikrein or a blocker of bradykinin [111]. Prophylactic treatment with low doses androgens or anti-fibrinolytics has been given successfully for many years [112, 113].

1.4.10 Other anti-inflammatory effects of C1-INH

It was claimed that C1-INH binds to the extracellular matrix by binding to type IV collagen, laminin and entactin [114]. These bindings are non-covalent bindings and mediate a locally increased concentration of C1-INH in extravascular sites of inflammation, probably to increase the regulation of the complement and contact system [115]. C1-INH binds to E- and P-selectin in endothelial cells to inhibit leukocytes rolling

and transmigration [116]. Cai and Davis claimed that C1-INH expresses a sialyl Lewis^x-related tetrasaccharide on the N-glycans on the N-terminal domain [116]. A sialyl Lewis^x-related tetrasaccharide is a fucose-containing tetrasaccharide that is expressed in several plasma and cell surface proteins [115]. All selectins recognize these saccharides and the binding between selectins and these moieties in C1-INH are assumed to cause the inhibition of leucocytes rolling and transmigration [116]. Liu et al. showed that the amino-terminal domain of C1-INH interacts with lipopolysaccharides [117]. C1-INH may also bind directly to several bacteria [118]. However, a recent study claimed that C1-INH does not have these sialyl Lewis^x-related tetrasaccharides and that earlier findings were caused by contamination from antichymotrypsin in the C1-INH preparations [119]. Does this contamination also explain the mechanisms behind several of the protease independent effects of C1-INH? This question remains to be resolved.

1.5 Toll-like receptors (TLRs)

TLRs are one of the most studied families of PRRs (Fig. 4). The TLRs consists of an N-terminal ligand recognition domain with repeating leucine-rich repeats (LRRs), a single transmembrane domain and a cytoplasmic tail with a toll-interleukin-1 receptor domain that recruit adapter molecules and start the signaling process [120]. In humans, 10 different TLRs have been found [121]. Some of them are placed in the plasma membrane, as TLR2 and TLR4, and some are placed intracellularly, such as TLR7 and 8 (Fig. 4 and Table 1). Most TLRs form homodimers, except TLR 2, which forms heterodimers with TLR1 or 6 [122].

LPS on Gram-negative bacteria is recognized by TLR4 [123]. The binding of myeloid differentiation protein 2 (MD2) to the extracellular domain is necessary for activation of intracellular signaling [124]. MD2 also helps TLR4 with recognition of LPS and localization in the cell [125]. CD14 is important in the transferring of LPS to MD2 [126]. TLR4 has other ligands, including PAMPs such as viral glycoproteins and taxol and DAMPs such as high mobility group box protein (HMGB1), fibronectin and fibrinogen [127]. The ligands for TLR2 are, for example, peptidoglycans, lipoteichoic acid, zymosan and lipoprotein [121]. However, TLR2 may also bind several DAMPs, including high mobility group box 1 (HMGB1), heat shock protein (Hsp 70) and hyalinuronic acid [127].

Binding to TLR4 activates the signaling both through NF-κB and the IRF family [121]. The signaling through NF-κB is a MyD88-dependent pathway and induces proinflammatory cytokines [6]. Internalized TLR4 activates IRF via Toll-interleukin receptor domain-containing adapter-inducing interferon-β- (TRIF) related adaptor molecule (TRAM) and TRIF in the endosome [122]. IRF initiates the production of interferon-β [128].

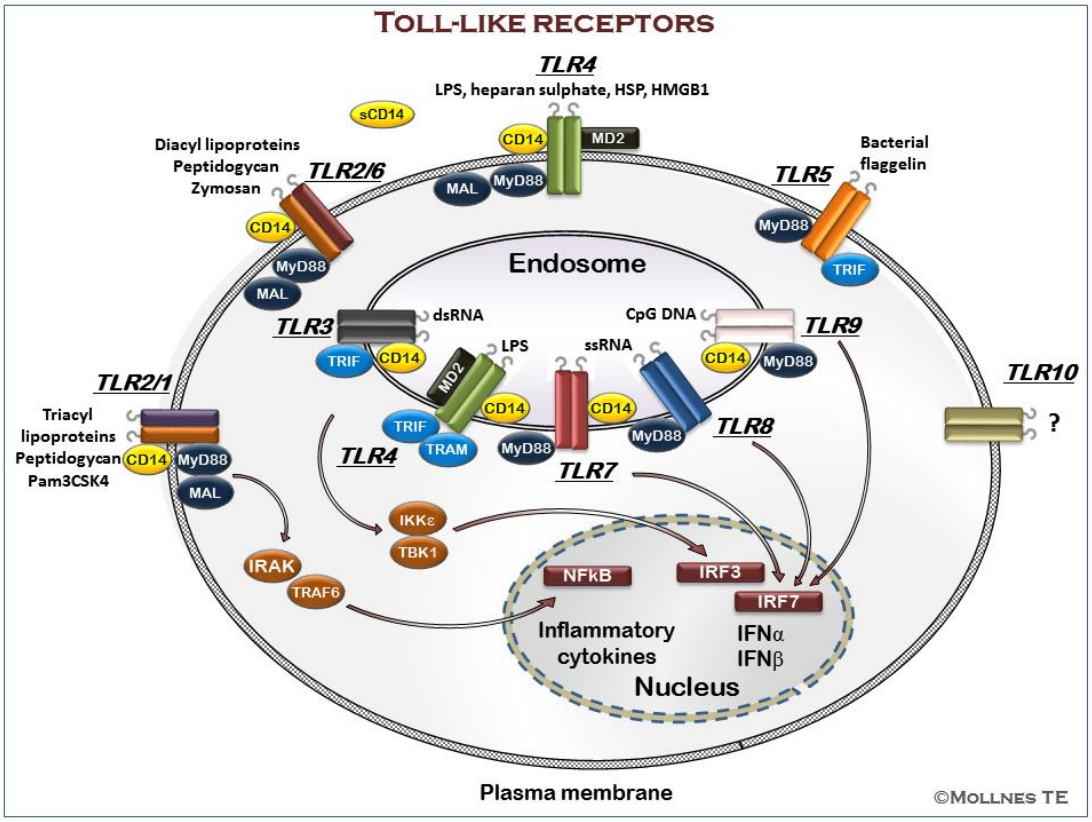

Fig. 4. An overview of the Toll-like receptors (TLRs) and their ligands. *TLRs are localized both extra-and intracellular. The intracellular TLRs in endosomes recognize ligand after endocytosis or phagocytosis. The binding induce intracellular signaling and transcription factors activate the transcription of genes. CD14 has been described as a coreceptor for murine TLR3, -7 and -9. Abbreviations: Ab = antibody, C = complement component, CD = cluster of differentiation, HMGB1 = high mobility group box 1, HSP = heat shock protein, IFN = interferon, IKK ε= inhibitor of nuclear factor kappa-B kinase subunit epsilon, IRF = interferon regulatory factor, IRAK = Interleukin-1 receptor (IL-1R)-associated kinases, LPS = lipopolysaccharide, MAL = MyD88 adapter-like, MBL = mannose-binding lectin, MD2 = myeloid differentiation protein 2,*

MyD88 = myeloid differentiation primary response gene 88, TBK1 = TRAF associated NFκB activator (TANK)-binding kinase 1, NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells, ss = single stranded, TLR = toll-like receptor, TRAF6 = TNF receptor-associated factor 6, TRIF = Toll-interleukin receptor (TIR)-domain-containing adapter-inducing interferon-β, TRAM = TRIF-related adaptor molecule. Reprinted with permission from T.E. Mollnes.

Several PAMPs activate both the TLR and complement system [129]. Studies have shown that there is a synergic effect between complement and TLRs [6, 129]. TLRs regulate the expression of complement factors and the expression or activation of complement receptors and in this way enhance or inhibit TLR-dependent responses [129].

CD14 is commonly used as a cell marker of monocytes. The glycoprotein is 55 kDa and expressed in myelomonocytic cells [130]. Membrane bound CD14 is a glycosylphosphatidylinositol (GPI)-anchored receptor but exist also in a soluble form (sCD14) [130]. Together with MD2 and TLR4 CD14 recognize LPS and a further intracellular signaling via NF-κB is mediated as described earlier and illustrated in Fig. 4 [130]. However, CD14 did also promote TLR4 endocytosis, activation of TRAM-TRIF pathway and IFN production [130]. CD14 is coreceptor also for TLR2 and 9 [6, 131].

Table 1. TLRs and their ligands

TLR	Ligands	Signaling adapter
Extracellular		
TLR1	Lipoprotein	MyD88
TLR2	LPS, Lipoprotein, HMGB	MyD88
TLR4	LPS, HMGB	MyD88, TRIF
TLR5	Flagelin	MyD88
TLR6	Zymosan, Protozoa	MyD88
TLR10*	Lipoprotein, HMGB	MyD88
Intracellular		
TLR3	Viral dsRNA	TRIF
TLR7	Viral ssRNA	MyD88
TLR8	Viral ssRNA	MyD88
TLR9	Unmethylated CpG bacterial DNA	MyD88

*Forms a heterodimer with TLR2 [132].

1.6 Hemostasis and coagulation

The blood has many functions in the body; nourishing, removing waste, gas exchange, defense against microbes, maintaining blood pressure and liquid balance and so on. These functions are dependent on a continuous stream of blood without hindrance or leaks. Hemostasis stops bleeding to prevent blood loss and maintain the circulation to organs [133]. This system has to be in balance to prevent undesirable thrombosis or bleeding. The hemostasis process has several steps, with many cellular and soluble factors involved. The first step to stop bleeding after an injury, i.e. to the skin, is vasoconstriction, which reduces the bloodstream to decrease blood loss. Blood vessel, platelets, coagulation factors, coagulation inhibitors and the fibrinolytic system are involved in the hemostasis. Platelets are activated during primary hemostasis by contact to the subendothelium that contain von Willebrand factor (vWF), collagen and TF, and the thrombin formed locally induce platelet to aggregation in the vicinity and adhere to the place of injury forming the platelet plug [11]. Secondary hemostasis refers to coagulation which results in deposition of fibrin at the platelet plug. The fibrin network gives the platelet plug more stability and strength. Tertiary hemostasis consists of the fibrinolytic system that breaks down fibrin clots.

1.6.1 Primary hemostasis

Primary hemostais is activated after vessel injury when blood is exposed to subendothelial collagen or TF-bearing cells. Platelets are 2-5 μm and without nuclei [134]. They live in the circulation for 7–10 days after formation and separation from the megakaryocytes in the bone marrow [135]. The youngest platelets are the largest and become smaller over time. Neutrophils and macrophages remove the old and fully activated platelets by transporting them to the spleen. The primary role of platelets is to maintain hemostasis and blood flow by preventing bleeding. The platelets adhere to the subendothelial extracellular matrix in case of injury or a vascular insult. The receptor GP1b/V/IX complex on the platelet surface binds to the vWF in the exposed subendothelial matrix. In addition, the GPVI and αIIβ receptors in platelets binds to collagen [136]. This binding induces a collagen-mediated platelet activation [137]. TF exposed in the subendothelial matrix and expressed in blood monocytes or endothelial cells further enhances the generation of thrombin and activates platelets through PAR4 [137]. The platelets have

several surface receptors and are involved in coagulation, complement activation, inflammation and angiogenesis [138]. There are three types of granula in the platelets. Each platelet contains 4-6 dense granula containing more than 200 small molecules such as calcium, ADP and polyphosphates which are released during platelet activation [135]. Many of these small molecules can induce signaling through receptors on the platelet surface. There are 60-80 alpha granula in one platelet, containing larger proteins, for example P-selectin, cytokines, chemokines and growth factors [135]. P-selectin tethers platelets to other cells [136]. Lysomal granula released during platelet activation release glycohydrolases involved in the degradation of glycoprotein, glycosaminoglycans and glycolipids [135]. Platelets have rough endoplasmatic retiliculum and ribosomes and are able to synthesize protein from mRNA [139].

1.6.2 Secondary hemostasis

The endothelium in the blood vessels has an anticoagulant effect that ensures the blood flow. When an injury occurs, the subendothelial cells, including TF exposing adventitial cells and matrix are available to plasma and activate several steps in the hemostasis. The coagulation cascade consists of serine proteases, which largely and normally circulate as inactive zymogens in plasma [140]. The activity of activated proteins is low, but binding to cofactors can increase the activity by five times or more. In addition, the cofactors circulate as inactive and have to be activated by proteolysis [140]. All the coagulation factors, except the vWF, TF, FIV (Ca^{2+}) and FVIII, are produced by the liver [141]. The coagulation system was previously and traditionally divided into the extrinsic, intrinsic and common pathway [142]. Serine protease FVIIa and the cell surface cofactor protein TF initiate the extrinsic pathway. The extrinsic tenase complex TF-FVIIa activates small amount of FIX and FX to FIXa and FXa [141]. The current model is the cell-based model of coagulation [143]. This model describes the activation of coagulation factors and the formation of the end product, fibrin (Fig. 5).

1.6.3 The cell-based model of coagulation

In 1992, Mann introduced a new theory of the coagulation cascade, later known as the cell-based model [142, 143]. There are three phases in this model (Fig. 5), including the

initiation, amplification and propagation phases [143]. When healthy blood vessels are injured, TF is exposed in TF bearing extravascular cells in arteries and veins. FVII binds to TF exposing cells [144]. Small amounts of FVIIa circulate in plasma in healthy individuals. The initiation phase starts when the TF-FVIIa complex is formed and activates small amount of FIX and FX to FIXa and FXa, and a small amount of thrombin is generated on the TF-bearing cells. FXa interacts with its cofactor FVa and this prothrombinase complex generates small amounts of thrombin on the TF exposing cells. FXa that dissociates from the TF-exposing cells to the fluid phase is rapidly inhibited by antithrombin and tissue factor pathway inhibitor (TFPI) localizing FXa activity to the TF bearing cell. In the amplification phase, thrombin is spread and activates the platelets in the vicinity, leading to platelet aggregation. In addition, the small amounts of thrombin activate FV, activate and dissociate FVIII from vWF and activate FXI. Platelets bind to collagen and vWF at the site of the vessel injury. The activated platelets get a procoagulant outer membrane and release granula creating a surface for assembling coagulation factors, including FXII and FXI for the propagation phase [145]. In the propagation phase, most of the thrombin needed to the local clot formation is formed. Thrombin activates FXI and FV to FXIa and FVa. Furthermore, FVIII is cleaved from the vWF and activated to generate FVIIIa [146]. The cofactors FVIIIa and FVa rapidly bind to the surface of activated platelets. FIXa generated by the TF exposing cells binds to the cofactor FVIIIa on the surface of activated platelets. In the propagation phase, the "intrinsic tenase" complex is generated, consisting of FIXa, FVIIIa and calcium [145]. This complex activates FX to FXa on the surface of activated platelets. The prothrombinase complex is a complex of FXa, FVa, phospholipids and calcium. The prothrombinase complex generates thrombin from prothrombin [147]. Thrombin has many functions; one is cleavage of fibrinogen to fibrin [147]. Thrombin removes two small peptides fibrinopeptides A and B from fibrinogen. Then, the fibrin can oligomerize to a fibrin network [148].

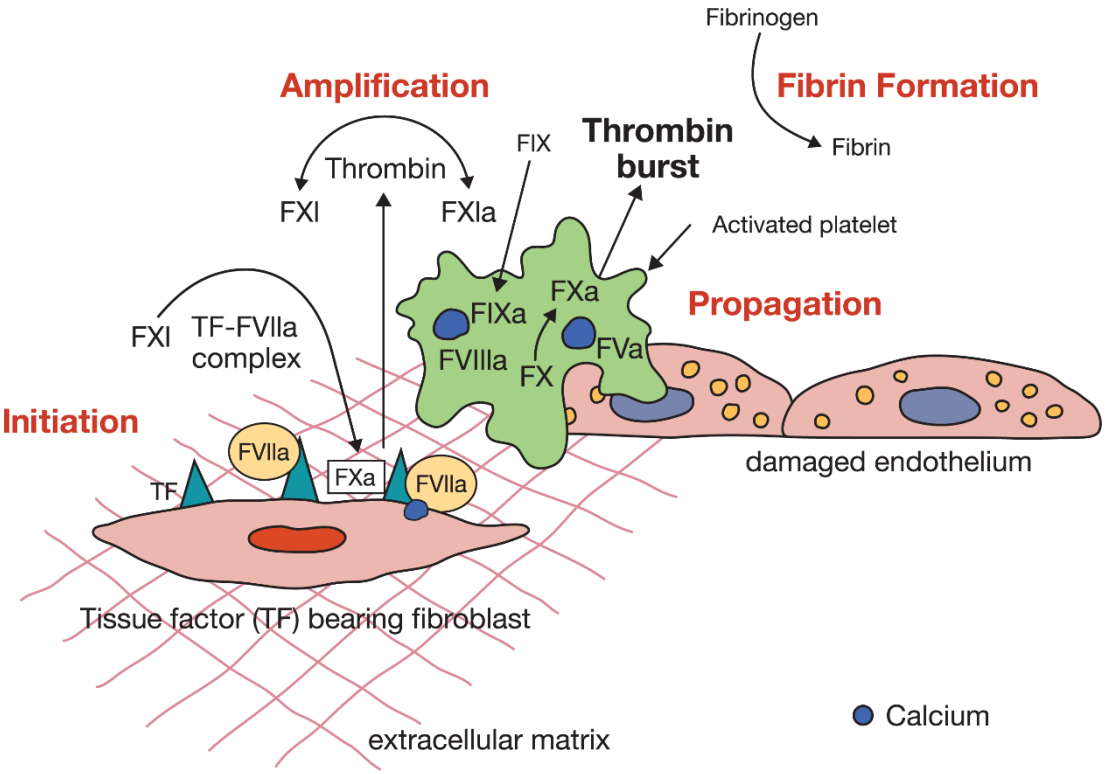

Fig. 5. The cell-based model of coagulation*. The cell-based model with three steps the initiation, amplification and propagation phase leading to fibrin formation**. Initiation after contact with fibroblasts that expressed tissue factor (TF), the small amount of thrombin is spread to platelets that became activated in the amplification phase. In the propagation phase tenase complex is formed and activate FX to FXa. Then the prothrombinase complex generates thrombin from prothrombin, and fibrin is produced by cleavage of fibrinogen by thrombin. Abbreviations: FIX = coagulation factor IX, FXa = coagulation factor X activated, TF = tissue factor. Reproduced from Anaesthesia and Intensive Care with the kind permission of the Australian Society of Anaesthetists [142] .*

1.6.4 Contact activation pathway

The contact activation system, previously called the intrinsic pathway, consists of FXII, FXI, prekallikrein and high molecular-weight kininogen (HK) [149]. Activation of the contact pathway induces activation of the intrinsic pathway and the kallikrein-kinin system [149]. The contact pathway factors are activated in blood when they encounter non-physiological negatively-charged surfaces such as kaolin, ellagic acid or glass surfaces of test tubes [149]. Other molecules and surfaces, including polyphosphates from bacteria and platelets, RNA, misfolded proteins, NETs and bacteria, can activate the contact activation system [149, 150].

