Crosstalk between inflammatory pathways and the blood coagulation cascade

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Manuscripts in Preparation

A novel interaction between coagulation Factor XI and its activated form, FXIa, with the complement protein properdin. S. L. Heal, L. J. Hardy, C. L. Wilson, M. Ali, R. A. S Ariëns, R. Foster, H. Philippou. *(In preparation)*

Identification of a novel interaction between (Pre)kallikrein and Factor FIX(a): Role in coagulation and fibrin formation. K. J. Kearney, J. Butler, O. M. Posada Estefan, C. L. Wilson, S. L. Heal, M. Ali, L. J. Hardy, J. Ahnström, D. Gailani, C. Longstaff, E. Hethershaw, H. Philippou. *(Submitted PNAS - in revision)*

Glycated albumin differentially modulates the contacts system and coagulation cascades. L. J. Hardy, S. L. Heal, E. Hethershaw, M. Ali, R. Foster, C. Longstaff, T. Renné, H. Philippou. *(In preparation)*

Abstract

Thromboinflammation occurs in many disorders due to a loss of normal antithrombotic and anti-inflammatory mechanisms, culminating in the dysregulation of inflammatory processes and clotting mechanisms. It becomes dangerous when thrombi appear in the vasculature; a well-recognised phenotype in the pathology of several disorders including sepsis and trauma.

Novel therapeutics are in high demand to treat thromboinflammatory conditions, such as disseminated intravascular coagulation (DIC). This project aimed to optimise a process for screening novel compounds targeting the alternative pathway of complement using surface plasmon resonance (SPR), enzyme linked immunosorbent assays (ELISAs) and Western blot techniques. It was revealed that compounds can be utilised to modulate the alternative pathway C3 convertase.

Crosstalk between complement and coagulation is still not well defined or understood. This thesis aimed to characterise the intercommunication between the two pathways using SPR, chromogenic assays, Western blots and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as well as whole blood and plasma clotting assays. A novel interaction between the alternative pathway of complement and the intrinsic pathway of coagulation was discovered. Factor (F)XI and its activated form FXIa bind to properdin (Factor P, FP) and C3b. The interaction with FP was further investigated and the functional consequences were found to be a surface dependent interaction. For the first time it was demonstrated that FP can be cleaved, by FXIa in the presence of dextran sulfate (500 kDa [DXS_{500kDa}]), suggesting a surface dependent change in substrate specificity of FXIa. The inhibition of FXIa by C1 esterase inhibitor (C1-INH) in the presence of DXS_{500kDa} and heparin sodium salt is modulated by FP.

These data reiterate the importance of further characterisation of crosstalk between inflammatory pathways and coagulation, as novel interactions are continuously being discovered. This has given insight into these interactions and may provide novel therapeutic targets for thrombinflammatory disorders.

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List of Abbreviations

$^{-/-}$	Gene knockout
A*number	Apple domain
A2AP	α2-antiplasmin
ABS	Antigen binding site
ACE	Angiotensin-converting enzyme
AD	Alzheimer's disease
ADME	Adsorption, distribution, metabolism and excretion
ADP	Adenosine diphosphate
aHUS	Atypical haemolytic uremic syndrome
AMD	Age-related macular degeneration
AMP	Antimicrobial peptide
AMR	Antimicrobial resistance
AMTR	Antibody-mediated transplant rejection
APC	Activated protein C
AP	Alternative pathway
APP	Acute phase protein
aPTT	Activated partial thromboplastin time
ATIII	Antithrombin III
ATP	Adenosine triphosphate
AUC	Area under the curve
B_1R	Bradykinin receptor 1
B_2R	Bradykinin receptor 2
BK	Bradykinin
BSA	Bovine serum albumin
BCSH	British Committee in Standards for Haematology

C1	Complement component 1
C1-INH	C1 esterase inhibitor
C2	Complement component 2
C3	Complement component 3
C3aR	Complement component C3a receptor
C4	Complement component 4
C4BP	C4b-binding protein
C5	Complement component 5
C5aR1	Complement component C5a receptor 1
C6	Complement component 6
C7	Complement component 7
C8	Complement component 8
C9	Complement component 9
CD	Cluster of differentiation
CDAMP	Cell death-associated molecular pattern
CLP	Cecal ligation and puncture
CNV	Choroidal neovascularisation
CP	Classical pathway
CR	Complement receptor
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DDD	Dense deposit disease
dH_2O	Distilled water
DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
DR5	Death receptor 5
DSA	Donor specific antibody