FXII binds to the surface and the binding induce a conformational change in FXII causing a non-proteolytic autoactivation to αFXIIa. αFXIIa can activate soluble FXII to β-FXIIa, which activates prekallikrein to kallikrein and FXI to FXIa. HK acts as a cofactor in both enzymatic cleavages [151]. Kallikrein increases its generation by activation of FXII to β-FXIIa. It can also cleave HK to bradykinin [151]. Bradykinin is a peptide with nine amino acids. Its half time in plasma is approximately 15 s [152]. The bradykinin 2 receptor (B2R) and bradykinin 1 receptor (B1R) both bind bradykinin. The bradykinin receptors are expressed by several cells; monocytes, machrophages, neutrophils, dendritic cells, lymphocytes, microglia, smooth muscle cells, endothelial cells, fibroblasts and several tumor cells [153]. B2R is continuously expressed, in contrast to B1R, which is only expressed in inflammatory conditions [154]. Binding to B2R induces vasodilation, and increases vascular permeability as well as pain and neutrophil chemotaxis [140]. The G-protein on the seven-transmembrane G-protein coupled receptor is involved in the release of several molecules, including nitric oxide, prostaglandin I_2 and superoxide [155].

The coagulation pathway was classically divided into three pathways, the extrinsic, intrinsic and final common pathway. The activation of FXII initiates the intrinsic pathway of the coagulation, which activates FXI to FXIa [156]. Furthermore, FXIa activates FIX to FIXa [11]. This step starts the common pathway, where the extrinsic and intrinsic pathways run together.

The prothrombinase complex consists of FXa and FVa, calcium, negatively charged phospholipids – mainly PS from tissue – and platelets, which cleave prothrombin to thrombin [141]. Thrombin cleaves fibrinogen to fibrin. However, thrombin also activates FXIII and provides a stable clot with covalent crosslinked fibrin polymers [141].

1.6.5 *The fibrinolytic system*

The fibrinolytic system prevents blood clotting in healthy vessels and dissolves clots during wound healing [133]. The protease tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) activate the zymogen plasminogen to

plasmin [133]. Plasmin cleaves fibrin. Plasminogen activator inhibitor 1 and 2 (PAI-1 and -2) inhibit both tPA and uPA, while alfa-2-antiplasmin inhibits plasmin [133].

1.6.6 Regulators of the coagulation cascade

The coagulation cascade is kept under control by multiple regulators. Thrombin binds to thrombomodulin (TM), a membrane-bound protein in the endothelium [157]. TM then activates protein C, which is bound to an endothelial protein C receptor (EPCR) [158]. Protein S works as a cofactor [159] and, in addition to vitamin K, the activated protein C (APC) is an activate anticoagulant that inhibits both FV and FVIII [160]. Antithrombin inhibits thrombin, FXa, FIXa and FXIa [133]. Protease inhibitors such as alpha-1-antitrypsin and α-macroglobulin also inhibit coagulation. TFPI inhibits the activity of FXa and the TF-FVIIa complex [140]. Plasmin is generated by cleavage of plasminogen and cleaves fibrin into fibrin degradation products [141].

1.6.7 Anticoagulants

EDTA, citrate, heparin and hirudin are commonly used anticoagulants to prevent clotting after blood sampling for diagnostic purposes [161]. Both EDTA and citrate inhibits coagulation through calcium binding [7]. The recombinant hirudin analogue lepirudin is a direct thrombin inhibitor and has no adverse effects on complement activation [7]. The antithrombin activity is increased approximately a thousand-fold by heparin [162]. Non-vitamin K antagonist or novel oral anticoagulants also named NOACs are a relative new drug group used to treat deep venous thrombosis (DVT) and pulmonary embolisms (PE) [163]. These drugs inhibit thrombin or FXa [163]. These drugs are more stable compared to warfarin which has a narrow therapeutic window, interacts with a lot of components and the patients therefore need a regular monitoring of the INR level, whereas monitoring of NOACs are generally not needed [164]. Inhibitors of platelet aggregation e.g. aspirin and clopidogrel are used to treat transient ischemic attacks, peripheral arterial disease and to prevent stroke [165].

1.7 Tissue factor (TF)

1.7.1 TF structure and function

TF is also called factor 3, CD142 or thromboplastin [140]. TF is an important key component in both coagulation and inflammation. The gene for TF is localized at chromosome 1 and consist of six exons [166]. The protein is a 47 kDa transmembran glycoprotein, and is composed of 219 amino acids in the extracellular domain, 23 amino acids in the transmembrane domain and 21 amino acids in the intracellular domain. Disulfide bridges between cysteine amino acids stabilize the protein [167]. TF belongs to the class 2 cytokine receptor family [168].

TF is largely expressed in the brain, lung, heart, kidney, uterus, placenta and testis [144, 168]. Cells that are in contact with blood do normally not express TF, in contrast to perivascular cells, including fibroblasts and adventitial pericytes that express TF [168]. TF activates coagulation if the endothelial barrier breaks and, therefore, ensures a hemostatic envelope around blood vessels [169]. Carmeliet et al. showed that TF knockout mice died at the embryo stadium because of extravasations of blood cells and abnormal blood circulation [170]. Thus, the main function of TF is to sustain hemostasis and vessel integrity. However, TF also has a function in apoptosis, wound healing, angiogenesis and proliferation [168].

TF is both a cofactor and receptor for FVII [171]. FVII is produced by the liver and is the only coagulation factor that also circulates in its activate form FVIIa, consisting of approximately 1% of the total FVII level [172]. TF becomes available for FVII in connection with a vessel injury. The complex between TF and FVII can activate FVII to FVIIa. The TF-FVIIa complex also activates FIX and FX to FIXa and FXa [173]. The prothrombinase complex, consisting of FXa and FVa activates the inactive prothrombin (FII) to FIIa (thrombin) and coagulation is activated. The end product of coagulation activation is fibrin formation from fibrinogen and the injury is healed [141].

TF is not normally expressed in blood cells, but may be expressed on blood monocytes during sepsis and some other inflammatory diseases [140]. Most of the intravascular TF is inactivated or encrypted and has to be activated or decrypted by mechanisms that are still partly unknown. There have been different theories to explain the TF activation

switch. The intracellular concentration of calcium and the extracellular surface expression of PS in the cell membrane are decisive for activation of functional TF activity [174, 175]. The plasma membrane normally has an asymmetric structure of phospholipids with negatively-charged phospholipids like PS in the inner leaf and neutral phospholipids, in the outer leaf. Cell activation changes this distribution, mediated by phospholipid scramblase that transfers the negatively-charged phospholipids, including PS and phosphatidylethanolamine to the outer leaf [176]. The redox-driven disulphide bonding between Cys186 and Cys 209 changes the decrypted TF to encrypted TF [177]. Protein disulfide isomerase is assumed to catalyze this reaction; however, this reaction has to be performed before the FVII binding to TF [176].

In 2003, an alternative spliced TF (asTF) was described [178]. This isoform of TF lacks the transmembrane domain, is soluble and circulates in the blood. The mRNA for asTF lacks exon 5; therefore, exon 4 is connected directly to exon 6 [178]. The role of asTF in coagulation has been a matter of discussion; however, asTF's affinity for FVIIa is low. Nonetheless, asTF induces pro-angiogenic stimulus through activation of the integrins α6β1 and αVβ3 [179]. Cell survival, cell proliferation, metastasis and migration are other effects induced by asTF [168].

1.7.2 TF and inflammation

TF is normally not available for FVII that circulates in the blood [169]. However, there are several cells that can express TF upon stimulation [176]. Inflammation activates monocytes to express TF [168]. However, the production and expression of TF by other cells, including platelets, neutrophils and eosinophils, is controversial [140]. LPS, TNF and IL-1β may induce TF expression on endothelial cells and monocytes *in vitro* [180-182]. However, the TF expression on endothelial cells is debated [183]. High LPS concentrations induce both TF expression and activity; however, the increased TF activity may be due to simultaneously increased PS surface expression on apotopic and necrotic cells [184]. In addition, C-reactive protein has been reported to induce TF expression in monocytes [185]. The *E. coli*-induced TF expression in monocytes is C5a dependent [186]. An earlier study showed that C5a-induced TF expression in endothelial cells [69]. Shear stress induces TF and asTF on endothelial cells *in vitro* [178, 187]. It is debated

whether platelets possess TF protein or acquire TF from monocytes [188-191]. Several studies conclude that TF-positive microparticles released from platelets originates from activated monocytes [183, 188]. Müller et al. detected TF protein located in the open canalicular system and in the alpha granula of nonactivated platelets using electron microscopy [192]. However, there was no TF in the megakaryocytes, indicating that the TF is acquired from other cells [192]. Another study using laser-assisted microdissection and manipulation to avoid contamination from leucocytes could not find platelet specific TF mRNA [193]. Others claim that platelets have TF mRNA [194] as well as de novo production of TF [190, 195]. It took around five minutes from pre-mRNA to observation of TF protein dependent on Cdc2-like kinase in response to platelet activation [195]. A live *E. coli* strain, O111, induced TF expression on platelets mediated by TLR4 [196]. Activated platelets bind to neutrophils and monocytes through P-selectin [197]. This interaction induced NF-κB and enhanced TF expression on monocytes [197].

1.7.3 Other effects of TF

The TF-FVIIa complex can activate the G-protein coupled seven helix receptor protease-activated receptor (PAR) 2 [179] (Fig. 6). The following Mitogen-activated protein (MAP) kinase, Phosphoinositide 3- (PI3) kinase and Rho-like GTPases activation induce cytoskeletal rearrangements, increased cytokines production (e.g. IL-8 and vascular endothelial growth factor (VEGF), gene transcription and cell survival [179].

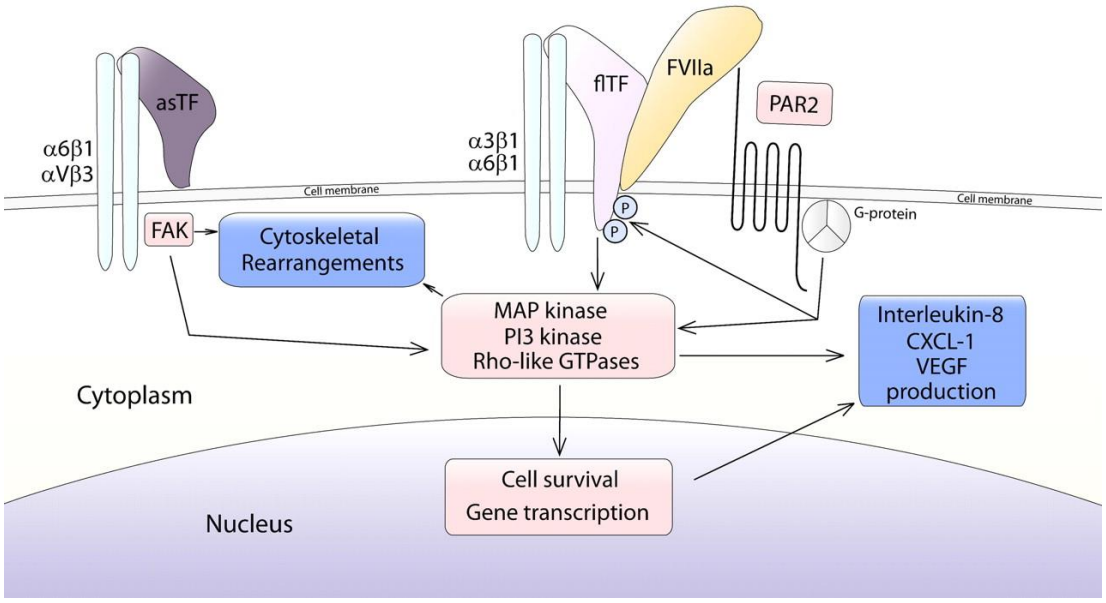

Fig. 6. Intracellular signaling induced by the Tissue factor (TF)-Factor (F)VIIa complex*. The complex of TF and FVIIa is bound to the G-protein coupled PAR2. The integrins α3β1 and α6β1 is necessary for intracellular signaling. asTF ligates these integrins and induce intracellular signaling through FAK. Abbreviations: asTF = alternatively spliced tissue factor, CXCL-1 = chemokine ligand-1, F = coagulation factor, FAK= focal adhesion kinase, flTF = full length TF, MAP = mitogen-activated protein, PAR = protease activated receptor, PI3 = phosphatidylinositol-3, Rho = Ras homologous, VEGF = vascular endothelial growth factor. Reproduced with permission of American Society of Hematology: van den Berg et al. [179].*

1.7.4 TF positive microparticles

Østerud and Bjørklid showed that monocytes were the only cells in the circulation that express TF, and that TF positive microparticles from activated monocytes can be transferred to other cells [183]. Øvstebø showed that LPS is necessary to induce TF activity by comparing *Neisseria meningiditis* with and without LPS [175]. The monocyte and monocyte derivated tissue positive microparticles expression were not dependent on LPS [175]. Over 90% of the TF activity in a human atherosclerotic plaques come from microparticles originating from monocytes and lymphocytes [198]. TF-MP level is increased by several types of cancer and in some of them the high level is associated with high risk of thrombosis [176].

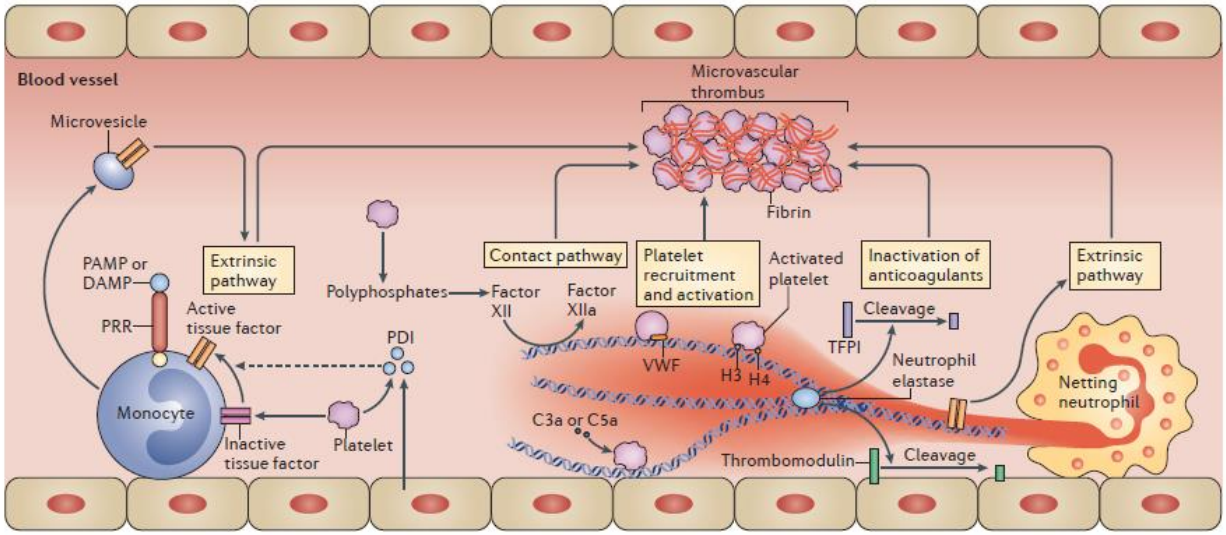

Fig. 7. Immunothrombosis. *The PRR on monocytes recognize DAMPs or PAMPs and activate the cells. TF is expressed on monocytes and microparticles are shedded. TF activate the coagulation. Neutrophils release NETs which also promote the immunothrombosis by histones and DNA. Enzymes in NETs cleave anticoagulants and the contact pathway is activated by activation of FXII. Platelet and endothelial cells derivated PDI promote fibrin generation, probably through TF activation; however, this mechanism is controversial.*

Abbreviations: DAMP = damage-associated molecular pattern, H = histone, NETs = neutrophil extracellular traps, PAMP = pathogen-associated molecular pattern, PDI = protein disulphide isomerase, PRR = pattern recognition receptor; TFPI = tissue factor pathway inhibitor; VWF = von Willebrand factor. From Engelmann and Massberg [10] *with permission to reprint from Springer.*

1.8 Thromboinflammation

1.8.1 Terms

The crosstalk between the complement, coagulation and other cascades is important to understand the mechanisms behind several diseases, and this interaction has been described in several terms. “Immunothrombosis” (Fig. 7) is a new term introduced by Engelmann and Massberg in 2013 [10]. The term describes the close connection between the coagulation and immune systems to detect and protect the host against pathogens in the vasculature [10]. Dysregulation of immunothrombosis is involved in the pathophysiology of many diseases, including myocardial infarction, pulmonary embolism and DIC [10]. Van der Poll et al. used and described the term

"immunothrombosis" as a thrombosis initiated by the immune system to provide local protection against an infection [13]. However, a dysregulated immunothrombosis can induce detrimental effects [10]. Thromboinflammation is defined as the results of cascades activation that mediate an activation of immune and endothelial cells in the vessels [11]. Activation of cells involved in innate immunity can induce pathological coagulation and thrombosis or chronic inflammation [11]. Thromboinflammation can also be activated as a result of contact with foreign surfaces, including biomaterials and drug delivery devices [151].

1.8.2 Platelets

Platelets are also able to interact directly with microbes since they have PPRs [10]. Platelets bind bacteria through receptors such as complement C1q receptor (gC1qR), which binds to bacteria coated with C1q [199]. Other receptors like glycoprotein 1b alpha polypeptide (GPIbα) and TLRs also mediate the interaction between platelets and bacteria [199]. In 2004, Shiraki et al. showed that platelets expressed TLR1 and TLR6 [200]. Cognasse et al. found that platelets also expressed TLR2, -4 and -9 [201]. During sepsis, the platelets are activated at a metabolic level, but the TLR expression on platelets is unchanged [202]. LPS stimulation does not induce granula release or phosphatidyle serine (PS) exposure [202]. Platelets, in contrast to monocytes, do not express CD14 [203], but TLR4 [204]. The platelets probably acquire sCD14 from plasma, and only high concentrations of LPS induce platelet responses [205]. The anucleate platelets contain all the proteins in the MyD88-dependent pathway for intracellular signaling following TLR4 activation [205]. The suggested results of this platelet activation are aggregation, granula secretion and cytokine production [205].

Platelets that bind and aggregate on leukocytes, forming leukocyte-platelet aggregates, play an important role in releasing NETs from neutrophils [18]. These aggregates and thrombocytopenia are often found in septic patients [18]. Leukocyte-platelet aggregates make platforms for thrombin production and a high level is associated with increased mortality [18]. Furthermore, platelets can modify the activity of different leukocytes [206].When platelets are activated, the contents of alpha and dense granula, including

platelet factor 4 (PF4) are released [207]. Lishko et al. showed that PF4 upregulates CR3 which is the receptor for PF4. Human neutrophils and monocytes express CR3 and PF4 binding increases the migration response mediated by CR3. PF4 also enhance the macrophages phagocytosis of *E. coli* in a CR3 dependent manner [208].

The microthrombi recognize and capture pathogens [10]. Thus, they inhibit pathogens from invading the tissue [209] and concentrate the antimicrobial peptides produced during the coagulation activation or released from platelets [10, 210].

1.8.3 Neutrophil extracellular traps

Activated neutrophils may release neutrophil extracellular traps (NETs) that consist of histones and DNA, as well as antibacterial components such as myeloperoxidase, lactoferrin, pentraxin and neutrophil elastase [10]. NETs also promote coagulation by stimulation and provide a scaffold for thrombus formation [211]. NETs have a negatively charged surface and activate FXII in the contact activation pathway [212], and binds vWF, which recruits platelets [213]. Furthermore, the binding of histones 3 and 4 to the NETs will promote platelet aggregation [211]. Furthermore, platelet activation is partly induced through TLR2 and TLR4 activation [214]. The NETs also contain enzymes including neutrophil elastase, which – together with extracellular nucleosomes from NETs or damaged endothelial cells – inactivate TFPI and promote- coagulation [209]. Neutrophil elastase was recently shown to decrease the inflammatory response through cleavage of cytokines, chemokines and TLRs [215].

1.8.4 Endothelial cells

TF expression on endothelial cells is induced by inflammatory cytokines such as TNF and IL-1 [180, 181]. LPS also induces TF expression in endothelial cells [216] and monocytes [217]. DAMPs, such as PS and protein disulphide isomerase (PDI)expressed on the cell surfaces or released from damaged host cells, can induce TF activation due to decryption of TF at the surface [10]. TF expression will activate FVII and promote coagulation activation as described earlier in the thesis [142]. The endothelial cells are covered by a 0.2-1 µm tick glycocalyx that maintains a normal permeability in addition

to an anti-inflammatory and anti-coagulative vessel wall [218]. Several conditions may disturb this protective layer, including cytokines, oxidants and bacterial endotoxins [219]. The complement-activation products C3a, C5a and C5b activate the endothelial cells to secrete vWF and increase expression of P-selectin [213]. Ultralarge vWF multimers promote platelet aggregation [206] and TF expression activated endothelial cells in the circulation mediate a procoagulant situation [168]. The shedding of the glycocalyx, that cover the endothelial cells, induce E-selectin and adhesion molecule 1 production and expression, that again recruite leucocytes [206]. The shedding of glycocalyx leads to edema due to enhanced vascular leakage and contributes to complications in sepsis, such as organ failure [219].

1.8.5 Other crosstalk between complement and coagulation

Multiple interactions between the complement and coagulation systems are known, but in this thesis only a few are described. C3a and C3a des-Arg induce platelet activation and aggregation [220]. Several coagulation factors, including FIX, FX, FXI and plasmin may cleave both C3 and C5 [221]. One study reported that thrombin may cleave C5 in a pure buffer system [222]. However, a recent study from our group indicates that thrombin may cleave C5 only at acidic pH (Nilsson et al. unpublished data). Eculizumab inhibits only the cleavage of C5 to C5a and C5b by C5 convertase [223]. The C5a and C5b-9 complex can induce TF expression on endothelial cells [224]. C5a may also induce TF expression on leukocytes [224]. Activated monocytes express TF that activates the coagulation system [225]. Activation of PAR2 by TF-FVIIa complex or FXa increased the IL-6, IL-8 and IL-1β generation in macrophages and neutrophils [226]. Fibrinogen and fibrin will recruit immune cells to the site of thromboinflammation supporting the fight against pathogens [227]. Activated protein C reduce LPS-induced TNF, IL-1β, IL-6 and IL-8 levels [197].

1.8.6 Inflammasomes and interaction with TF and complement

PPRs such as TLRs, Nod-like receptors (NLRs) and retinoic acid inducible gene 1 (RIG-I) can induce activation of inflammasomes by ligand binding [228]. The inflammasome is an intracellular multiprotein platform that activates caspase-1, inducing inflammation

or cell death [229]. Activated caspase-1 induces maturation of proinflammatory cytokines IL-1β and IL-18 [229]. This process requires a minimum of two signals: a priming signal from a receptor that initiates gene transcription of Nucleotide-binding oligomerization domain, leucine-rich repeat and pyrine domain-containing 3 (NLRP3) inflammasome and IL-1β via NF-κB, and an exogenous signal such as products from bacteria and virus-like toxins and glycans, environmental polluters and disease-associated accumulation of altered protein complex [228]. The second signal can also be endogenous, such as reactive oxygen species [228]. The C5a is an important signal 1 for NLRP3 activation in monocytes. Furthermore, the C5a-induced generation of reactive oxygen species regulates NLRP3 activity [228]. Rothmeier et al. found that ATP stimulation through the macrophage P2X7 induced a caspase-1 dependent actin remodeling. The result of this remodeling was procoagulant TF positive microparticles [230].

1.8.7 Long-pentraxin 3 (PTX3) and interaction with TF and complement

PTX3 is an acute phase protein that belongs to the pentraxin family [231]. C-reactive protein (CRP) and serum amyloid P component (SAP) are short pentraxins; while PTX3 is a long pentraxin [232]. The gene is localized at chromosome 3, q24-q26 [231]. The carboxy terminal domain has structural homogeneity with the short pentraxins, however, the amino terminal domain has no homogeneity with other known proteins [231, 233]. PTX3 is a 45 kDa glycoprotein, bound together with disulfide bindings [41]. Several cells, like monocytes, macrophages, alveolar epithelial cells, dendritic cells and fibroblasts, can express PTX3 [234]. The PTX3 expression is increased by stimuli such as IL-1β, TNF, LPS or microorganisms [234]. Neutrophils do not produce PTX3, but the granula contain monomers of PTX3 that form multimer PTX3 in the extracellular environment [235].

Normally, the PTX3 concentration is below 2 ng/mL, but increases to 200 –800 ng/mL due to infection, inflammation, sepsis or septic shock [233]. Several studies have shown correlations between the PTX3-levels and the severity of critically ill patients with sepsis, SIRS or septic shock [236-238]. Lee et al. performed a metastudy of 16 publications and found that the PTX3 level was significantly higher in patients with severe sepsis compared to patients with less severe sepsis and in non-survivors compared to survivors [239].

PTX3 opsonization of fungi, virus and some bacteria types increase phagocytosis activity [233]. However, PTX3 delays the inflammatory endocytosis of late apoptotic cells and, in this way, inhibits antigen presentation of self protein to protect against autoimmunity disease [240]. Genetic polymorphisms of PTX3 in humans are associated with susceptibility to tuberculosis, fungal infections and *E. coli*-induced urinary tract infections [241].

PTX3 interacts with C1q in the classical complement pathway [242]. In the lectin complement pathways, PTX3 interacts with MBL [243] and ficolin-M, ficolin-1 and ficolin-2 [241]. Endothelial cells that were stimulated with PTX3 and other stimuli such as LPS, IL-1β, or TNF increased the expression of TF [244]. LPS- and PTX3 increased the phosphorylation of IκBx in monocytes and the transcription of TF [182].

1.9 Aims of the study

The overall aims of the study were to examine some of the interactions between three important systems in the body, the complement, CD14/TLRs and their effect on bacteria-induced coagulation and inflammation. These systems are involved in many diseases, for example sepsis. We need new knowledge to understand and develop new therapeutic principles to treat sepsis.

The aim of the first study was to investigate how the selective or combined inhibition of complement C3 and CD14 affects the LPS- and *E. coli*-induced TF mRNA, TF surface expression on monocytes, TF-MP and coagulation. Blood from healthy donors was added selective inhibitors of complement C3 and CD14 alone or in combination and stimulated with *E. coli* ultrapurified LPS or *E. coli* bacteria and the effects on TF mRNA, TF surface expression, TF-MP and coagulation was measured.

The aim of the next study was to study the effect of supraphysiological doses of C1-INH on coagulation kinetics and fibrinolysis in fresh human whole blood using ROTEM®. Blood from healthy donors was added increasing concentrations of C1-INH and coagulation kinetics were analyzed using ROTEM®. We also aimed to analyse the effect of C1-INH on the *E. coli*-induced coagulation kinetics.

The aims of the third study were to examine the role of complement C5 both in normal coagulation kinetics using ROTEM® and the role in *E. coli*-induced TF upregulation and coagulation activation. We used blood from both a C5 deficient individual (C5D) and healthy donors, and added purified complement C5 to the C5D blood or the specific C5 inhibitor eculizumab to the healthy controls. In addition, we aimed to examine the effect of selective and combined inhibition of complement C5, CD14 and TLR4 on the *E. coli*-induced TF surface expression, TF-MP and coagulation activation.

The aim of the last study was to compare the effects of a specific TLR4-MD2 inhibitor (eritoran) and anti-CD14 (clone r18D11) on ultrapurified *E. coli* LPS, *S. aureus* and *E. coli*.-induced cytokine release, phagocytosis and CD35 expression. The blocking anti-CD14 antibody was used since we aimed to inhibit CD14, a coreceptor of several extra- and intracellular TLRs. The selective CD14, TLR4 inhibitors were used alone and in combination with the specific C3 inhibitor compstatin using the whole blood model of inflammation.

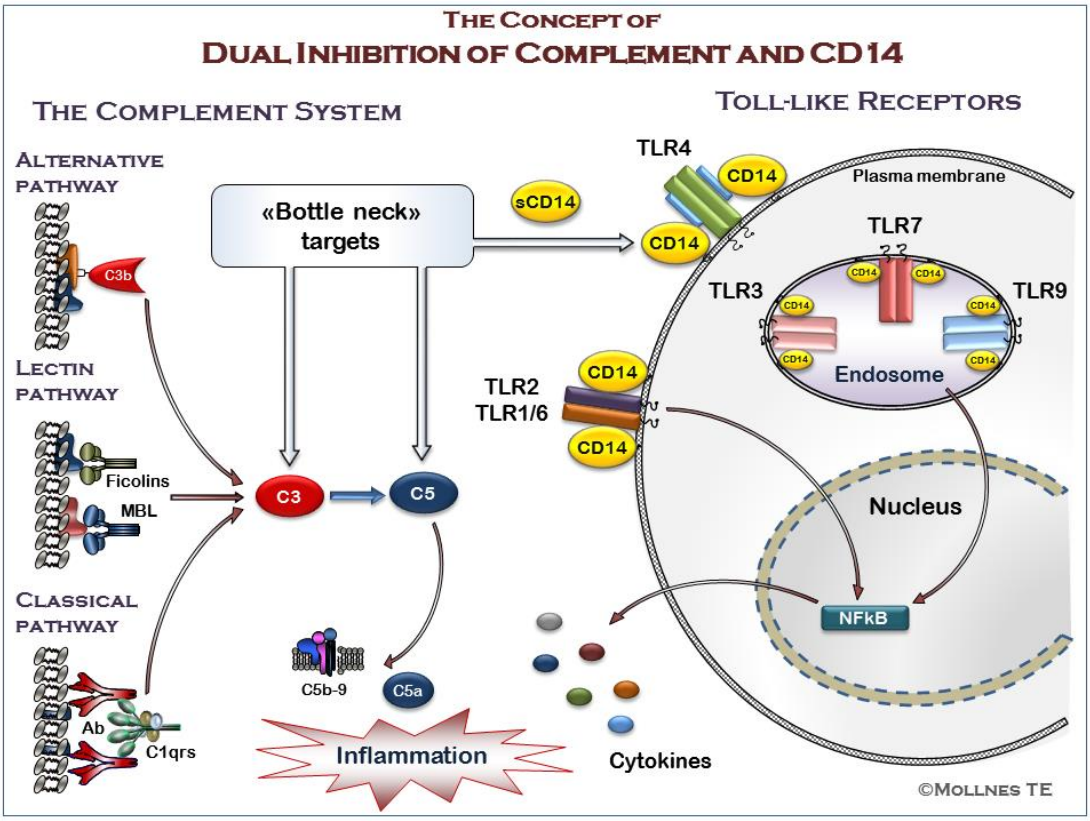

Fig. 8. Illustration of the combined inhibition of complement and TLR. *The "bottle neck" targets are C3, C5 and CD14. An inhibition of the complement and TLR can reduce the inflammatory responses and may be an additional treatment in many diseases.*
Abbreviations: Ab = antibody, C = complement component, CD = cluster of differentiation, MBL = mannose-binding lectin, NFκb = nuclear factor kappa-light-chain-enhancer of activated B cells, TLR = toll-like receptor.
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1.10 Hypothesis

In 2008, Mollnes et al. published that the combined inhibition of the complement and TLR system by anti-CD14 (Fig. 8) could be a successful strategy to avoid a dangerous overactivation of the immune system [4]. The Norwegian complement group has worked with different aspects of this main hypothesis [5, 245-251]. In this thesis, the effects of selective and combined inhibition of complement and CD14/TLRs on the bacteria-induced coagulation activation and inflammation were studied.

Paper 1:
H0: Selective and combined inhibition of complement C3 and CD14 has no effect on bacteria-induced coagulation and upregulation of TF.
HA: Selective and combined inhibition of complement C3 and CD14 has effect on bacteria-induced coagulation and upregulation of TF.

Paper 2:
H0: C1-INH has no effect on coagulation kinetic in the absence or presence of *E. coli* bacteria.
HA: C1-INH has effect on coagulation kinetic in the absence or presence of *E. coli* bacteria.

Paper 3:
H0: Complement component C5 has no effect on normal hemostasis and coagulation kinetics in fresh human whole blood.

HA: Complement component C5 has effect on normal hemostasis and coagulation kinetics in fresh human whole blood.

H0: Complement component C5 has no effect on *E. coli*-induced TF mRNA, TF surface expression on monocytes, TF-MP and coagulation.
HA: Complement component C5 has effect on *E. coli*-induced TF mRNA, TF surface expression on monocytes, TF-MP and coagulation.

Paper 4:
H0: Selective and combined inhibition of CD14 or TLR4/MD2 and complement C3 has no effect on the bacteria-induced inflammatory response.
HA: Selective and combined inhibition of CD14 or TLR4/MD2 and complement C3 has effects on the bacteria-induced inflammatory response.

2.1 Whole blood model of inflammation

2.1.1 Ethics

The project was approved by the regional ethics committee of the Northern Norway Regional Health Authority (PREK NORD 32_2004, PREK NORD 1141_2010, PREK NORD 1801_2013). The blood donors provided written informed consent. In the third paper we used blood from a C5D individual. There are only a few known cases of this defect, about 40 in the world [50]. Therefore, it is difficult to increase the number of observations from several C5D individuals. However, the experiments were done on two different days to avoid random results. Furthermore, control experiments using eculizumab added to blood from healthy donors were used to confirm the results.

2.1.2 Whole blood model of inflammation

The whole blood model of inflammation was developed in our research group [7] to allow us to study the interaction between several cascade systems, including complement and coagulation in blood, without affecting complement activation. However, the coagulation must be inhibited to avoid clotting, and lepirudin was chosen as an anticoagulant since it did not affect complement activation [7]. Lepirudin, a hirudin analogue, specifically inhibits thrombin. The lepirudin passed its expiry date in the last experiments, but it was stored as a powder and as recommended by the producer. As a control, the anticoagulant effect of lepirudin was examined. Blood samples anticoagulated with citrate and added lepirudin did not coagulate in the ROTEM® instrument using NATEM reagents and recalcification using $CaCl_2$. Furthermore, no clot was detected after 120 minutes incubation of lepirudin anticoagulated whole blood on the bench. The purity of lepirudin was also analyzed using HPLC-MS/MS and the result was one major peak corresponding to the expected molecular mass of lepirudin. Therefore, we concluded that the lepirudin was functional and stable. The traditional anticoagulants, such as EDTA and citrate, bind Ca^{2+} and inhibited complement activation and other mechanisms in blood [7]. Heparin in low concentration [252] enhanced complement activation, and high concentrations inhibited complement activation [7, 253]. Fresh human whole blood was distributed into several tubes containing PBS or inhibitors and was incubated for a few minutes, before adding activators. The tubes were incubated at 37°C in 2 hours or for longer time periods

in a rotator. The time point 2 hours was chosen after initial dose-response and time course experiments with ultrapurified *E. coli* LPS and *E. coli* bacteria. After incubation, EDTA or citrate was added to the tubes to stop further complement activation. The further processing of the tubes was dependent on the analyte of interest.

2.1.3 Whole blood model and human umbilical endothelial cells (HUVEC)

Nymo et al. has developed a modified version of the whole blood model, with endothelial cells to examine the effects of cholesterol crystals in these cells [254]. HUVEC were seeded in 48-wells plates with 7.5% fetal calf serum and growth supplements. The cells were used in passage 2–5. Prior to the experiments the monolayer of HUVEC were washed before adding lepirudin blood from healthy donors. Inhibitors were added in the blood and were pre-incubated five minutes before the activators or phosphate-buffered saline (PBS) was added as a negative control. The plate was incubates at 37°C for 4 hours with 5% CO_2 and shaking. After incubation the blood was removed, centrifuged and the plasma stored in a freezer at -80°C. The cells were washed and stained before trypsin detachment and flowcytometric analyzing.

2.1.4 Inhibitors and antibodies

In the first paper we used a commercial antibody, a $F(ab´)_2$ fragments of an blocking anti-CD14 antibody from Diatec. In the other papers, we used an antibody produced in-house against CD14, as described by Lau et al. [255]. This anti-CD14 blocking antibody is recombinant and consists of an IgG2/IgG4 hybrid Fc region, such as eculizumab. This structure induces a minimal complement activation (IgG4) and does not bind to the Fcγ receptor (IgG2) [256]. Anti-CD14 blocking mAbs inhibits the binding between LPS and the lipopolysaccharide binding protein (LBP) complex and CD14. In this way the signal pathway through TLR4 and other extra- and intracellular TLRs using CD14 as a coreceptor is inhibited [257]. *In vivo* studies with piglets have shown that anti-CD14 reduced the proinflammatory cytokine levels and abolished the *E. coli*-induced procoagulant situation [258]. One meta-study showed that soluble CD14, also named presepsin, has a prognostic value for sepsis [259]. The non-survivors had higher presepsin values on the first day during sepsis than the survivors [259].

We used a plasma-derived C1-INH from Berinert (Berinert® P C1 esterase inhibitor from ZLB Behring GmbH Marburg, Germany). The supply of this medication is limited and there is a small risk of infections as compared to the recombinant C1-INH [260]. However, the halftime of the plasma derived C1-INH is longer due to the glycosylation [260].

Eritoran is a synthetic analogue of lipid A, which inhibits lipid A from binding to MD2; the signaling through TLR4 is inhibited [261]. The TLR4 inhibitor eritoran was suggested to be a candidate for sepsis treatment, however, the phase III study showed that there was no significant difference between the eritoran and placebo groups [261]. The effect of eritoran is useful for research purposes to differentiate between the effects of TLR4 and CD14. CD14 collaborates with several TLRs, such as TLR2 and is not specific for TLR4 [6].

Eculizumab, also named Soliris®, binds to C5 and inhibits cleavage to C5a and C5b [37]. It was approved by the U.S. Food and Drug Administration and European Medicines Agency for PNH in 2011 [223] and aHUS in 2007 [262]. Eculizumab treatment has recently been recommended for Myasthenia gravis [263]. Eculizumab inhibits only the C5 convertase-mediated C5 cleavage [223]. However, C5 cleavage can possibly be mediated by other proteins, including thrombin, plasmin and FXa, at least in purified buffer systems [221].

PMX53 (AcF-[OPdChaWR]) is a specific C5aR1 antagonist that inhibit the C5a binding to CaR1 and was used in paper III. PMX53 was synthesized as described [264], and purified by reverse phase high-performance liquid chromatography.

Compstatin has a high specificity for C3 and protects it from the C3 convertase [37]. The peptide was found by a screening of clones from a phage-displayed library of C3b binding peptides and consists of 13 amino acids [265]. Compstatin is under constant development to provide better properties [266]. In this work, we have used two different batches of compstatin, i.e. Cp20 in paper I and Cp40 in paper IV. Compstatin inhibits C3 in the complement cascade and thus inhibits all the three pathways at the level of C3 [37].

Furthermore, the complement opsonization is inhibited with decreased C3b and enhanced C4b opsonization which is an important mechanism in immunity defense [5]. Little is known about the effects of long-term use of compstatin, including susceptibility to pyrogenic infections, which is observed in primary C3-deficient individuals [267]. Compstatin is a promising therapeutic option for several clinical conditions with persistent opsonization on host surfaces or complement dysregulation such as PNH, age-related macular degeneration (AMD), renal disease and organ transplantation [267].

In the third study we used eculizumab in blood from healthy donors, and blood from a C5-deficient individual. To examine the role of C5 in hemostasis and *E. coli*-induced coagulation, the blood from the C5D was analyzed before and after reconstitution with purified human C5 (Complement Technology, Tyler, TX).