DXS	Dextran sulfate
ECM	Extracellular matrix
EDC	1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EGF	Endothelial growth factor
ELISA	Enzyme linked immunosorbent assay
EPCR	Endothelial protein C receptor
ETP	Endogenous thrombin potential
FB	Complement factor B
FC	Flow channel
FD	Complement factor D
FDP	Fibrin degradation product
FH	Complement factor H
FI	Complement factor I
FP	Complement factor P/properdin
FC	Flow channel
Fc	Fragment crystallisable
Fib	Fibronectin domain
fM	Femtomolar
fMLP	N-formylmethionyl-leucyl-phenylalanine
g	Grams
GAG	Glycosaminoglycan
GP	Glycoprotein
GPCR	G-protein coupled receptor
GPRP	Glycine-Proline-Arginine-Proline
HAE	Hereditary angioedema
HBS	HEPES buffered saline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HiPBST	Phosphate buffered saline with high salt and Tween®20
HK	High molecular weight kininogen
HLA	Human leukocyte antigen
HSS	Heparin sodium salt
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ISTH	International Society on Thrombosis and Haemostasis
JAAM	Japanese Association for Acute Medicine
kDa	Kilo Dalton
KKS	Kallikrein-kinin system
LMWH	Low molecular weight heparin
LOS	Lipo-oligosaccharide
LP	Mannose-binding lectin pathway
LPS	Lipopolysaccharide
M	Molar
MAC	Membrane attack complex
MACPF	MAC/perforin
MASP	MBL-associated serine protease
MBL	Mannose-binding lectin
MCP	Membrane cofactor protein
µg	Micrograms
mg	Milligrams
MG	Macroglobulin
MHC	Major histocompatibility complex
µM	Micromolar
mM	Millimolar

MOD	Multiple organ dysfunction
NET	Neutrophil extracellular trap
ng	Nanograms
NHS	N-hydroxysuccinimide
NK cell	Natural killer cell
nM	Nanomolar
NPP	Platelet-poor normal pooled plasma
OD	Optical density
OPD	o-Phenylenediamine
PAI	Plasminogen activator inhibitor
PAMP	Pathogen associated molecular pattern
PAR	Protease-activated receptor
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween®20
PCH	Polycarboxylate high capacity sensor-chip
pg	Picograms
PK	Prekallikrein
PKa	Kallikrein
PL	Phospholipid
pM	Picomolar
PMN	Polymorphonuclear
PNH	Paroxysmal nocturnal haemoglobinuria
PolyP	Polyphosphate
PPB	Plasma protein binding
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PRR	Pattern recognition receptor

PS	Phosphatidylserine
PT	Prothrombin time
PTT-a	PTT-automate 5 silica activator
PVDF	Polyvinylidene difluoride
RA	Rheumatoid arthritis
RB	Running buffer
RBT	RB with Tween®20
RBTDM	RBTM with DMSO
RBTM	RBT with $MgCl_2$
RBTM++	RBTM + $CaCl_2$ + $ZnCl_2$
RCL	Reactive centre loop
ROS	Reactive oxygen species
ROTEM	Rotational thromboelastometry
RU	Response units
s	second
s^{-1}	Per second
SARS-COV-2	Severe acute respiratory syndrome coronavirus 2
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERPIN	Serine protease inhibitor
SIRS	systemic inflammatory response syndrome
SLE	Systemic lupus erythematosus
SPR	Surface plasmon resonance
SM	Small molecule
SSC	Scientific and Standardisation Committee
SST	Serum separator tube
TAFI	Thrombin activated fibrinolysis inhibitor

TAT	Thrombin-antithrombin
TBST	Tris buffered saline with Tween®20
TED	Thioester-containing domain
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TGF	Tissue growth factor
TLR	Toll-Like receptor
TNF-α	Tumour necrosis factor-α
tPA	Tissue plasminogen activator
TSR	Thrombospondin type-1 repeat
UFH	Unfractionated heparin
uPA	Urokinase plasminogen activator
(v/v)	(volume/volume)
VBG	Veronal buffer with gelatin
VBG++	VBG + $CaCl_2$ + $MgCl_2$
VTE	Venous thromboembolism
vWD	von Willebrand disease
vWF	von Willebrand factor
WB	Whole blood
WBC	White blood cell
(w/v)	(weight/volume)
WT	Wild type

Chapter 1 Introduction

1.1 Immunology and Inflammation

1.1.1 The Immune System

Exposure to potential pathogens and allergens is a regular occurrence, through inhalation, direct contact and ingestion. The immune system has evolved to recognise these potentially dangerous pathogens/allergens and can form an immunological memory to decrease response time if the same exposure reoccurs. Host defence mechanisms are important for protection against infectious disease by detecting and eradicating foreign, harmful agents (Sattler, 2017). This complex system is divided into two subcomponents; innate immunity and adaptive immunity, in which innate immunity can activate and regulate adaptive immunity (Romo et al., 2016) and both rely on the activities of leukocytes. Innate immunity acts as the initial response where surveillance proteins and cells detect pathogens and label them for phagocytosis and destruction (Dunkelberger and Song, 2010). Adaptive immunity is a more specific response where antibodies are produced specific to a pathogen and a strong and rapid immune response can be elicited if the pathogen was to infect for a second time (Wing and Remington, 1977). Through the immune system, the body is capable of distinguishing the differences between self and nonself substances, allowing elimination of potentially dangerous pathogens and even cancerous cells (Chaplin, 2010). This system is comprised of many subsystems including different cell types (Table 1.1), protein complexes and protease cascades, which all lead to an inflammatory response. The immune system is a major target for novel therapeutics for managing a number of diseases and syndromes including cancer, infection and autoimmune disorders (Parkin and Cohen, 2001).

1.1.1.1 Innate Immunity

Innate immune responses act as the first line of defence, triggering inflammation when innate immune cells (Table 1.1) and surveillance proteins detect trauma or infection (Newton and Dixit, 2012). There are two types of innate immune response;

inflammatory responses e.g. complement activation and cytokine release, and phagocytosis. The innate immune system is stimulated immediately or within a few hours of trauma or antigen presentation and involves surveillance cells recognising conserved features found on the surfaces of pathogens through pattern recognition receptors (PRRs). These receptors include Toll-like receptors (TLRs) which are found on a variety of granulocytes, mast cells and other immune cells (Table 1.1) (Taghavi et al., 2017). Inflammatory proteins including those in the complement system can opsonise pathogens via these conserved features to initiate their destruction through disruption of their membranes or by phagocytosis by neutrophils and macrophages (Dunkelberger and Song, 2010). During phagocytosis, assorted lytic enzymes along with antimicrobial peptides (AMPs) and reactive oxygen species (ROS), kill the invading pathogen (Lim et al., 2017). Antimicrobial peptides called defensins are found in mucus layers, in epithelial cells and in neutrophils and can kill or inactivate Gram-positive and Gram-negative bacteria as well as other pathogens including parasites and viruses. Defensins also act as signalling molecules which aid in the modulation of angiogenesis, wound healing and the inflammatory response (Kudryashova et al., 2017). Cytokines are also released from innate immune cells, epithelial cells and other cell types (Shiozawa et al., 2015) and are modulators of the immune response. These soluble, intercellular messengers can initiate inflammation and are able to limit inflammatory responses to pathogens and sites of trauma, decreasing the risk of host damage (Lacy and Stow, 2011). Cytokines are a family of intercellular messengers, some of which have specific actions and are specialised. Types of cytokines include interleukins (IL), interferons (IFN), tumour necrosis factors (TNF), tissue growth factors (TGF) and miscellaneous haematopoietins (Cohen et al., 1974). Chemokines are chemotactic cytokines, which act as chemo-attractant molecules to help traffic cells to sites of inflammation and infection (Cavalera and Frangogiannis, 2014). The innate response can then initiate the adaptive immune

response, which is more specific to the invading pathogens and is responsible for the majority of the phenomenon of immunological memory, aiding in faster immune responses if the same pathogen was to successfully invade once again (Simon et al., 2015).