The blocking antibody against human TF was a murine IgG1 monoclonal antibody (Sekisui 4509 from American Diagnostica Inc, Stamford, CT) and was used to inhibit the effect of TF and, thus, identify the quantitative role of TF in the *E. coli*-induced coagulation activation (Paper I).

2.1.5 *Controls*

The human serum albumin was used as a control for C1-INH. Albumin was used as control and was added in the same molar concentrations as C1-INH and had no apparent effects compared with those of C1-INH.

A control antibody, NHDL, produced in our laboratory, was used as control for eculizumab and aCD14 (Lau et al. unpublished). It has the same molecular design as these antibodies. In paper III, the control antibody was used in a high concentration. However, the control antibody produced some unexpected results. NHDL significantly increased the *E. coli*-induced TF-MP level. Monocyte surface expression of TF and prothrombin fragment F 1 + 2 (PTF1.2) was also visibly increased, but this increase was not statistically significant. This effect is probably associated with the Fc-domain and is abolished by C5 inhibition. NHDL is an IG2/4 antibody that does not bind C1q or most Fc gamma receptors [255].The glycosylation profile may explain the ability to activate

complement, Fc gamma receptors or coagulation (Lau et al., unpublished). However, the control antibody NHDL had no inhibitory effects like eculizumab and, therefore, we accepted this result. Nevertheless, in the statistical tests, we compared the results of inhibitors with the samples with added PBS and activator.

2.1.6 Activators

LPS is the major component of the outer membrane of Gram- negative bacteria [268]. LPS consists of three different domains that are covalent-bound to each other: a glycan or O-antigen in the outer layer, a core oligosaccharide and a glycolipid called lipid A in the inner layer [269]. Lipid A is the biologically active part of LPS [270]. Lipid A is also responsible for LPS-induced TF activity [217]. LPS is differentiated into smooth or rough according to the colony surface of the corresponding bacteria [130]. Smooth LPS contains the whole LPS, while rough LPS is a mutant form that lacks the O-specific chain [269]. Huber et al. showed that rough LPS induced TNF expression in a CD14-independent way in mast cells in contrast to smooth LPS which is CD14-dependent [271]. LBP binds LPS [272]. LBP is suggested to split LPS to monomeric molecules [273]. CD14 transports LPS from LBP to MD2 [124] and TLR4 [123]. This initiates an intracellular signal pathway via MyD88 and NF-κB that induces expression of pro-inflammatory cytokines and TRIF and IRF3 leading to IFN release [121]. We used ultrapurified *E. coli* LPS as a control stimulus in some experiments in the human whole blood model of inflammation. Purified *E. coli* LPS alone did not activate complement measured as sTCC in concentrations below approximately 1 µg/mL [5].

In this project we used the heat-inactivated *E. coli* strain LE392, i.e. a Gram-negative bacterium with smooth LPS (ATCC33572). This strain is complement sensitive and is killed in lepirudin plasma. *E. coli* activates both the alternative and classical pathways [7]. Furthermore, *E. coli* may activate the extrinsic pathway through TF upregulation [274] and contact pathway due to bacterial polyphosphates [275] or FXII binding to the negatively charged surfaces [268]. The bacteria were grown in a lactose agar at 37°C overnight. Then, some colonies were transferred to a growth medium (LB-medium) and incubated at 37°C overnight. The bacteria were heat-inactivated by incubating at 60°C in 60 minutes. To remove most of the free LPS in the bacterial preparations, the bacteria

were washed nine times in PBS without Ca^{2+} and Mg^{2+} [245]. They were counted by flowcytometric analysis [5], distributed in tubes with 7 x 10^9 bacteria/mL and stored at 4°C. *E. coli* and *Pseudomonas* are the most common Gram-negative bacteria that cause sepsis [2]. The concentration of *E. coli* used was mostly 1 x 10^7/mL. However, in the ROTEM® analysis, the *E. coli* was used at a concentration of 1 x 10^8/mL. These concentrations were high, but pathophysiologically relevant [7]. *E. coli* (and also *S. aureus*) is immobilizated in clots *in vivo* by FXIII [268].

S. aureus Cowan strain 1 (ATCC 12598) was used in paper IV. *S. aureus* is the most frequent Gram-positive bacteria causing sepsis [2]. The bacteria were grown on blood agar petri dishes, and some colonies were transferred to DMEM F-12 (Gibco, Thermo Fisher Scientific, NY, US). After incubation overnight the bacteria were washed and counted similar to the *E. coli bacteria* [5, 246]. *S. aureus* has a great number of virulence factors and two of the most important are the staphylocoagulase which activates coagulation and von Willebrand factor binding protein that inhibit coagulation [276]. These protects the bacteria against the immune system and allows them to grow unhindered in blood [276].

2.2 Enzyme-linked immunosorbent assays (ELISA)

ELISA is a commonly used method for detecting the level of antigen or antibody. In a sandwich ELISA, the antibody is attached to, for example, the microtiter well. To prevent unspecific binding, a blocking step is often necessary before the sample is added. The specific antigen binds to the attached antibody and the unbound specimen is washed away. A secondary enzyme-tagged antibody is added, and a sandwich is made consisting of antibody-antigen-antibody. The unbound specimen is again washed away. The enzyme cleaves an added substrate to a measurable signal that is proportional with the antigen in the sample. In competing analysis, the antigen level is reverse proportional to the signal, which can be measured by spectrophotometric-, fluorescence- or luminescence methods. The concentration of the specific analyte is found by using a standard curve. Several samples with known concentrations are analysed to obtain the standard curve. In this project, we used commercial ELISA kits for PTF1.2, PTX3 and TF-MP detection. TCC in plasma was analyzed using an antibody against a specific C9 neoepitope in the TCC

complex. The ELISA method was developed and modified by our laboratory [277, 278]. Cytokines were analyzed using multiplex technology. Magnetic beads with a size of 6.5 micron were coated with different antibodies against cytokines and 27 cytokines were detected and quantified at the same time. The beads were analyzed by flow cytometry and identified by unique infrared dye and unique red dye concentrations. Each cytokine had one specific bead. A biotynilated-secondary antibody was used to quantify the cytokines. An added standard enables quantification of each cytokine.

2.3 Quantitatative polymerase chain reaction (qPCR)

The analytic process changed from the first to the third paper. In the first paper, the mRNA was isolated from PAXgene® tubes by a half automatic procedure using AB6100 Nucleic Acid Prep (Applied Biosystems, Warrington, UK), and reverse transcriptase was used to make complementary deoxyribonucleic acid (cDNA). Further, the cDNA was used for qPCR. In the third paper, the isolation process was changed to Tempus™ tubes and the RNA was isolated by magnetic beads and MagMax™ for Stabilized Blood Tubes RNA isolation kit (Thermo Fisher Scientific, Vilnius, Lithuania). The qPCR of TF mRNA in whole blood was done using a commercial kit, where reverse transcription and amplification is done in one step. The probe is an oligonucleotide that is labeled with a fluorescent reporter dye at the 5'end and a quencher at the 3'end. The quencher prevents the fluorescence signal as long as the probe is intact. The probe and the target-specific primers will be attached to the target sequence if it is present. The 5'nuclease activity of Taq DNA polymerase will cleave the probe during the primer extension which increases the signal from the fluorescence reporter dye. A high number of the target sequence gives a high fluorescence signal. The signal is compared to the signal from the endogen control and the TF mRNA results were calculated by the delta-delta Ct-method. The endogen control human beta-2-microglobulin was stably expressed over time in the whole blood model [250]. The TF mRNA measurement includes all the blood cells, in contrast, the flowcytometric analysis of TF is given as monocyte surface expression due to gating of these cells.

2.4 Flow cytometric analysis

The extra cellular expression of TF, CD11b and CD35 were analyzed using flow cytometry (Paper I, III, IV). Monocytes and granulocytes were localized in different places in the plot with forward scatter (FSC) at the X-axis and side scatter (SSC) at the Y-axis. FSC separates the cells by size, and SSC in regards to the complexity or granularity. In addition, fluorochrome-labeled antibodies were used to separate cells, the antibody against CD45 to stain the leukocytes and anti-CD14 to label the monocytes (Fig. 9). The fluorescent antibodies were also used to quantify the amount of different markers of interest. The fluorochrome was excited when the light from a laser met the labeled antibody. When the excited molecule returned to the base, the energy was emitted as light, and this is called fluorescence. Fluorescein isothiocyanate (FITC) absorbs blue light at 488 nm and emits green light at 530 nm. Phycoerythrin (PE) emits a yellow light at 575 nm. Peridinin chlorophyll protein complex (PerCP) emits at

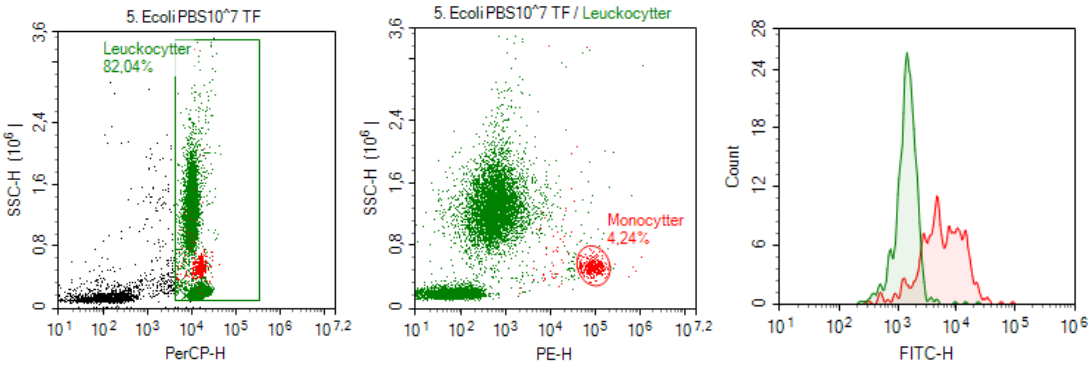

Fig. 9. One example of gating strategy in flow cytometric analysis. *Whole blood leukocytes are stained with Peridinin chlorophyll protein complex (PerCP)-labeled anti-CD45 which differentiated leukocytes from noise and debris (left panel). Monocytes are gated using Phycoerythrin (PE)-labeled anti-CD14 (middle panel). The histogram (right panel) shows monocyte tissue factor (TF)-expression using a Fluorescein isothiocyanate (FITC)-labeled anti-TF (red curve) and the isotype control in green color of a whole blood sample stimulated 2 hours with Escherichia coli bacteria (1 x 10^7/mL).*

FITC-labeled anti-human TF antibody (product number 4508CJ, clone VD8; American Diagnostica, Inc., Stamford, CT) was used to analyze the TF surface expression on monocytes (Paper I, III). This antibody and three others against TF were tested, considering their ability to detect and block TF [279]. Basavara et al. found that the clone VD8 were able to detect TF with high specificity [279]. The TF median fluorescence

(MFI) results were adjusted against a gamma isotype control antibody, to correct for unspecific binding and the autofluorescence from monocytes due to the weak signal from TF. All this to ensure that the TF increase is real.

CD11b expression was analyzed using a PE-labeled anti-CD11b antibody (Becton Dickinson, San Jose, CA) (Paper III, IV). In paper IV, a FITC-labeled anti-CD35 (clone E11, Becton, Dickinson and Company, San Jose, CA) and a PE-labeled anti-CD11b antibody (clone D12, Becton, Dickinson) were used, in addition to anti-CD45-PerCP (clone 2D1) and anti-CD14-PerCP and anti-CD14-PE (clone MΦP9). Phagocytosis was also quantified by flow cytometry (Paper IV). The results were given in MFI. In the phagocytosis analysis the bacteria were stained with Alexa Fluor 488. All the results were compensated to avoid overlap from the different fluorochrome signals. During the incubation at 37ºC, the number of monocytes decreased for unknown reasons. Perhaps they attached to the wall of the plastic tubes or were destroyed after activation. Furthermore, the activation changes the cells and may make the gating more difficult. Therefore, the incubation time was no longer than 120 minutes in most experiments to ensure a sufficient number of monocytes in the flow cytometric analysis. However, this time probably reflected a longer time *in vivo,* because the cells were not replaced with new cells, which is what happens in real life.

2.5 TF functional activity in plasma microparticles

In paper I, the TF-MP analysis was performed in Tromsø at prof. Bjarne Østerud's research laboratory. The method was earlier described by Engstad et al. [280]. TF functional activity was measured in platelet-free plasma using a two stage amidolytic assay. The basic principle in this test is the ability of TF-FVIIa complex to activate FX. FXa activates further prothrombin to generate thrombin [280]. In paper III, the TF-MP was measured by a commercial kit, Zymuphen MP-TF kit (Aniara Diagnostica, West Chester, OH). The plasma sample was added to a microplate coated with murine monoclonal antibody against human TF. FVIIa and FX were added and TF was the limiting factor. A substrate for FXa was added and the following yellow substrate was measured by an MRX microplate reader (Dynex Technologies, Denkendorf, Germany). Both methods measured the functional activity of TF in plasma microparticles.

2.6 Thromboelastometry

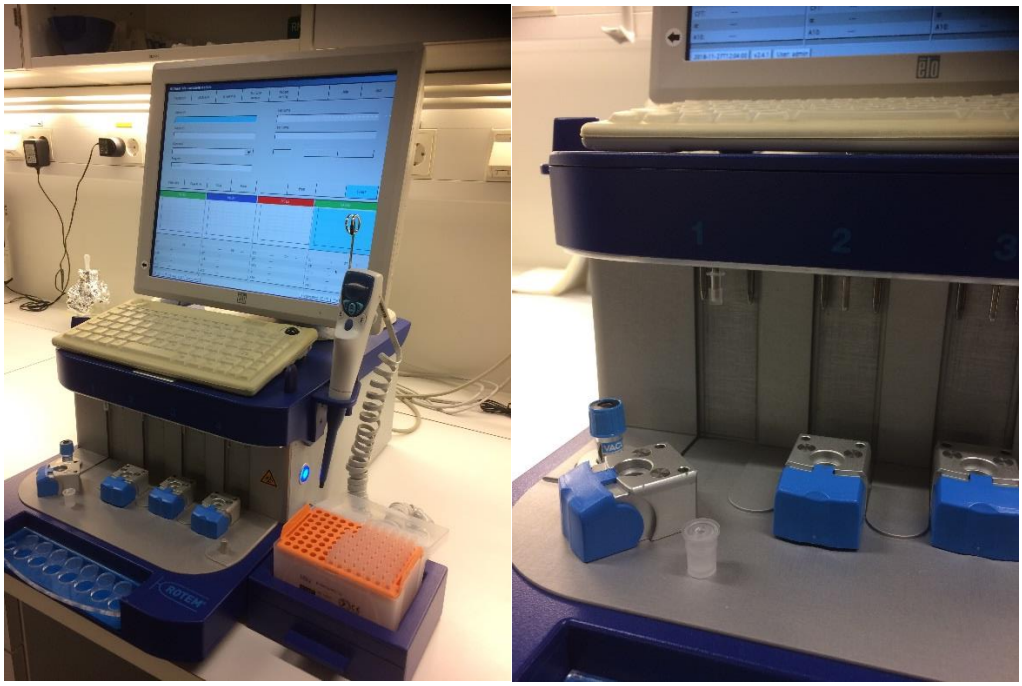

Fig. 10. Illustration of the ROTEM® instrument *(Tem Innovations GmbH, Munich, Germany).*

The first thromboelastography instrument was described by H. Hartert in 1948 [281]. The instrument examines the viscoelastic properties of coagulation and coagulation kinetics in human whole blood [282]. The analysis is conducted at a low shear pressure (0.1/s) similar to the bloodstream in the vena cava [283]. The blood is added to a preheated cup (37°C). In a similar method named thromboelastography the cup rotate and the pin is stationary [282].

Rotational thromboelastometry (ROTEM®) (Fig. 10) is a modification of the original TEG technology [283]. In ROTEM®, the pin rotates back and forth at an angle of 4.75°. It is connected to a sensor that registers movement and reflects coagulation development. The resistance increases with increasing clot size [283]. ROTEM® is more stable and less sensitive to vibrations than thromboelastography instruments. Futhermore, ROTEM® has an automatic pipette. The results are presented as both graph and numbers and ROTEM parameters are described below (Fig. 11 and Table 2). ROTEM® has four channels and several different reagents for different analysis. In comparison, thromboelastography instruments have only two channels [283].

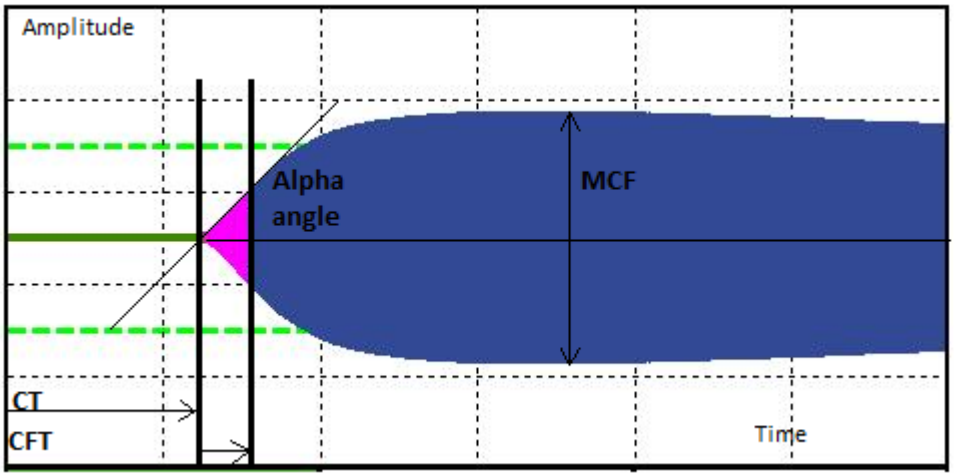

Fig. 11. ROTEM® parameters placed in the graphic illustration.

Table 2. Description of various parameters of the ROTEM®.

ROTEM® parameter	Description of each parameter
CT	Coagulation time. Time from activator is added to the amplitude reach 2 mm, given in seconds.
CFT	Clot formation time. Time from 2–20 mm amplitude, given in seconds.
α	Alpha angle. Coagulation kinetics indicating the initial speed of clot formation, given in degrees.
MCF	Maximum clot formation. Indicate the strength of the clot, the maximum amplitude given in mm.
LY60	Clot lysis 60 minutes after CT. LY60 is given in relation to the amplitude of the MCF.

In this project we have used three different ROTEM® reagents, including non-activated thromboelastometry (NATEM), extrinsic thromboelastometry (EXTEM) and intrinsic thromboelastometry (INTEM). The venous blood samples were anticoagulated with citrate and were recalcified before analyzing clot kinetics. In NATEM the only addition

was the start reagent containing $CaCl_2$ used for recalcification. In EXTEM, the extrinsic pathway was activated by adding TF. In the INTEM test, the intrinsic pathway was activated by adding in-tem reagents containing phospholipids from rabbit brains and ellagic acid [283].

The NATEM test is more suitable for analyzing the possible effect of activators such as bacteria and several inhibitors than the INTEM and EXTEM, which contain commercial activators of the intrinsic and extrinsic pathways. However, control experiments showed that the NATEM clotting time (CT) was reduced over time. Samples for EXTEM or INTEM analysis were durable for 4 hours, according to the producer. A study of stability and reproducibility of samples analyzed by ROTEM® concluded that the samples were stable over 120 minutes regarding these tests [284]. However, samples for NATEM analysis had a much lower durability [285]. Therefore, we decided to standardize the time from venipuncture to analyzing NATEM CT, etc. In this way, reduction in CT results caused by different time points for NATEM analysis was avoided. All samples were stored in a heatblock at 37ºC, since temperature affects the results [286]. The instrument was controlled in regard to the stability of the device temperature and the quality of the analytical system, by measuring the electronic CCD (charge coupled device) chip values, which were the detector [283]. ROTEM® is less sensitive to external vibration then TEG [287]; however, we were careful to place the ROTEM® in a location without centrifuges or other disturbing conditions.