1.1.1.2 Pathogen-Associated and Damage-Associated Molecular Pattern Molecules

Pathogens (bacteria, viruses, fungi and protozoa) express evolutionarily conserved structures at their surfaces, known as pathogen-associated molecular patterns (PAMPs). These structures are recognised by immune cells through pattern recognition receptors (PRRs) expressed at their surface (Janeway and Medzhitov, 2002). Once PRRs are bound to PAMPs, intracellular signalling events are triggered, leading to the expression of many proinflammatory molecules. These molecules, including cytokines and chemokines, cell adhesion molecules and immunoreceptors, are released and the early innate host response to infection is initiated, leading to subsequent activation of the adaptive immune response and pathogen clearance (Medzhitov, 2007). PAMPs are essential for pathogen survival and are easily distinguished from self-molecules (Kurup and Tarleton, 2013). Host factors can also be recognised in some instances, when abnormal complexes are expressed under inflammatory states such as apoptosis (Amarante-Mendes et al., 2018).

Apoptosis, pyroptosis, necroptosis and autophagy are variants of programmed cell death. Cell death is a natural way to clear aging cells and plays a vital role in the regulation of the immune system. These mechanisms are initiated through infection, injury, cellular stress and exposure to chemotherapeutics. Cell death via necrosis is dangerous and uncontrolled, culminating in cells releasing their contents into the local environment which can lead to toxicity, inducing inflammation, and tissue damage occurs as a result (D'Arcy, 2019). Cell death is induced in several ways, including ischemia (Thornton et al., 2017), exposure to toxins (Hickman and Boyle,

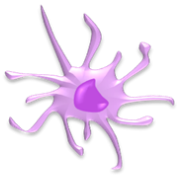
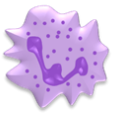
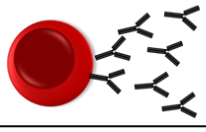
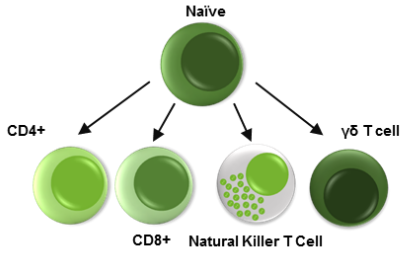
1997) and adenosine triphosphate (ATP)-depletion (Eguchi et al., 1997). A variety of endogenous proteins and molecules from different cellular compartments and structures, including the nucleus, mitochondria and the cytosol are released upon cell deterioration. These proteins and molecules are known as danger-associated molecular patterns (DAMPs), cell death-associated molecular patterns (CDAMPs) and alarmins. These molecular patterns are expressed in many different cell types and work synergistically with cytokines and antibiotic peptides (Sangiuliano et al., 2014). When cell death becomes dysregulated, this leads to many issues including increased lymphocytes, inability to clear infected phagocytes, autoimmunity and malignancy (Tang et al., 2019).

1.1.1.3 Adaptive Immunity

The adaptive immune system is much more specific and sophisticated than the innate immune system. The innate immune system is crucial for initiating the adaptive immune response, and both responses work together to eliminate pathogens, however the adaptive response is highly specific to the invading pathogen (Jain and Pasare, 2017). Two main classes of adaptive immunity exist: the antibody response and the cell-mediated response. Both responses are performed by lymphocytes, a type of leukocyte. The antibody response is responsible for antibody secretion from activated lymphocytes known as B cells, which recognise antigens presented on immune cells such as dendritic cells and macrophages. Antibodies are immunoglobulins (Ig) and can bind to the specific foreign antigen that initiated their secretion. There are five isotypes: IgM, IgG, IgA, IgE and IgD (Stavnezer et al., 2008). They can work in a variety of ways by either neutralising viral activity (Krauss, 2016) or by marking pathogens for phagocytosis or initiating complement activation (Overdijk et al., 2015). The cell-mediated response involves naive T cells recognising a foreign antigen in the form of the major histocompatibility complex (MHC) on the surface of antigen presenting cells such as dendritic cells (DCs), either at the site of infection or within the lymph nodes, creating effector T cells which eliminate the infected cell, preventing replication and dissemination of the pathogen. Effector T cells can also produce cytokines, activating macrophages and thus phagocytosis, and induce antibody production by B cells. Memory T cells are also formed, to aid in production of a rapid immune response to the original antigen if the pathogen were to invade again (Table 1.1) (Wing and Remington, 1977).

Table 1.1 Immune cells and their functions.

The immune system is only functional with the support of a variety of cells to detect and destroy pathogens and to produce important antibodies (Parkin and Cohen, 2001).

Mast Cell		• Long lived, containing granules. • Found between tissue and external environment, i.e. mucosal barriers, skin. • Activated by pathogens, physiological mediators and allergens.
Basophil		• Granular, mononucleocytes. • Only circulating WBC to contain histamine. • Similar to mast cells. • Activation leads to rapid degranulation. • Activated by complement anaphylatoxins and chemokines.
Macrophage		• Monocyte involved in eradication of bacteria and other harmful organisms. • Antigen presenting. • Release cytokines. • Migrate to and circulate in nearly all tissues to eliminate apoptotic cells and pathogens.
Eosinophil		• Important in defence against nematodes and other parasites. • Can be detrimental as part of allergic responses e.g. asthma. • Bi-lobed nucleus. • Granules containing cytokines, chemokines and destructive enzymes.
Dendritic Cell		• Most potent type of antigen presenting WBC. • Capture and process antigens to present MHC molecules for recognition by T cells. • Responsible for initiation of adaptive immune response. • Continuously sample their environment for rapid pathogen detection.
Neutrophil		• Granulocytes, polymorphonuclear. • Most abundant WBC in humans and mice, phagocytosis of pathogens. • Multi-lobed nucleus. • The first WBCs recruited to sites of acute inflammation.
Natural Killer Cell		• Same family as T and B cells. • Kill host cells infected with viruses and detect cancer cells. • Secrete cytokines. • Constant contact with other cells.
B Cell		• Centre of adaptive response and mediate antibody production. • Undergo antigen dependent and independent selection. • Can differentiate in to memory B cells. • Can aid in apoptosis.
T Cell		• CD4+/T helper cells – 2 types, one fights viruses and some bacteria, one fights larger organisms. Also a role in allergic response. • CD8+/cytotoxic T cells recognise MHC Class I molecules and are important for recognising pathogens within host cells. They release cytotoxic granules, also found in NK cells. • NKT cells can produce large amounts of cytokines aiding in immune response. • γδ T cells recognise infected and transformed cells by cytokine and chemokine production.

1.2 The Complement System

1.2.1 Complement in Immunity

The complement system is an important protease cascade involved in inflammation. It is triggered in response to infection and trauma, and can also play a role in apoptosis (Nauta et al., 2003). Complement activation was first seen as a product of the antibody response, however it has since been found to activate during early infection in the presence of pathogens before antibodies are produced, indicating that it may have evolved as part of the innate immune system (Ricklin et al., 2010). The complement cascade (Figure 1.1) is comprised of many plasma proteins (zymogens) that can be activated through sequential proteolytic cleavage leading to formation of specific complexes, resulting in opsonisation of pathogens and recruitment of inflammatory cells. The zymogens are generally produced in the liver, and can be found throughout the body in tissues and fluids (Qin and Gao, 2006). At sites of infection these zymogens are activated via a series of three pathways: the classical pathway (CP), the mannose-binding lectin pathway (LP) and the alternative pathway (AP). As the complement cascade begins with activation of a zymogen, this leads to cleavage of a substrate, often another zymogen. Each zymogen that is activated leads to amplification of the cascade, resulting in a rapid generation of complement activation (Chakraborty et al., 2018). Most complement components are denoted as “C” followed by a number e.g. C1. All three pathways lead to a common, terminal pathway which involves the cleavage of the central C3, leading to opsonisation of pathogens by C3b and the release of anaphylatoxin C3a. Ultimately, the membrane attack complex (MAC), a large complex of complement proteins, is formed which comes together to form a cytolytic pore in the pathogen (or infected or malignant host cell) membrane (Elvington et al., 2016). MAC integration into cell membranes causes lysis, destroying the pathogen, however unregulated MAC assembly can be detrimental to the host (Serna et al., 2016). There are many regulatory processes

involved to prevent uncontrolled complement activation, and host targeted complement activation both of which would be detrimental if allowed to occur. These processes involve specific proteins which are present at higher concentrations than the zymogens, and some are present on the surface of host cells (Wijeyewickrema et al., 2016).

Complement proteins can sense danger signals and transmit messages through opsonins and anaphylatoxins, products of zymogen cleavages. Many components of the complement system can recognise PAMPs: including mannose-binding lectin (MBL) and C3b, with some evidence showing that the C1q subcomponent of the C1 complex can also directly bind to PAMPs, thus leading to potential activation of all three pathways (Degn and Thiel, 2013).