2.7 Platelet function analyzer

The platelet function analyser Siemens PFA200 was used [288]. The blood was drawn from a reservoir with a constant vacuum through a capillary and a hole in the test membrane [288]. This membrane contains platelet agonist and the high shear rates induced platelet attachment, activation, aggregation and a clot that stopped the flow through the hole [288]. Prolonged clotting time and comparison of different PFA200 tests can indicate hemostatic disorders or drug-mediated effects, although the instrument has some limitations [288]. However, some conditions such as low hematocrites, low platelet numbers and pregnancy [288], affects the results. We used collagen/ADP cassettes where the membrane is coated with the agonist collagen and ADP (Paper III).

2.8 Statistical methods

Statistical tests were used to determine whether the null hypothesis was to be retained or rejected. The null hypothesis (H_0) states that there is no effect or difference between the groups. The alternative hypothesis (H_A) states the opposite; that there is an effect or difference between the groups.

In this project, the sample size (n) never exceeded six. However, a calculation of sample power showed that this number of observations was enough to find statistically significant differences between the activated sample and the samples that were added inhibitor(s). For example using IBM® SPSS® Sample Power®3 n = 3 was enough to identify a significant increase in NATEM CT from 789 ± 97s in PBS sample to 2025 ± 264 s in samples added C1-INH (47.6 µM) with a power of 96 % and an alpha = 0.05 using a paired Student's t-test. If you cause great effects, you only need a small n, in contrast to small differences or effects that need a large n.

Before the statistical data analyses, the data was tested to see if the results were normally distributed or not. The most suitable test for a low number of specimens is the Shapiro-Wiik test. The normal distributed test results were analyzed by parametric tests. The results that also failed in the normality test after logarithm transformation were analyzed using non-parametric tests.

The result of the statistical analysis is a *p*-value. This value is the probability to do a type 1 error when the H_0 hypothesis is correct. In other words, the probability to conclude that there is an effect or difference which does not exist. Alternatively, the p-value is the probability for that your results are due to a random case and not a due to a real difference between the groups. The borderline for accepting a type I error is the α-value and it is often set to 0.05 [289]. A p-value lower than 0.05, indicate that it is improbable that the H_0 hypothesis is the case. H_0 was rejected and H_A was probably applicable [290]. In this case, the result is statistically significantly different. Type II errors occur if you keep the H_0 hypothesis and conclude with no effects while the opposite is true – there is an effect (Table 3). The risk of type I errors increases with an increased threshold, and the risk for

type II errors increases with a reduced threshold. A significance level of 0.05 provides a balance between these risks.

Table 3. Different outcomes of statististic decisions

	H_0 true	H_0 False
Reject H_0	Type I error False positive	Correct
Fail to reject H_0	Correct	Type II error False negative

In this project, several statistical tests were used. A paired Student's t-test was used to compare two groups with normally distributed numbers. In comparison, a Wilcoxon matched-pair signed rank test was used for results not distributed normally. To compare several groups against one control group, a one-way repeated measures analysis of variance (ANOVA) was used with Dunnett's multiple comparisons test as a post-hoc test for parametric distributions, and a Friedman test with Dunn's multiple comparisons test as a post-hoc test for nonparametric results. The post-hoc test identifies which groups are significantly different from the control group. A two-ways ANOVA and Sidak's multiple comparisons test were used to compare the effects over time. A correlation analysis was performed using the Pearson correlation on normally distributed data.

2.9 The candidate's role in the study

The experiments were planned together with the supervisors. The candidate performed many of the experiments using the whole blood model. However, sometimes the experiments were performed together with many colleagues due to a heavy work-load. The candidate performed all ROTEM® and PFA200 analysis. In addition, mRNA isolation and TF mRNA quantification by qPCR were done by the candidate. Flowcytometric analysis of the samples was done mostly by the candidate. The candidate did some of the ELISA analysis, but most were conducted by the candidate's colleagues. The days with venipuncture and analysis of the C5-deficient individual were busy, and the experiments were performed with the help of the candidate's colleagues. The

candidate analyzed the data and did all the statistical tests. The first draft of the papers was written by the candidate and reviewed by all the coauthors. The candidate changed the manuscripts in line with the feedback and submitted the manuscripts. The candidate is the second author of the last manuscript and contributed to the flowcytometric analysis. The candidate read and commented on the manuscript before submission.

3 Summary of main results

In the first paper we showed that the combined inhibition of complement and CD14 reduced ultrapurified *E. coli* LPS- and *E. coli*-induced coagulation in human whole blood. Coagulation was measured as plasma PTF1.2. In addition, qPCR was used to analyse TF mRNA levels, TF surface expression on monocytes was analysed by flow cytometry and TF function in plasma MPs was analysed using a two-stage amidolytic assay [280]. TF mRNA, TF-MP and TF expression on monocytes were largely dependent on both complement and CD14. We found that a TF-blocking antibody nearly abolished the LPS and *E. coli*-induced PTF1.2, indicating that TF is responsible for both the LPS and *E. coli*-induced coagulation. The study gave important information about the interaction of complement and CD14 and useful information for further experiments to find a potential new therapy for sepsis-induced coagulation activation.

In the second paper we studied the effects of C1-INH on coagulation kinetics in human whole blood. C1-INH is a serine protease that inhibits or interacts with both the complement and coagulation systems. Previous studies indicated that C1-INH may have procoagulant effects [101, 291]. In paper II, supraphysiological concentrations of C1-INH were added to blood from healthy donors. The samples were analysed using ROTEM® and NATEM, EXTEM and INTEM reagents. The results indicated that C1-INH had an anticoagulant effect at very high and supraphysiological concentrations. C1-INH at a very high concentration also abolished the *E. coli*-induced reduction in NATEM CT. Another interesting result was the effect of C1-INH on fibrinolysis. C1-INH at a very high concentration also delayed the fibrinolysis measured as lysis index 60 by ROTEM®. The sum of effects of C1-INH on coagulation *in vivo* has to be further investigated.

In the third paper, the role of C5 in hemostasis *in vitro* and *E. coli*-induced coagulation was examined in human whole blood. C5 had no role in the normal coagulation kinetic in human whole blood. In contrast, the *E. coli*-induced TF mRNA and TF-MP levels were partially C5-dependent. We used fresh blood from a C5 deficient individual and healthy donors. C5 was added to the blood samples from the C5D individual and eculizumab was added to blood from the healthy control, and we compared the effects of coagulation. The combined inhibition by eculizumab and anti-CD14 or eritoran most efficiently reduced the monocyte TF surface expression, TF-MP and PTF1.2 levels. Then, we wanted to study

if C5a could induce TF surface expression on monocytes alone. We used recombinant C5a as an activator, but this was not sufficient to induce TF surface expression on monocytes alone. However, C5a addition induced CD11b expression on monocytes, which was inhibited by the specific C5aR1 inhibitor PMX53, indicating that the C5a-induced CD11b expression is dependent on C5aR1.

In the last paper IV, the effect of eritoran, anti-CD14 and the C3 inhibitor compstatin (Cp40) was compared in the *E. coli*, LPS-, *E. coli*- or *S. aureus*- induced inflammation. Eritoran blocked the LPS-induced cytokines to a greater extent than anti-CD14; however, the differences between the inhibitors were significant only for IL-8. CD14 showed a broader effect on whole bacteria-induced inflammation probably explained by its co-receptor function of several TLRs [6]. In comparison, eritoran only inhibits LPS binding to MD2 and blocks the signaling through TLR4 [292]. In addition, the combined inhibition of eritoran and anti-CD14 with compstatin (Cp40) was studied. Both for the *E. coli*- and *S. aureus*-induced CD11b and CD35 expression on monocytes, combined inhibition with Cp40 and anti-CD14 was most effective. There were no differences between the combined inhibition of Cp40 and anti-CD14 and Cp40 and eritoran on the *E. coli*-induced granulocyte expression of CD11b and CD35. The phagocytosis in both monocytes and granulocytes of *E. coli* and *S. aureus* was inhibited to the same extent as anti-CD14 and eritoran in combination with Cp40. This study confirms earlier studies indicating that combined inhibition of CD14, TLR4 and complement may be a new therapeutic treatment for sepsis-induced inflammation.

The studies in this thesis examined the interactions between the complement system, CD14 and TLRs on coagulation in human whole blood. Complement and coagulation is closely connected. However, the TLRs are also involved in the crosstalk between these systems. We used different specific inhibitors to block keypoints in the complement, CD14, TLRs and coagulation cascades and used our whole blood model of inflammation [7]. The discussion is structured as a combined discussion of all four papers in view of the different inhibition strategies.

4.1 Whole blood model of inflammation

The main advantage of the model is the use of the anticoagulant lepirudin which has no adverse effects on complement activation [7]. In contrast to heparin, citrate and EDTA, lepirudin did not affect the complement cascade [7]. Lepirudin is a hirudin analogue and a specific thrombin inhibitor. It is therefore not possible to evaluate the effect of thrombin in this whole blood model. Thrombin is activated by FXa, and activates FX in a positive feedback mechanism. It also activates FXI and the cofactors FV and FVIII [293] and cleaves fibrinogen to fibrin [19]. However, thrombin has also several effects in the immune system [19]. It activates platelets through the protease-activated receptor (PAR) 1 and 2 [294]. Thrombin-induced PAR1 activation mediates increased cytokine release in addition to increased expression of selectins (E- and P-), ICAM-1 and VCAM-1 on endothelial cells [295]. PAR1 signaling can also induce apoptosis in endothelial cells through activation of caspases. By vascular injury, PAR1 activation contributes to rapid platelet aggregation [295]. PAR4 requires a higher concentration of thrombin to induce proinflammatory response in endothelial cells compared to PAR1 [295]. The C-terminal peptides in thrombin belong to the host defense peptides (HDPs). The HDPs have anti-inflammatory properties and are bactericidal [296]. Thrombin cleaves fibrinogen to fibrin; however, this process also generate fibrinopeptide A and B, both of which are chemoattractants [268]. Thrombin also plays a role in anticoagulation by binding to thrombomodulin and activation of protein C [297]. Huber-Lang et al. has shown that thrombin is able to cleave C5 in a pure buffer [222]. However, a new study by our group indicates that thrombin was not able to cleave C5 in plasma at a physiological pH, but

cleavage was possible at an acidic pH (Nilsson et al. 2018 submitted). In conclusion, results from experiments with whole blood models are still relevant.

In this model we always add the inhibitors before the activators [7]. In real life, this is impossible in sepsis patients. However, the combined inhibition of complement and CD14 or TLRs is a potential new therapeutic treatment for patients with a high risk of developing sepsis after surgical procedures or after large traumas as prophylaxis. *In vitro* experiments have indicated that there is a therapeutic window after exposure to the bacteria [298]. Compstatin added to blood after bacteria stopped further complement activation and combined inhibition with compstatin and anti-CD14 was most efficient. This may indicate that combined inhibition could also be administered in a short period of time after sepsis onset and still reduce sepsis-induced inflammation [298].

The whole blood model allows all cells to interact in contrast to other models using isolated cells [7]. However, endothelial cells are not included, and the movement and shear stress of the blood is not the same as *in vivo*. Under physiological conditions there is a flow that brings new platelets, cells, coagulation factors and other important contributors to the site of inflammation. In our whole blood model, the blood tubes are in continuous movement, but the cells and other "players" are not replaced. Thus, the lack of shear stress and normal blood flow are limitations in the whole blood model. Therefore, incubation of the whole blood for more than two hours is normally not recommended, due to a reduction in the number of leukocytes. The blood cells are activated over time, and this may induce a change in shape and possibly the death of some cells. To get reliable results in flow cytometry analysis, it is advantageous to analyze many cellular events, which may be reduced by increasing incubation times. The gating is more difficult and leukocyte number is reduced due to several reasons.

4.2 Effect of C1-inhibitor on coagulation kinetics

C1-INH is a multifunctional protease inhibitor, which is involved in complement, coagulation and contact activation systems [91]. In addition, the C1-INH has anti-inflammatory effect which makes C1-INH a promising treatment option for several diseases [118]. Cai and Davis showed that C1-INH has siayl Lewis^{x} tetrasaccharide, and

they assumed that this could explain the interaction between C1-INH and E- or P-selectins [116]. In paper II, C1-INH in a supraphysiological concentration, abolished the *E. coli*-induced reduction in NATEM CT. In the discussion, we referred to the anti-inflammatory effect of C1-INH. However, a recent study claimed that C1-INH did not express Lewis^x tetrasaccharide as shown earlier. The C1-INH reagent was shown to be contaminated with α1-antichymotrypsin which expresses this Lewis^x tetrasaccharide [119]. Therefore, the anti-inflammatory effects of C1-INH are unclear; and it is possible that they originate from the α1-antichymotrypsin? The C1-INH ability to bind LPS is dependent on glycosylations in the N-terminal domain [118]. We also used C1-INH from Berinert like Cai et al., therefore, the results of our experiments also have to be taken with reservations regarding contamination of the C1-INH reagent. New studies will have to be conducted with recombinant C1-INH preparations to answer this question. However, there are several studies showing that C1-INH may have valuable characteristics. One study from Russia shows promising results with C1-INH as treatment in sepsis patients [299]. C1-INH reduced the ischemi/reperfusion (I/R) injury compared to controls after limbs amputation and replantation in pigs [300].

We showed that C1-INH at supraphysiological doses had a moderate anticoagulant effect *in vitro* (Paper II). Earlier studies have concluded that C1-INH was a procoagulant and infusion increased the risk for thrombotic events [101]. We found that the C1-INH dose-dependently delayed the coagulation kinetics (Paper II). However, the mechanism for this is not known. In an earlier study, we showed that C1-INH inhibited *E. coli*-induced TF mRNA generation [274]. TF is the starter of the extrinsic coagulation pathway [141, 171]. C1-INH is a known inhibitor of FXII. FXII is important in the contact activation pathway and, in this study; FXIIa is one of the possible initiators of coagulation activation by the plastic cup on the ROTEM® instrument [151]. Incubation of citrated blood for 60, 120 or 180 minutes at 37°C with the specific FXIIa inhibitor infestin, showed no effect on NATEM CT, CFT and alpha angle (unpublished data). This indicates that FXIIa probably not activate coagulation on the ROTEM® instrument. We found that C1-INH abolished *E. coli*-induced coagulation. Furthermore, infestin had no effect on the *E. coli*-induced reduction in NATEM CT indicating that FXIIa is not involved. These results indicate that C1-INH inhibition of FXIIa probably not explains the effects on ROTEM®. However, the doses of C1-INH used were supraphysiological and the physiolological relevance of these

doses are unclear. The platelet release C1-INH from their granula and induce an increased local C1-INH concentration upto 100 µM [85, 86]. This indicates a possible relevance for also the high C1-INH doses.

We used thromboelastometry on ROTEM® to examinate the effect of C1-INH on coagulation kinetics (Paper II). The advantage of studying coagulation and fibrinolysis in a global system by ROTEM® is that all blood cells, coagulation factors and inhibitors are present [283]. However, real life is more complex, and several factors differ from this instrument in the *in vivo* situation. Results from the ROTEM® analysis thus have to be confirmed in the *in vivo* situation. *In vitro* testing of coagulation activation in whole blood must be performed using anticoagulants, and citrate is the recommended anticoagulant in ROTEM®. Citrate reduce complement activation due to the Ca^{2+} and Mg^{2+} binding [301]. In addition, the blood flow is different and low in the cup, and endothelial cells are not included. In contrast to traditional coagulation tests that only measure the functional activity or antigen concentration of one coagulation factor, thromboelastometry gives a more composed picture of the total hemostasis and coagulation kinetics in whole blood.

4.3 Complement inhibition

The complement system is involved and activated locally or systemically in many diseases [37]. The complement system can be inhibited at different levels of the cascades by specific complement inhibitors [37]. At present, only the anti-C5 mAb eculizumab is available to inhibit complement activation *in vivo* and at a high cost [37].

Compstatin was used as a specific C3 inhibitor in paper I and IV. Selective inhibition of C3 reduced the *E. coli*-induced TF mRNA levels, TF surface expression on monocytes and coagulation activation (paper I). In comparison, selective C3 inhibition had no effects on the *E. coli*-induced CD11b and CD35 upregulation on monocytes, while the CD11b and CD35 upregulation on granulocytes were reduced (paper IV). The *S. aureus*-induced cytokine release was more complement dependent than the *E. coli*-induced cytokine release (paper IV), in line with other studies [245, 246]. Inhibition at the level of C3 inhibits further activation of complement, including the formation of C3a and TCC formation [37]. Individuals with C3 deficiency have an increased risk for infections [302].

Compstatin did not only reduce the C3b opsonization of microbes but increased the C4b opsonization which protects humans against infections [5, 303]. In some conditions, the need for treatment is time-limited and antibiotics can be given as prophylaxis. However, C3 inhibition over a long time may potentially increase the bacterial growth and thus increase the risk of infections-induced inflammation [6]. However, unpublished results from studies in monkeys receiving Cp20, a compstatin analogue, for one month indicate that there was no increased risk of infections [52]. They also claimed that a person with a fully developed immune system does not have any increased risk of infections by Cp20 treatment, but vaccinations will be given as prophylaxis [37]. Cp40 has shown promising results for conditions such as PNH, hemorrhagic shock, sepsis-associated organ damage and other disease models involving complement activation [52]. Furthermore, compstatin significantly reduced coagulation activation and organ damage in an *E. coli* sepsis model in baboons [304].

Another currently used complement inhibitor in the clinic is C5 inhibition by eculizumab [37]. C5 is a key component in the interaction between complement and coagulation as described earlier [9]. C5 inhibition is, therefore, a potential new therapeutic option for the treatment of sepsis [305]. There are several possible ways to inhibit at the level of C5 [37]. One advantage of C5 inhibition is that opsonization by C3b is preserved and this is probably more appropriate for long-term treatment [6]. However, the choice of treatment is also dependent on the disease mechanism [37]. Eculizumab inhibits the cleavage of C5, including the formation of C5a and C5b-9 [256]. RA101295 is another inhibitor of C5 cleavage that has given successful results in *E. coli*-induced sepsis in baboons with reduced organ damage and mortality [306]. C5aR1 antagonists inhibits the binding of C5a to C5aR1 and preserve the terminal complement complex pathway [307]. In paper III, eculizumab was used to examine the effect of C5 in normal physiological hemostasis and in *E. coli*-induced coagulation in human whole blood. Eculizumab significantly reduced TF mRNA and TF-MP levels, but did not completely abolish the *E. coli*-induced increase (Paper III). In comparison, C5 inhibition combined with anti-CD14 or TLR4 inhibitor eritoran completely inhibited the *E. coli*-induced monocyte TF, TF-MP and plasma PTF1.2. These results indicate that eculizumab reduces the bacteria-induced coagulation and inflammatory response and may be an additional advantage for individuals under treatment with eculizumab [308].