Complement activation culminates in the destruction of pathogens, through MAC formation and lysis, or through phagocytosis by immune cells. Complement products such as anaphylatoxin C5a can initiate the release of more complement proteins into the local environment from immune cells such as neutrophils, leading to heightened complement activation. It is important that this is tightly regulated, by fluid phase and surface-bound proteins, to prevent complement activation on host tissues. Dysregulated complement activation can lead to a plethora of issues, including autoimmune disorders, with the kidneys being highly vulnerable (Liszewski et al., 2017).

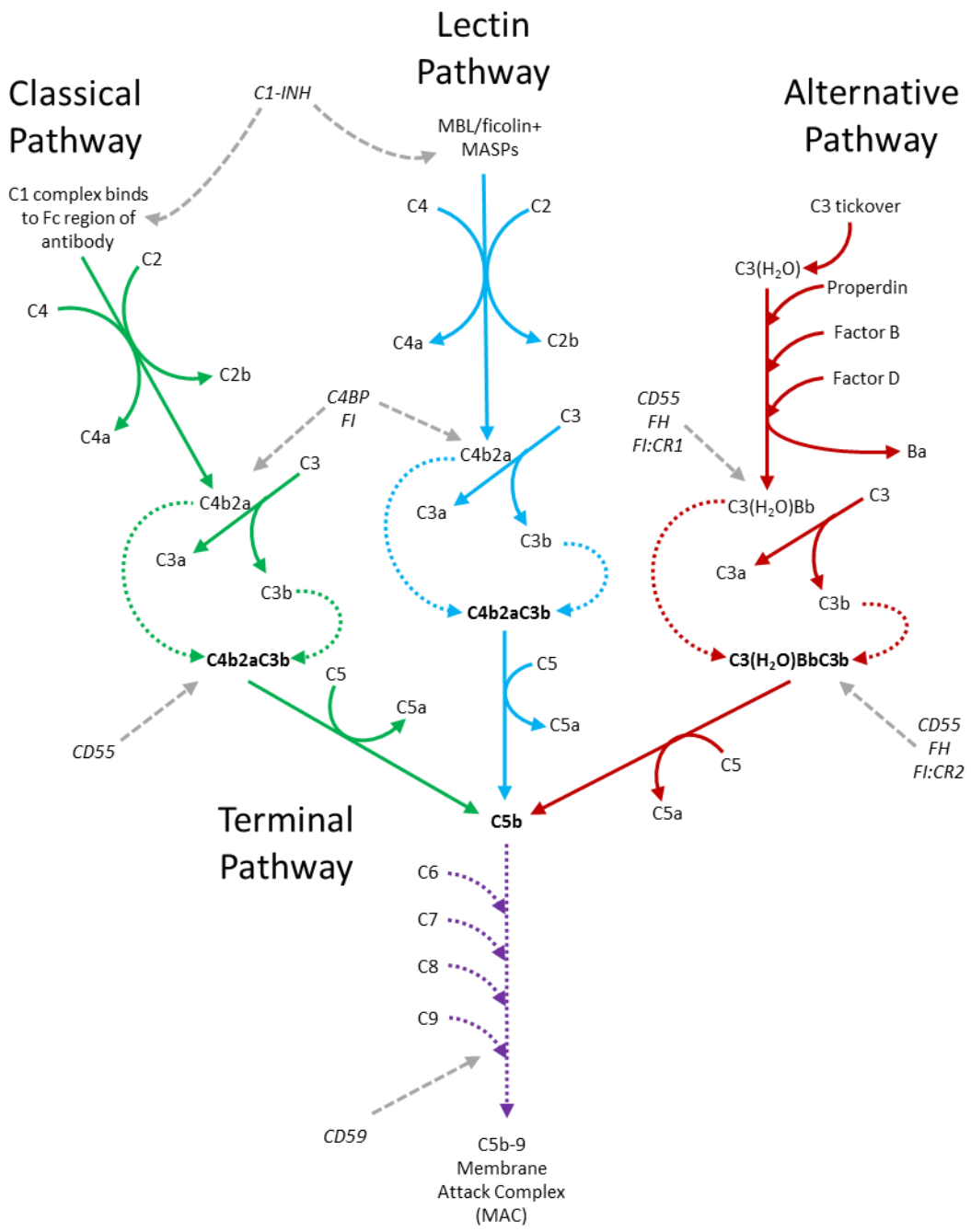


Figure 1.1 The complement cascade.