In our study, we also included an individual with C5 deficiency. Individuals with complement deficiencies are natural knockouts that are of great value for research purposes [56]. The role of a specific complement component becomes visible. However, deficiencies of complement C5 are extremely rare [50]. The only symptoms our C5D individual had was repeated *Neisseria meningococci* infections [50]. The C5D individual included in our paper has been vaccinated with a meningococcal oligosaccharide vaccine [50]. However, there are an increasing number of functional C5-defect individuals due to increasing use of eculizmab. Furthermore, several cases of meningococcal infections are reported in those using eculizumab despite vaccination. The cause may be that eculizumab inhibits C5aR activation and following CR3 expression on monocytes and granulocytes, which reduce the opsonization, phagocytosis and oxidative burst [309]. In contrast to our results, a recent study on anti-phospholipid syndrome showed that the procoagulant state induced by TF activation and anti-phospholipid antibodies induced thrombosis was C3 dependent and C5 independent [310].

Keshari et al. showed that blocking of C5 can reduce the level of free LPS in the plasma due to reduced lysis of the bacteria [306]. We used only heat-inactivated bacteria in our studies and therefore we could not examine the effect of eculizumab on their viability and lysis. However, these possible effects of inhibiting C5a and TCC generation on LPS release support the tight connections between the complement and the TLR system.

4.4 Inhibition of CD14 and TLR4

CD14 is a coreceptor of several human TLRs, including TLR2,-4 and -9 [6]. Anti-CD14 efficiently reduced the LPS-induced TF surface expression on monocytes, PTX3 and PTF1.2, but not TF mRNA upregulation and TF-MP (Paper I). In comparison, specific inhibition of TLR4 using eritoran significantly reduced the LPS-induced TF on monocytes, TF-MP and PTF1.2 (Paper III). However, the different results using anti-CD14 and selective TLR4 inhibition on LPS-induced TF-MP may be due to a high biological variation of the TF-MP results in paper 1. However, the *E. coli*-induced TF expression on monocytes, TF-MP and PTF1.2 were not significantly reduced by specific TLR4 inhibition or anti-CD14 (Paper III). This indicate that other molecules than LPS,

CD14 and MD2/TLR4 are probably involved in the *E. coli*-induced coagulation. This is in line with the findings by Øvstebø et al. that a LPS deficient *Neisseria meningitidis* induced TF surface expression on monocytes [175]. The LPS-induced increase of inflammatory parameters; TNF, IL-1β, IL-6, IL-8, CD11b and CD35 on monocytes were significantly reduced by both anti-CD14 and the specific TLR4 inhibitor eritoran (Paper IV). This indicate that the LPS-induced inflammatory cytokines, CD11b and CD35 upregulation are mainly mediated by CD14 and MD2/TLR4 activation. Furthermore, selective inhibition of CD14 and TLR4 reduced the cytokine release induced by whole whole bacteria (Paper IV). In comparison, anti-CD14 reduced the *E. coli*-induced leukocyte activation markers CD11b and CD35 on monocytes while eritoran had no effect, indicating a broader effect of blocking CD14 (Paper IV). Eritoran was suggested to be a new treatment for sepsis, but eritoran did not improve the survival in a phase III study [261]. The inhibition of CD14 has been shown to be more efficient than selective MD2 inhibition of *E. coli*-induced inflammation using the *in vitro* whole blood model, possibly due to inhibition of several TLRs using CD14 as coreceptor [311]. However, anti-CD14 alone reduced both inflammatory parameters and reversed the procoagulant state in a porcine model of *E. coli* sepsis [258]. In paper I, we showed that the combined inhibition of CD14 and complement C3 reduced the *E. coli*-induced TF surface expression on monocytes, TF-MP and PTF1.2 significantly more than the selective inhibition. These findings support the main hypothesis that combined upstream inhibition of complement and CD14/TLRs is necessary to inhibit bacteria-induced coagulation.

The early cytokine response and antibacterial defense were highly dependent on TLR4, but the survival of TLR4 knockout mice were not increased in an *E. coli* sepsis model [312]. This support that single inhibition of MD2/TLR4 is not sufficient to inhibit whole bacteria-induced responses. TLRs activation are involved in several disease, e.g. TLR2 is associated with systemic lupus erythematosus (SLE) and sepsis [313]. TLR2 knockout mice showed reduced autoantibody level, and increased survival during bacterial inflammation, respectively [313]. However, there is no TLR inhibitors in clinical use. A possible explaination is that several TLRs interact with the same ligand and that a specific TLR inhibitor did not provide satisfactory results [313].

4.5 Coagulation inhibition

Sepsis is a complex and heterogeneous condition with, in some cases, enhanced risk of microvascular thrombosis and bleeding [314]. TF is responsible for sepsis mediated DIC [315]. Several studies have been performed to recreate the balance by using anti-TF and recombinant anticoagulants to inhibit the overactivation of coagulation and limit organ damage [19]. We found that the *E. coli*-induced coagulation was TF dependent (Paper I). TF inhibition in sepsis has been successful in animals to reduce the coagulopathy measured as fibrinogen, the cardiovascular collapse measured as mean systemic arterial pressure (MSAP) and cell injury measured as creatine [316]. TF expression is vital as indicated by the finding that $TF^{-/-}$ knockout mice did not survive [170]. A phase I study using recombinant FVIIa to inhibit the complex between TF and FVII was terminated due to increased bleeding and tendency to increased mortality compared with the control group [317]; therefore, a state with no or little TF expression is probably associated with increased risk of bleeding. Perhaps it is better to inhibit further upstream in the cascades to inhibit bacteria- and inflammation-induced TF. Therefore, we checked whether the combined inhibition also inhibited the TF expression on the level of TF mRNA, TF protein surface expression on monocytes or TF functional activity in microparticles (Paper I, III). However, we directly inhibited TF by a blocking anti-TF mAb to examine the TF-dependent coagulation. Anti-TF reduced LPS- and *E. coli*-induced PTF1.2 by 76–81 % (Paper I). These results indicate that TF is the major contributor for LPS- and *E. coli*-induced coagulation. The correlation between PTF1.2 and TF-MP also confirmed that TF is an important key for inflammatory-induced coagulation in paper III.

Tissue factor pathway inhibitor (TFPI) is a natural inhibitor to TF-FVIIa complex in an FXa-dependent way and inhibits FXa directly [318]. In a septic state, the anticoagulant is reduced due to reduced production and increased consumption [19]. Recombinant TFPI (rTFPI) was given to patients with severe sepsis in a single-blind randomized controlled trial [319]. The study showed a trend toward reduced mortality, but a larger study could not confirm these results and showed no reduction in mortality and increased events of bleeding [320].

The anticoagulant-activated protein C (APC) is reduced in sepsis patients, and a low level is associated with increased mortality [321]. APC reduced endotoxin-induced TNF, IL-

1β, IL-6 and IL-8 [321]. Yuksel et al. showed that the reduction of LPS-induced TNF was due to APC-induced blocking of NF-κB [322]. In a large multicenter randomized study, recombinant APC, called drotrecogin alpha activated (commercially known as Xigris), was given to sepsis patients [323]. Xigris reduced mortality, but mediated a possible increased risk of serious bleeding [323]. However, the promising results shown by Bernard et al. were not confirmed since the mortality at 28 days was not significantly different between the placebo and Xigris groups in an unpublished study called PROWESS-SHOCK [324]. Due to these findings, Xigris was removed from the market [324].

Thrombomodulin (TM) is reduced under a sepsis state and recombinant TM may be a component for sepsis treatment. TM binds thrombin and, in this way, reduces the thrombin level. The protein C is activated to APC by TM. APC has both anticoagulant and anti-inflammatory effects. Thrombin bound to TM deactivates the thrombin activatable fibrinolysis inhibitor (TAFI), which reduces both the complement activation and bradykinin [325]. A meta-study concluded that recombinant TM therapy for sepsis patients can reduce mortality and there was no difference between the control group and recombinant TM group considering bleeding events. However, the results have to be confirmed with studies including a larger number of patients [326].

4.6 Combined inhibition of complement and TLRs in thromboinflammation

In the previous section, the challenges in inhibiting of coagulation were described regarding treatment of sepsis. We argue for another approach. In 2008, Mollnes et al. published a hypothesis paper about the combined upstream inhibition of complement and TLR systems [4]. The immune system is complex and several reports have indicated a synergic effect between the complement and TLR systems [6, 129]. Both systems must be inhibited to efficiently block the huge activation seen in sepsis [247]. Eritoran is one example of single inhibition that did not work in humans with several sepsis [261]. Combined inhibition has been successfully used in different combinations, i.e. inhibition of complement at the level of C3 or C5 in combination with anti-CD14 or TLR antagonists [6]. In 2007, Brekke et al. showed that monocyte activation measured as CD11b upregulation, phagocytosis and oxidative burst was mostly CD14 dependent in

contrast to the activation of granulocytes, which was mostly complement-dependent. However, the combined inhibition of complement and CD14 inhibition completely blocked *E. coli* induced activation in both cells [5]. The combined inhibition has blocked the inflammatory response measured as cytokines, complement activations products and TF, in studies using *in vitro* whole blood models [245, 327] and in animal models *in vivo* [246, 248, 249]. Combined inhibition of C3 and CD14 reversed the transcription of 70% of 2335 genes changed by *E. coli* incubation in human whole blood [250]. Furthermore, the combined inhibition with C3 and CD14 or TLR4 inhibition efficiently reduced the *E. coli*-induced phagocytosis, and CD11b and CD35 upregulation (paper IV). In comparison the selective inhibition of C3 and CD14 was much less efficient indicating crosstalk between complement and TLRs/CD14 [250]. Such experiments using combined complement and CD14/TLR inhibition have been performed in different porcine and mice models, with promising results on survival [6, 247, 251]. Inhibition of C5 and CD14 in a meconium-induced inflammation model with piglets reduced local and systematic inflammation. The treatment reduced MPO level in bronchoalveolavar lavage (BAL) fluid and IL-1β in plasma [328].

In this thesis, we have used this combined inhibition to examine the effect of selective complement and CD14/TLR inhibition and combined inhibition on *E. coli*-induced coagulation activation, leukocyte activation and cytokine release (Paper I, III, IV). The results indicate that combined inhibition of both complement at the level of C3 or C5, and inhibition of CD14/TLR4 is more efficient to inhibit *E. coli*-induced TF and coagulation compared to selective inhibition of complement or CD14/TLR4. Inflammation induced by *E. coli* and *S. aureus* bacteria was inhibited more efficiently by anti-CD14 than TLR4 inhibition, especially when combined with C3 inhibition. Furthermore, phagocytosis of *E. coli* and *S. aureus* by both monocytes and granulocytes was significantly reduced by the combined inhibition described in paper IV. Phagocytosis involves several TLRs due to intracellular degradation of the bacteria and contact with intracellular TLRs [29].

The combined inhibition efficiently reduced all TF read-outs, PTF1.2 levels in plasma (Paper I, III), leukocyte activation markers and cytokine release (Paper IV). PTF1.2 is released at the last step of the coagulation cascade and before thrombin inhibition by the anticoagulant used, namely lepirudin. The combined inhibition of complement and TLR

seems to be a good approach to inhibit LPS and *E. coli*-induced coagulation and, in this way, improve the prognosis of sepsis and other inflammatory states.

4.7 Future perspectives

Several *in vitro* studies have shown promising results for the combined inhibition of complement and TLR [6]. Furthermore, *in vivo* studies in porcine and mice models have been published [6]. However, there are still questions that need to be answered before the combined inhibition of complement and CD14/TLRs may be a part of the treatment of patients with sepsis. First of all, we need more knowledge about how this selective and combined treatment of complement and CD14/TLRs affects bacterial growth using live bacteria. Next, we need more studies on the combined inhibition of complement and CD14/TLRs as adjunct treatment in combination with antibiotics and other commonly used supportive therapies in sepsis.

Platelets interact with leukocytes and are an active part of the immunity system with several functions as earlier described [43]. The effect of the combined inhibition of complement and TLRs on platelet activation remains to be studied both *ex vivo* in the whole blood models and *in vivo* in relevant animal models. The results may give important information about the crosstalk between complement, TLRs and coagulation activation since platelets are involved. Platelets ability to generate TF is controversial [329] and hopefully a question that could be answered in the near future. Platelets have C1-INH in their alpha granula, and platelet activation with granula release increases the local C1-INH concentration greatly. The effect of C1-INH and other contact activation inhibitors on platelet activation should be studied.

In addition, further development of the whole blood model of inflammation is needed. The use of anticoagulants that do not inhibit thrombin, i.e. specific fibrinogen inhibitors should be examined. This will make it possible to study the role of complement, TLRs and thrombin in platelet and coagulation activation. Furthermore, the role of complement activation and TLRs in the activation of coagulation and inflammatory response can be studied with thrombin fully active. Finally, the inclusion of endothelial cells in such a

model would be interesting, allowing the study of interaction between endothelial cells, blood cells and platelets in complement and coagulation cascades.

5 Conclusions

The immunity and hemostatic systems are closely connected. Knowledge about the connection between the different systems is necessary to design an optimal treatment to reduce the mortality of sepsis and other diseases. The complement and TLR systems work synergically and several studies have shown that a combined inhibition of these is needed to inhibit bacteria-induced inflammation and TF upregulation. This study indicates a role of complement, CD14 and TLRs in bacteria-induced TF upregulation. Furthermore, it suggests that the combined inhibition of complement and CD14/TLRs may reduce the bacteria-induced expression of TF mRNA, TF protein surface on monocytes and microparticles. The combined inhibition may thus reduces the bacteria-induced immune response and coagulation, but has to be used together with traditional sepsis treatment, including antibiotics, intravenous fluid, vasopressor medications and oxygen supply [330]. Several studies in animal models etc. are thus needed to determine if this combined treatment of complement and CD14/TLRs may be a potentially adjuvant therapy for human sepsis in the future.

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Papers

PAPER I

PAPER II

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C1-inhibitor efficiently delays clot development in normal human whole blood and inhibits *Escherichia coli*-induced coagulation measured by thromboelastometry

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ABSTRACT

Introduction: C1-inhibitor (C1-INH), a serine protease inhibitor in plasma plays a central role in the cross-talk among the complement, coagulation, fibrinolytic and kallikrein-kinin systems. However, previous reports indicate thrombotic risks in children following supraphysiological dosing with C1-INH. Objective: To investigate the role of supraphysiological C1-INH concentrations in clot development with and without addition of *Escherichia coli* (*E. coli*) in fresh human whole blood using thromboelastometry.
Materials and methods: Blood was collected in citrate tubes, and C1-INH (3.0 to 47.6 μM) or human serum albumin (HSA) was added as a control. Activated partial thromboplastin time (aPTT) was analysed in the plasma. The analyses non-activated thromboelastometry (NATEM), extrinsic (EXTEM) or intrinsic thromboelastometry (INTEM) were performed using rotational thromboelastometry.
Results: C1-INH increased aPTT 1.8-fold ($p < 0.05$), whereas HSA had no effect. C1-INH increased NATEM clotting time (CT) from 789 s to 2025 s ($p < 0.05$) in a dose-dependent manner. C1-INH reduced the NATEM alpha angle from 47 to 28° ($p < 0.05$) and increased the NATEM clot formation time from 261 s to 595 s ($p < 0.05$). *E. coli* significantly reduced the NATEM CT after 120 min of incubation. C1-INH prevented *E. coli*-induced activation ($p < 0.05$). C1-INH significantly increased the INTEM CT ($p < 0.05$), but had no effect on EXTEM CT. C1-INH (47.6 μM) significantly reduced fibrinolysis measured as NATEM and EXTEM lysis indices LI60.
Conclusions: Supraphysiological C1-INH concentrations have dose-dependent anticoagulant effects in human whole blood *in vitro*. At very high levels C1-INH also inhibits fibrinolysis.

1. Introduction

C1-inhibitor (C1-INH) is a serine protease inhibitor in the complement, coagulation, fibrinolytic and kallikrein-kinin systems. The C1-INH gene is localized on chromosome 11 (p11.2-q13) [1], and the protein has a molecular weight of 105 kDa [2]. The carboxy-terminal of C1-INH contains a protease recognition site that forms covalent complexes with proteases. Under normal conditions, the plasma concentration of C1-INH is approximately 0.25 g/l (2.38 μM) [3]. C1-INH efficiently inhibits contact activation by complexing with activated coagulation Factor XII (FXIIa), kallikrein [4] and FXIa in the intrinsic coagulation pathway [5]. C1-INH inhibits thrombin and the presence of endothelial cells increases this inhibition [6,7]. In the fibrinolytic system, C1-INH inhibits plasmin and tissue plasminogen activator (tPA) [5]. Supraphysiological C1-INH concentrations efficiently inhibit the up-regulation of *Escherichia coli* (*E. coli*)-induced tissue factor (TF) which activates the extrinsic coagulation system [8]. C1-INH can also bind Gram-negative bacteria and LPS *via* non-covalent binding to its heavily glycosylated amino-terminal end [9,10]. C1-INH regulates both the classic pathway by inhibiting C1r and C1s and the lectin pathway of the complement system [5]. Interestingly, C1-INH was recently reported to have beneficial effects on the survival of patients with sepsis [11].

Abbreviations: aPTT, activated partial thromboplastin time; C1-INH, C1-inhibitor; *E. coli*, *Escherichia coli*; EXTEM, extrinsic thromboelastometry; HAE, hereditary angioedema; HSA, human serum albumin; INTEM, intrinsic thromboelastometry; LI, lysis index; min, minute; NATEM, non-activated thromboelastometry; PBS, phosphate buffered saline; ROTEM, rotational thromboelastometry; tPA, tissue plasminogen activator; TF, tissue factor.

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C1-INH deficiency increases vascular permeability, and hereditary angioedema (HAE) patients have reduced functional C1-INH concentrations [12]. C1-INH deficiency increases the ability of kallikrein to separate bradykinin from the high molecular weight kininogen. Previous studies have shown that FVIIa and thrombin levels are increased during HAE attacks, indicating activation of coagulation [13–15]. During these attacks, HAE-patients develop oedema of the skin and mucosa, which can typically be reversed by an infusion of 20 Units/kg of C1-INH. One Unit (U) is the activity of C1-INH in 1 ml of citrated plasma, which corresponds to 0.25 mg (2.38 nmol) C1-INH. To our knowledge, the precise relationship between infused C1-INH per kg of body weight and the resulting increase in plasma concentration of C1-INH has not been published. If one assumes a 20% body weight distribution volume of C1-INH into both the plasma and the extra-cellular fluids, 20 U/kg of C1-INH corresponds to 0.24 μmol/l of C1-INH. If only distributed in plasma, a dose of 20 U/kg C1-INH corresponds to approximately 1.13 μmol/l. In the latter case a 20 U/kg dose should theoretically increase the normal plasma concentration of C1-INH by 50% from 2.38 μM.

When newborns with serious hearts defects were given a large dose of 500 U/kg C1-INH to prevent capillary leakage after cardiopulmonary bypass, they also had thrombosis [16]. An intravenous dose of purified human C1-INH 40 U/kg reduced myocardial infarctions in pigs without side effects, but 200 U/kg resulted in thrombosis [17], suggesting a bell-shaped side effect curve. These observations suggested that C1-INH has prothrombotic effects. However, Lucca et al. showed that 250 U/kg recombinant human C1-INH was well tolerated and prevented haemorrhage-induced inflammation in pigs to a greater extent than 100 U/kg [18]. Thus, the dose-effect in humans remains unclear.

Rotational thromboelastometry provides a comprehensive analysis of the coagulation kinetics in human whole blood. Thrombelastometry provides important data on clot strength and fibrinolysis [19,20]. Thromboelastometry is increasingly used in emergency departments and intensive care units to guide treatment [21]. Recently, a study in rabbits indicated that infusion of up to 800 U/kg C1-INH significantly reduced coagulation analysed by thromboelastometry [22]. Thus, to add additional information the aim of this study was to use thromboelastometry to examine the effect of supraphysiological doses of C1-INH on clot development in native human whole blood and after addition of *E. coli in vitro*.

2. Materials and methods

2.1. Reagents and bacteria

Purified human C1-INH (Berinert® P) was obtained from ZLB Behring GmbH, Germany. Human serum albumin (HSA) (Albuminativ, Octapharma, Stockholm, Sweden) was used as a negative control. *E. coli* (strain LE392, ATCC33572, American Type Culture Collection, Manassas, VA) was grown and counted and inactivated by incubation at 60 °C for 60 min.

2.2. Blood sampling and whole blood experiments

This study was approved by the regional ethics committee of the Northern Norway Regional Health Authority. The blood donors provided written informed consent. The donors were both females and males males with ages ranging from 34 to 61 years. They had not taken any drugs. Fresh venous blood was collected using Vacuette tubes with 3.2% citrate (Greiner Bio-One GmbH, Kremensmünster, Austria). Blood (5 parts) was added to the C1-INH in Dulbecco's Phosphate buffered saline with $CaCl_2$ and $MgCl_2$ (PBS, Sigma-Aldrich, St. Louis, MO), or HSA (1 part) in polypropylene tubes (Nunc, Roskilde, Denmark). After 5 min at 37 °C, the activator, PBS control, *E. coli* or silica based clot activator in Vacuette rapid coagulating tubes (1 part) was added as previously described [23]. Thromboelastometry analyses were then performed immediately. In the time course experiments with *E. coli*, thromboelastometry analyses were performed after 0, 120 and 240 min of incubation. In some experiments, the tubes were centrifuged at 3000 ×*g* for 15 min at 4 °C, and PT-INR and activated partial thromboplastin time (aPTT) were analysed immediately in the plasma. The remaining plasma was stored at −80 °C for subsequent analysis.

2.3. Coagulation analyses

All donors were tested for disorders in the coagulation system. PT-INR and aPTT were analysed in citrated plasma using STA-R Evolution (Diagnostica Stago, Asniéres, France). The reagents used for clot detection were STA® -SPA+ for PT-INR and STA®-PPT for aPTT. Protein C and antithrombin were assayed colorimetrically using STACHROM®Protein C and STACHROM®ATIII kits from Diagnostica Stago. Protein S and activated protein C resistance were analysed in clotting assays using the STACLOT®Protein S kit from Diagnostica Stago and the COATEST®APC™ Resistance V kit (Chromogenix, Bedford, MA, USA), respectively.

2.4. Thromboelastometry

ROTEM® delta (Tem Innovations GmbH, Munich, Germany) was used to analyse the kinetics and properties of clot formation. This analysis was performed according to the instruction manual. The samples were analysed within 15 min after blood sampling. For the non-activated thromboelastometry (NATEM) analyses, only the re-calcification reagent star-tem® containing $CaCl_2$ (Tem Innovations GmbH) was used. Star-tem® (20 μl), followed by 300 μl of citrated human whole blood, was added to a preheated plastic cup with an automatic pipette [19,21]. Intrinsic thromboelastometry (INTEM) analysis was used to assess the intrinsic pathway and in-tem® reagent containing ellagic acid was added (Tem Innovations GmbH). Extrinsic thromboelastometry (EXTEM) analysis was used to analyse the extrinsic pathway. The ROTEM tests were run from 1 h and 15 min (INTEM and EXTEM) to 8 h (NATEM).

2.5. Platelet function

Fresh citrated human whole blood (3.2% sodium citrate from Vacuette, Greiner Bio-One GmbH) was obtained from healthy blood donors and stored for at least ten min at room temperature. PBS, HSA or increasing C1-INH concentrations in 44 μl of PBS were added to the citrated blood (831 μl) before analysis. Platelet function was analysed on an INNOVANCE® PFA-200 system (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany) using a Dade PFA Collagen/ADP Test Cartridge from Siemens. Haematocrit and platelet counts were analysed in all blood donors because abnormal values may affect the PFA-200 results [19,24]. Donors with an enhanced closure time were excluded from the study. One of the donors for platelet function later proved to have a heterozygous Leiden mutation. The results were as expected and were therefore included in our results.

2.6. Statistical analyses

The Shapiro-Wiik test in IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, NY, USA) was used for verifying normal distribution. GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA) was used for the statistical analysis. Skewed results were either log-transformed to a normal distribution if possible or analysed using non parametric tests. Normally distributed data were analysed using a one-way repeated measurements analysis of variance (ANOVA), and a Dunnett's multiple comparisons test was conducted using PBS as a control. The HSA results were compared with an equimolar concentration of C1-INH using paired Student's *t*-test. Two-way repeated measurements ANOVA and Sidak's multiple comparisons test were used to compare samples to which PBS or *E.coli* were added at several time points. If log-transformation of skewed data failed to reach

normality, data were instead analysed using the non-parametric Friedman test followed by Dunn's multiple comparisons test. Wilcoxon matched-pairs signed rank test was used to compare the HSA results to an equimolar concentration of C1-INH. Statistical significance was defined as *P*-values < 0.05. To identify a significant increase in NATEM CT from 789 ± 97 s (mean ± SD) after the addition of PBS to 2025 ± 264 s after the addition of C1-INH (47.6 μM), this would require 3 blood donors with a power of 96% and an alpha = 0.05 using a paired Student's *t*-test according to power analysis using IBM®SPSS® Sample Power® 3 (SPSS Inc., Chicago, Il, USA).

3. Results

3.1. C1-INH increased aPTT, wereas PT-INR was unaffected

C1-INH significantly and dose-dependently increased aPTT from 44 s in the control to 79 s at the highest C1-INH concentration (47.6 μM) (Fig. 1a) ($p < 0.05$). The highest C1-INH concentration (47.6 μM) significantly increased the aPTT compared with 47.6 μM HSA ($p = 0.0002$). With the exception of the highest C1-INH concentration where a slight increase was observed, C1-INH had no effects on PT-INR (Fig. 1b).

3.2. C1-INH reduced native clot development

C1-INH dose-dependently increased the NATEM CT from 789 s in the PBS control to 2015 s ($p < 0.05$) at the highest concentration (Fig. 2a), indicating delayed coagulation. The highest C1-INH concentration (47.6 μM) significantly increased the NATEM CT compared with HSA ($p < 0.0001$). C1-INH dose-dependently reduced the NATEM alpha angle, reflecting the velocity of clot development, from 47° in the control to 28° ($p < 0.05$) at the highest C1-INH concentration (Fig. 2b). C1-INH (47.6 μM) also significantly reduced the alpha angle compared with HSA ($p = 0.0313$). C1-INH dose-dependently increased the NATEM CFT from 261 s to 595 s ($p < 0.05$) at the highest C1-INH concentration (Fig. 2c). C1-INH (47.6 μM) significantly increased the NATEM CFT compared with HSA ($p = 0.0313$). The NATEM MCF was only slightly, but significantly increased ($p < 0.05$) by C1-INH (Fig. 2d). C1-INH (47.6 μM) significantly increased NATEM MCF compared with HSA ($p < 0.0001$).

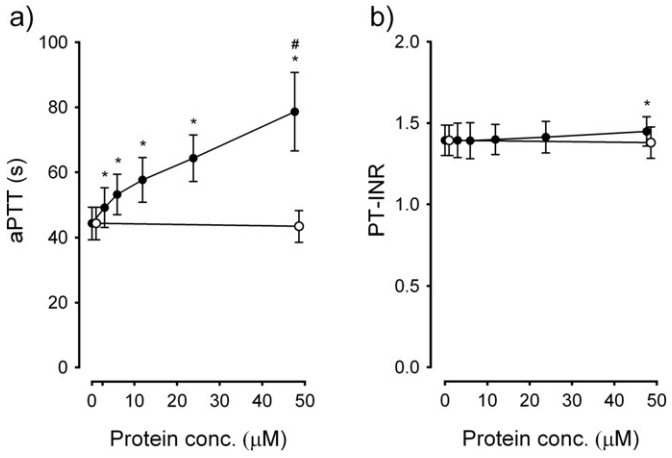

Fig. 1. The effect of C1-inhibitor (C1-INH) on (a) activated prothrombin time (aPTT) and (b) prothrombin time international normalized ratio (PT-INR). C1-INH (●) was added to fresh citrated human whole blood for final concentrations of 0, 3.0, 6.0, 11.9, 23.8 and 47.6 μM. Human serum albumin (HSA) (47.6 μM) was used as a control (○). The samples were centrifuged, and aPTT and PT-INR were analysed in plasma using STA-R Evolution. The aPTT results are given in seconds (s). The PT-INR results are given as a ratio between the donors' prothrombin time and the control plasma. Values are given as means ± standard deviation (SDs) from single experiments with 6 different donors. $^{*}p < 0.05$ compared with the samples without C1-INH using a one-way, repeated measurements ANOVA. $^{\#}p = 0.0002$ when C1-INH (47.6 μM) was compared with the samples to which HSA was added using paired Student's *t*-test.

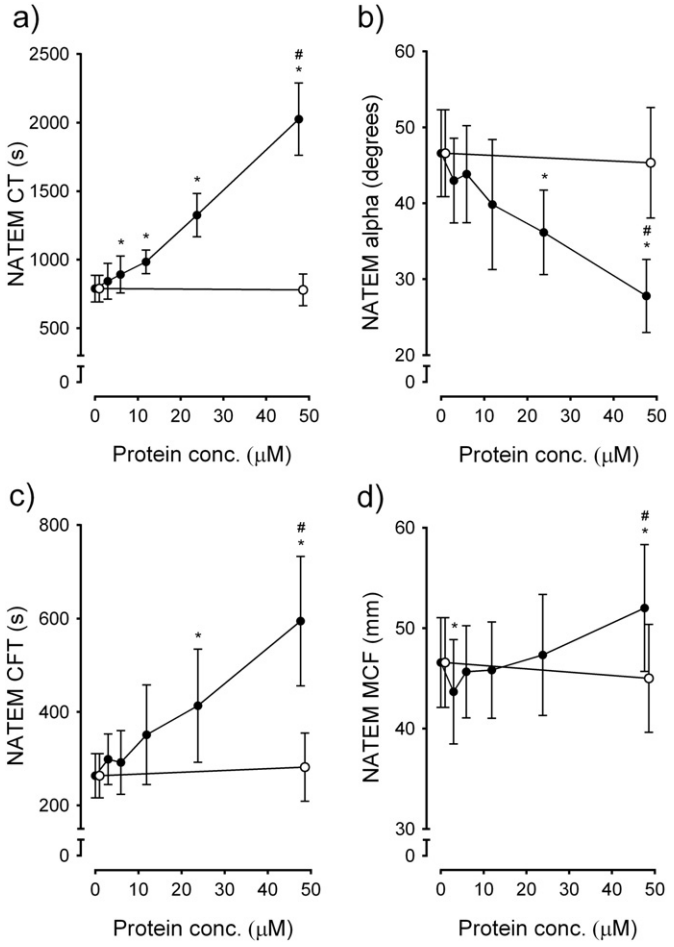

Fig. 2. The effect of C1-inhibitor (C1-INH) on coagulation was analysed using thromboelastometry and non-activated thromboelastometry (NATEM). C1-INH (●) was added to fresh citrated human whole blood to for final concentrations of 0, 3.0, 6.0, 11.9, 23.8 and 47.6 μM. HSA was used as a control (○). The samples were re-calcified and immediately analysed. (a) The effect of C1-INH on NATEM coagulation time (CT) is given in seconds (s), and (b) the NATEM alpha angle is given in degrees. (c) The effect of C1-INH on NATEM clot formation time (CFT) is given in seconds, and (d) the effect of C1-INH on NATEM maximal clot firmness is given in millimetres (mm). Data are given as means ± SDs from single experiments with 6 different blood donors. $^{*}p < 0.05$ compared with the samples without C1-INH using a one-way, repeated measurements ANOVA. Friedman test and Dunn's multiple comparisons test were used for skewed data. $^{\#}$C1-INH (47.6 μM) was compared with the samples to which HSA was added using paired Student's *t*-test or Wilcoxon matched-pairs signed rank test for skewed data: in panel a) $p < 0.0001$, b) $p = 0.0313$, c) $p = 0.0313$ and d) $p < 0.0001$.

3.3. C1-INH delayed clot development following activation of the intrinsic coagulation pathway

C1-INH dose-dependently increased the INTEM CT from 191 s to 309 s ($p < 0.05$) at the highest C1-INH concentration (Fig. 3a) and dose-dependently reduced the INTEM alpha angle compared with the PBS control ($p < 0.05$), indicating that the velocity of clot formation was reduced. C1-INH (47.6 μM) also significantly increased the INTEM CT compared with HSA ($p < 0.0001$). The highest C1-INH concentration (47.6 μM) reduced the alpha angle from 73° with HSA to 62° ($p < 0.0001$) (Fig. 3b). C1-INH dose-dependently increased the INTEM CFT from 98 s in the PBS control to 152 s ($p < 0.05$). C1-INH (47.6 μM) also significantly increased the INTEM CFT compared with HSA ($p < 0.0001$). C1-INH had no effect on the INTEM MCF (Fig. 3d).

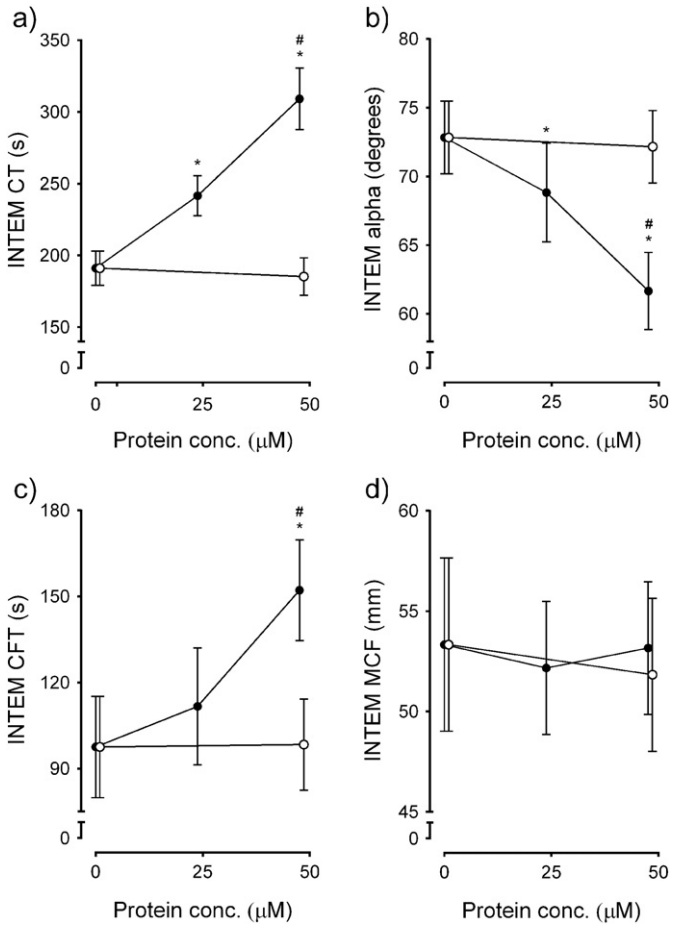

Fig. 3. The effect of the C1-inhibitor (C1-INH) on the intrinsic coagulation pathway was analysed using thromboelastometry and intrinsic thromboelastometry (INTEM) analyses. C1-INH (●) was added to fresh citrated human whole blood for final concentrations of 23.8 and 47.6 μM. HSA was used as a control (○). The samples were re-calcified and immediately analysed by INTEM analyses with phospholipids and ellagic acid. (a) The effect of C1-INH on INTEM coagulation time (CT) is given in seconds (s). (b) The effect of C1-INH on INTEM alpha angle or coagulation kinetics is given in degrees. (c) The effect of C1-INH on INTEM clot formation time (CFT) is given in seconds. (d) The effect of C1-INH on INTEM maximal clot firmness (MCF) is given in millimetres (mm). Values are given as means ± SDs from single experiments with 6 different donors. $^{*}p < 0.05$ compared with the samples without C1-INH using a one-way, repeated measurements ANOVA. $^{\#}$C1-INH (47.6 μM) was compared with samples added HSA using a paired Student's *t*-test, for panel a) $p < 0.0001$, b) $p < 0.0001$, c) $p < 0.0001$ and d) $p = 0.0624$.

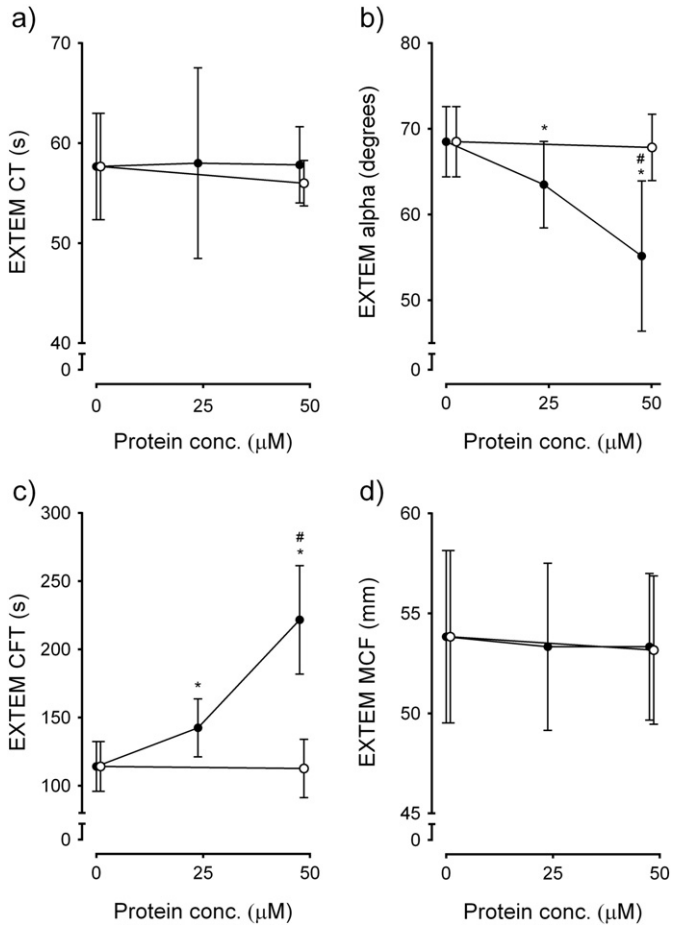

Fig. 4. Effect of C1-inhibitor (C1-INH) on the extrinsic coagulation pathway was analysed using thromboelastometry and extrinsic thromboelastometry (EXTEM). C1-INH (●) was added to fresh citrated human whole blood for final concentrations of 23.8 and 47.6 μM. HSA was used as a control (○). The samples were re-calcified and immediately analysed using EXTEM with activated tissue factor (TF). (a) The effect of C1-INH on the EXTEM coagulation time (CT) is given in seconds (s). (b) The effect of C1-INH on the EXTEM alpha angle is given in degrees. (c) The effect of C1-INH on the EXTEM clot formation time (CFT) is given in seconds. (d) The effect of C1-INH on the EXTEM maximal clot firmness (MCF) is given in millimetres (mm). The values are given as means ± SDs from single experiments with 6 different donors. $^{*}p < 0.05$ compared with the samples without C1-INH using a one-way, repeated measurements ANOVA. $^{\#}$C1-INH (47.6 μM) was compared with samples to which HSA was added using paired Student's *t*-test, in panel b) $p = 0.0030$, c) $p < 0.0001$.

3.4. C1-INH reduced the EXTEM alpha angle and increased the CFT

The EXTEM CT was not affected by the addition of C1-INH (Fig. 4a). However, C1-INH dose-dependently decreased the EXTEM alpha angle from 69 degrees in the PBS control to 55° ($p < 0.05$) at the highest C1-INH concentration. C1-INH dose-dependently increased the EXTEM CFT from 114 s to 222 s ($p < 0.05$) (Fig. 4c), whereas it had no effect on EXTEM MCF (Fig. 4d). C1-INH (47.6 μM) also significantly reduced the EXTEM alpha angle ($p = 0.003$), and increased the EXTEM CFT ($p < 0.0001$) compared with the HSA control.

3.5. Suphraphysiological doses of C1-INH reduced fibrinolysis

The highest C1-INH concentration (47.6 μM) significantly reduced fibrinolysis, as measured with lysis indices 60 (LI60) in the analyses NATEM EXTEM compared with the sample to which HSA was added (Table 1).

3.6. E. coli bacteria increased the native clot formation, as measured by the NATEM CT and alpha angle

E. coli (1×10^8/ml) reduced the NATEM CT from 622 s in the PBS control to 120 s ($p < 0.05$) after 240 min of incubation (Fig. 5a). In comparison, the clot activator (positive control) reduced the NATEM CT from 873 s to 500 s ($p = 0.0192$) (Fig. 5a), indicating that *E. coli* was a highly potent activator of coagulation. *E. coli* increased the NATEM alpha angle from 44° in the PBS control to 66° after a 240 min of incubation ($p < 0.05$), indicating that coagulation kinetics was increased (Fig. 5b). *E. coli* also significantly decreased the NATEM CFT ($p < 0.05$) and increased the NATEM MCF ($p < 0.05$) compared with the PBS control after 240 min of incubation (Fig. 5c, d).

3.7. C1-INH abolished E. coli-induced coagulation and the reduction in NATEM CT

E. coli decreased the NATEM CT from 805 s to 330 s ($p = 0.0088$), but, at the highest concentration (47.6 μM), C1-INH completely reversed this

Table 1
Effects of C1-inhibitor on fibrinolysis.

Test	Addition	Concentration	NATEM	*p*-Value	INTEM	*p*-Value	EXTEM	*p*-Value
LI45	PBS		97.6 (±1.0)		96.0 (±1.7)		95.7 (±1.2)	
	HSA	47.6 μM	97.2 (±1.2)		95.3 (±1.5)		94.8 (±1.7)	
	C1-INH	23.8 μM	98.3 (±1.2)	>0.05[a]	96.5 (±1.2)	>0.05[b]	97.2 (±1.2)	>0.05[a]
	C1-INH	47.6 μM	100 (±0)	>0.05[a]	97.2 (±0.8)	>0.05[b]	99.8 (±0.4)	<**0.05**[a]
				0.06[c]		**0.02**[d]		**0.03**[c]
LI60	PBS		95.7 (±2.2)		93.2 (±1.5)		91.7 (±1.9)	
	HSA	47.6 μM	94.0 (±2.4)		92.5 (±2.0)		90.5 (±1.9)	
	C1-INH	23.8 μM	96.7 (±2.3)	>0.05[a]	93.7 (±1.4)	>0.05[a]	93.2 (±1.6)	>0.05[a]
	C1-INH	47.6 μM	99.5 (±0.8)	<**0.05**[a]	94.0 (±0.9)	>0.05[a]	97.5 (±0.5)	<**0.05**[a]
				0.03[c]		0.13[c]		**0.03**[c]

Results are given in percentage, mean (±standard deviations) ($n = 6$).
Abbreviations: C1-INH; C1-inhibitor, EXTEM; extrinsic thromboelastometry, HSA; human serum albumin, INTEM; intrinsic thromboelastometry, LI; lysis index, NATEM; non-activated thromboelastometry, PBS; phosphate buffered saline.
p-Values in bold indicate $p < 0.05$.
[a] Friedman test and Dunn's multiple comparisons test between C1-INH and PBS.
[b] One-way repeated measurement ANOVA and Dunnett's multiple comparisons test between C1-INH and PBS.
[c] Wilcoxon matched-pairs signed rank test between HSA and C1-INH 47.6 μM.
[d] Paired Student's *t*-test between HSA and C1-INH 47.6 μM.

reduction in NATEM CT (Fig. 6). The highest C1-INH concentration did not significantly change the NATEM alpha angle, CFT or MCF after the addition of *E. coli* (data not shown).

3.8. C1-INH reduced the platelet function as measured by closure time

C1-INH dose-dependently and significantly ($p < 0.05$) increased the closure time by 63% compared with the PBS control (Fig. 7). However, the increase was not significant ($p = 0.064$) when the highest C1-INH concentration (23.8 μM) was compared with the HSA control.

4. Discussion

In this study, we show for the first time that supraphysiological doses of C1-INH significantly and dose-dependently delayed coagulation in nearly all the parameters examined using thromboelastometry in fresh human whole blood. The effects were observed during normal coagulation and in the *E. coli*-enhanced coagulation, which was virtually abolished by C1-INH. The highest C1-INH doses also inhibited fibrinolysis.

C1-INH dose-dependently increased aPTT values in accordance with a study in rabbits [22]. The most important mechanism underlying the enhancement of aPTT values by C1-INH most likely involves the binding of C1-INH to FXIIa [25]. The slightly increased baseline aPTT values are most likely due to the dilution of whole blood with PBS. The sizeable effects on aPTT values observed in our study are most likely due to the supraphysiological dose of C1-INH. Consistent with these findings, a previous report on HAE patients treated with C1-INH infusion showed moderately increased aPTT values compared to the baseline values [26]. The more moderate effects on the aPTT values in C1-INH substituted HAE patients is probably explained by a lower total C1-INH level. Even if 20 U/kg of C1-INH is administered it would not bring the HAE patients' plasma concentration of C1-INH from under 20% to above normal levels. Interestingly, HAE patients display increased levels of both thrombin-antithrombin and plasmin-antiplasmin complexes under attacks but do not suffer from thrombosis nor bleeding [13]. The increase in the NATEM CT due to C1-INH indicates an anticoagulant effect on the native coagulation. At supraphysiological concentrations, C1-INH also dose-dependently inhibited clot development after intrinsic pathway activation, most likely due to the binding of FXII. Thus, part of the anticoagulant effect of C1-INH observed in this study may be due to the inhibition of contact activation induced by plastic surfaces in the cups used for thromboelastometry. This effect can also be explained by the inhibitory effects of C1-INH on thrombin [6]. However, C1-INH has a minor role in inhibition of thrombin under physiological conditions. Cassia et al. discussed in their paper that the inhibitory effects of C1-INH on thrombin could be relevant in severe infection and inflammation when other anticoagulant pathways are reduced and when C1-INH is increased [7]. This could possibly also be the case in our study since high C1-INH doses were used.

C1-INH had no effects on EXTEM CT, indicating that the TF-induced coagulation time was not affected. Schürmann et al. also found that C1-INH has no effect on EXTEM CT and MCF in rabbits [22]. This is consistent with the minimal effect we found of C1-INH on PT-INR levels. The surface of activated platelets may induce FXII-dependent contact activation [27], which in turn could be reduced by high doses of C1-INH. In normal plasma, without extra C1-INH, antithrombin is more important [27]. C1-INH effects on FXIa may also at least partially explain the results because C1-INH inhibits FXIa both directly and indirectly *via* thrombin [5,6], and because thrombin activates FXI [28].

NATEM MCF reflects the stability of the clot formed in the whole blood. C1-INH slightly increased NATEM MCF, but had no effects on INTEM or EXTEM MCF. This is in contrast to the findings obtained by Levy et al. who noted a slight reduction in INTEM MCF at lower C1-INH doses up to 7 U/ml [29]. This finding corresponds to approximately seven times the physiological concentration, *i.e.*, a concentration similar to one of the lower concentrations used in our study. MCF is generally affected by fibrinogen concentration, FXIII and platelet numbers and function. Of these factors, only the platelet function may potentially be influenced by C1-INH [20,30]. The presence of a minimal, but bell shaped effect of a large C1-INH dose on clot firmness is unexpected and its significance must be confirmed. As C1-INH in large doses also reduced the clot formation, *vide infra*, the significance may be small.

To our knowledge, this is the first report showing that high levels of C1-INH significantly reduced fibrinolysis as measured by thromboelastometry. Our results suggest that the effect of C1-INH on fibrinolysis is slightly more pronounced after activation of the extrinsic pathway. C1-INH affects the fibrinolytic system through the inhibition of FXIIa, plasmin and tPA [5], potentially causing a thrombotic effect. Relan et al. found no change in the thrombin-antithrombin complex, *d*-dimer and plasmin alpha-2 antiplasmin complex levels after recombinant C1-INH infusion to HAE-patients but the resulting amount of C1-INH was low [26]. Alpha-2 antiplasmin and plasminogen activator inhibitor-1 are the main inhibitors of plasmin and t-PA, respectively, whereas C1-INH plays a minor role in this process. According to the inhibition rate constants, even a ten-fold increase in the C1-INH concentration would increase plasmin inhibition by only a few percent [26]. Crowther et al. recently reviewed the prothrombotic effects of up to 100 U/kg of C1-INH and described them as rare complications [31]. However, limited animal and clinical data suggested that C1-INH, particularly at high doses of up to 500 U/kg may be prothrombotic [31]. Although the calculation is not precise, one may administer as

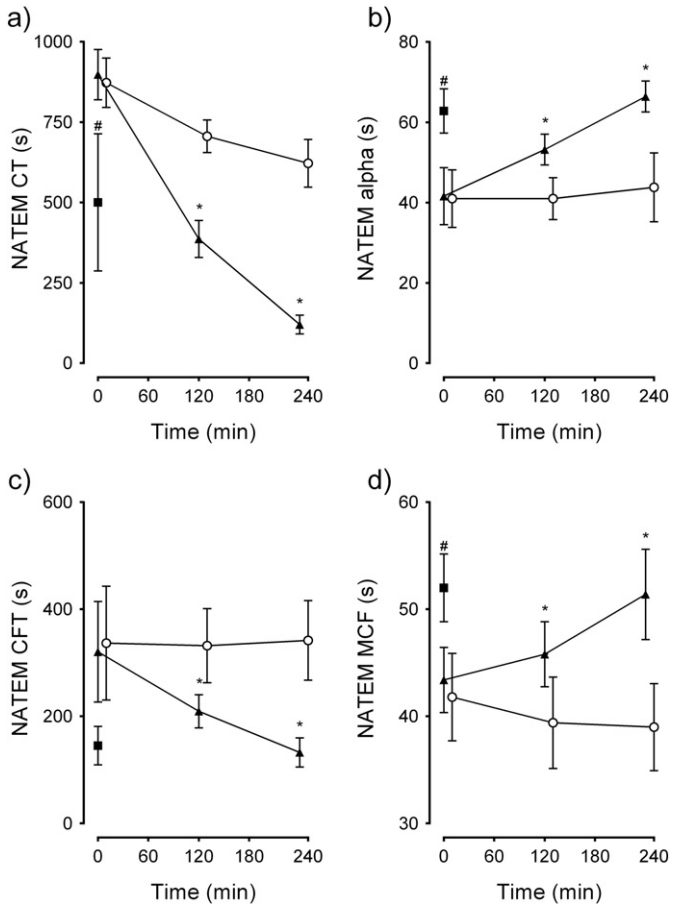

Fig. 5. Effect of *E. coli* (1×10^8/ml, ▲) on the coagulation kinetics in fresh human whole blood was analysed using non-activated thromboelastometry (NATEM). The tubes were incubated for 0, 120 or 240 min with *E. coli* or Phosphate buffered saline (PBS) before analysis. PBS was used as a negative control (○) and the clot activator at time zero as the positive control (■). (a) The effect of *E. coli*, clot activator and PBS control on NATEM coagulation time (CT) is given in seconds (s). (b) The effect of *E. coli*, clot activator and PBS control on coagulation kinetics evaluated as the alpha angle is given in degrees. (c) The effect of *E. coli*, clot activator and PBS control on clot formation time (CFT) is given in seconds. (d) The effect of *E. coli*, clot activator and PBS control on maximal clot firmness (MCF) is given in millimetres. The data are given as means ± SDs from single experiments using five different blood donors. *Samples to which *E.coli* was added compared with the PBS control using two-way repeated measurements ANOVA: panel a) T120 $p < 0.05$, T240 $p < 0.05$, b) T120 $p < 0.05$, T240 $p < 0.05$, c) T120 $p < 0.05$, T240 $p < 0.05$ and d) T120 $p < 0.05$, T240 $p < 0.05$. #$p < 0.05$ compared with the negative PBS control using paired Student's *t*-test or Wilcoxon matched-pairs signed rank test for non-normally distributed data.

much as 1000 U/kg C1-INH to obtain the levels used in this study. In addition, as both coagulation and fibrinolysis can be influenced at high C1-INH levels, we do not know the sum of these effects.

The anticoagulant effect of C1-INH was further confirmed by the observation that C1-INH inhibited the coagulation activation induced by *E. coli* in fresh human whole blood. The rationale for the *E. coli* experiments was that we aimed to study the effect of C1-INH on clot development after coagulation activation with bacteria which is a stimulus related to Gram-negative sepsis. Interestingly, C1-INH abolished the *E. coli*-induced decrease in NATEM CT. In the time course study of the effect of *E.coli* bacteria on NATEM CT we analysed the samples after 0, 60, 120 and 240 min. The stability of NATEM analysis is short and Meesters et al. showed that NATEM CT decrease from 1226 s to 903 s after 90 min storage [32]. Therefore, we included a PBS control and compared the decrease in samples added *E.coli* with the samples added PBS only. We have previously reported that C1-INH efficiently reduced *E. coli*-induced coagulation as measured by prothrombin F1.2 levels in the plasma [8]. By binding to both LPS and bacteria,

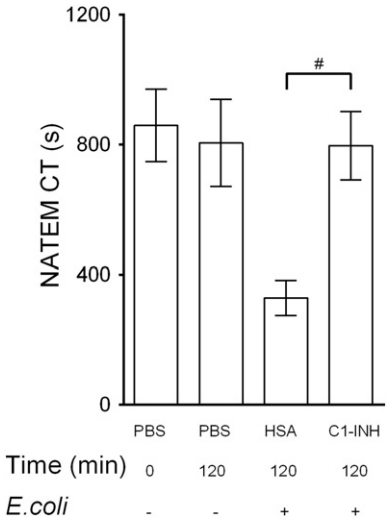

Fig. 6. Effect of C1-inhibitor (C1-INH) on *E. coli*-induced non-activated thromboelastometry (NATEM) coagulation time (CT) was analysed in fresh human whole blood using thromboelastometry. Before incubation, HSA or C1-INH was added to the samples. *E. coli* (1×10^8/ml) or HSA was incubated for 120 min. The NATEM CT values are given in seconds (s). The results are given as means ± SDs from single experiments with 3 different blood donors. #$p = 0.0088$ compared with the HSA control using paired Student's *t*-test.

independently of the protease inhibitor domain, C1-INH also has several anti-inflammatory effects [33,34]. C1-INH may inhibit *E. coli*-induced coagulation by interfering with the interaction between endotoxin and its receptor complex on macrophages [33,34]. We have previously also shown that C1-INH efficiently inhibited *E. coli*-induced TF mRNA and TF surface up regulation on monocytes [8]. The study by Jansen et al. in baboons supported that C1-INH may improve the outcome of *E.coli* sepsis, possibly by reducing prekallikrein and FXII consumption [35]. Interestingly, C1-INH infusion increased the C1-INH levels two to three times the physiological C1-INH concentration and reduced human mortality during sepsis [11]. It is tempting to speculate that

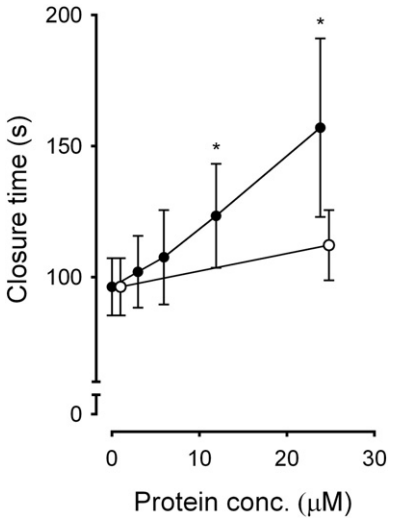

Fig. 7. Effect of C1-inhibitor (C1-INH) on platelet function in fresh human whole blood. Increasing concentrations of C1-INH (●) were added to fresh citrated human whole blood. The final C1-INH concentrations were 3.0, 6.0, 11.9 and 23.8 μM. PBS and human serum albumin (HSA) (○) were used as controls. The results were expressed as the closure time and are given in seconds (s). The results are given as means ± SDs from single experiments with six different donors, with the exception of HSA ($n = 3$). *$p < 0.05$ compared with the samples without C1-INH using a one-way, repeated measurements ANOVA. No significant difference was found when C1-INH (23.8 μM) was compared with HSA using a paired Student's *t*-test.

the anticoagulant effect of C1-INH attenuated the sepsis-induced intravascular coagulation in these patients.

The finding that C1-INH dose-dependently reduced the platelet plug formation further confirmed the anticoagulant effect of C1-INH. This result confirms earlier studies performed with other platelet function analysers [30,36]. C1-INH from the alpha-granules in platelets can be released upon platelet activation, and the local concentrations of C1-INH may increase in the range 20 to 100 μM [37,38]. HAE patients released a lower amount of platelet C1-INH than healthy controls and have an increased platelet aggregation [36,37]. C1-INH infusion normalized platelet function in HAE patients [30]. This finding suggests that C1-INH inhibits platelet function *in vivo*.

The whole blood model used in this study has some limitations. First, it does not include endothelial cells. Second, the contact activation induced by the plastic surfaces in the sample tubes and ROTEM cups may have affected the results. The relatively long time that elapsed between drawing the blood and performing the NATEM CT analysis in the time course experiments with *E. coli* may also have affected the results. Third, in this study we examined only the effect of C1-INH *in vitro*. The *in vivo* condition is more complex than our *in vitro* model. The ROTEM analysis may not reflect the clinical situation for several reasons. In the ROTEM chamber the blood flow is only induced by the rotation of the pin. In addition, the same blood cells are present for a long time in the ROTEM chamber in contrast to the *in vivo* situation. Furthermore, the ROTEM cuvette is probably not tight because the pin needs room to move, so there may be some space in between so air can get inside. However, we did not get any warnings from the instrument that leakage of air was a problem in our study. Furthermore, there is not always accordance between *in vivo* conditions and *in vitro* coagulation results. One example involves individuals with lupus anticoagulant who have prolonged aPTT but have increased thrombotic risk [39]. However, the whole blood model also has several advantages concerning the interaction between different cells including platelets, leukocytes and plasma factors participating in clot development [23]. The anticoagulant effect of therapeutic C1-INH concentrations should be confirmed in studies in humans *in vivo*.

In conclusion, native C1-INH in concentrations that are sometimes administered as an experimental therapy can delay clot development and have anticoagulant effects in fresh human whole blood *in vitro*. Furthermore C1-INH abolished *E. coli*-induced coagulation, indicating that the use of C1-INH as a supplementary adjuvant treatment for sepsis in humans merits further exploration. This effect have to be confirmed in an *in vivo* model, due to several limitations with this *in vitro* model. At high doses, C1-INH inhibits both clot development and fibrinolysis and the sum of these effects should be clarified in future studies.

Contribution

OLB, EWN and TEMs have designed this study. AL has performed the experiments and analysed the data. AL, HF, EWN, TEM and OLB have interpreted the data and written the manuscript.

Conflict of interest

The authors declare no competing financial interests.

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PAPER III

PAPER IV