The classical pathway (green), lectin pathway (blue) and alternative pathway (red) all converge at the terminal pathway (purple) where C5 is cleaved to C5a and C5b, and C5b can recruit the terminal complement proteins to form the membrane attack complex. Image adapted from (Van Roey et al., 2017).

1.2.2 Classical Pathway

Classical pathway activation involves the binding of IgM or IgG antibodies to antigens on the pathogen surface (Diebolder et al., 2014). The first CP component is C1, a complex of 3 subunits: C1q, C1r and C1s (Figure 1.2). The C1q subunit is responsible for binding to the antibodies that are already bound to the antigens (Gaboriaud et al., 2004). IgM is a large complex of antibodies and binding sites for C1q are exposed after antigen binding. Successful activation of CP by IgG requires many IgG molecules to bind in close proximity for sufficient C1q interactions (Basiglio et al., 2010). Once the globular domains of C1q have bound to the fragment crystallisable (Fc) regions of the antibody (Poon and Schumaker, 1991), a conformational change occurs moving the “8” shaped form of C1r/C1s (Figure 1.2) into an “S” shaped form, allowing C1r to cleave C1s, beginning the sequential activation cascade (Arlaud et al., 2002). C1s can then cleave C4 and C2 into small fragments C4a and C2b which may have some pro-inflammatory properties, and larger fragments C4b and C2a which interact to form the enzymatic complex C4b2a. C4b2a is the CP C3 convertase which cleaves C3, and leads to the terminal pathway and MAC formation (Mortensen et al., 2016). C1q can also be activated during apoptosis by acute phase proteins (APPs) (Nauta et al., 2003).

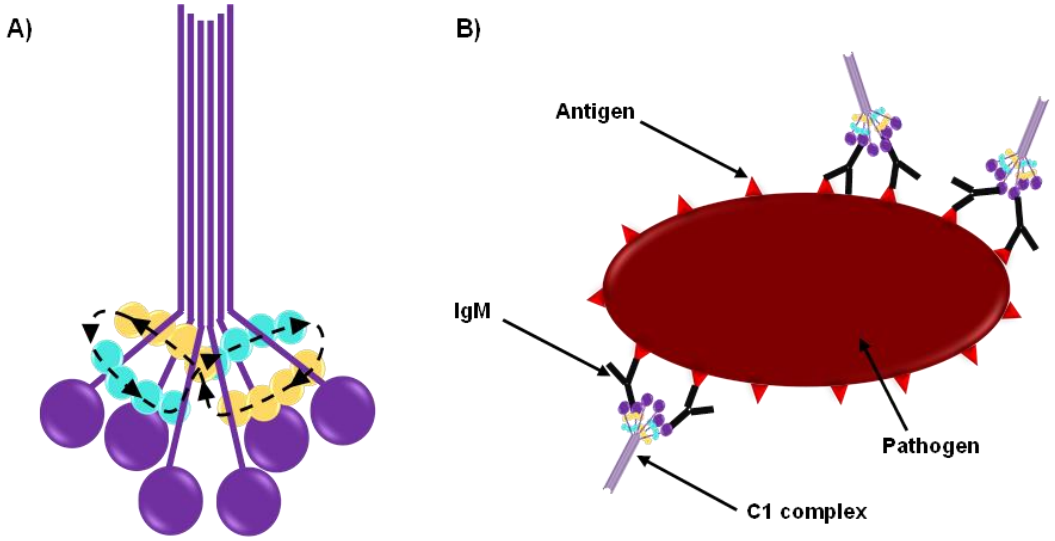


Figure 1.2 Initiation of CP by the binding of the C1 complex to antibodies at the pathogen surface.

A) Schematic diagram of the C1 complex adapted from (Basiglio et al., 2010). Showing the three subunits; C1q in purple, C1r in blue and C1s in yellow. Dashed line with arrows represents the 8 shaped form of C1r and C1s. B) A diagram to show complement fixation. Fab regions of antibodies binding to the antigens at the pathogen surface, and the C1 complex binding to the Fc region of the antibodies.

1.2.3 Mannose-Binding Lectin Pathway

Lectin pathway activation leads to a similar cascade to CP activation. They both initiate the formation of the C3 convertase complex, C4b2a (Sikora et al., 2018). The LP requires pattern recognition molecules, MBL and ficolins, to recognise polysaccharides associated with the surfaces of pathogens (Kjaer et al., 2013). The structure of MBL is similar to C1q (Vorup-Jensen et al., 2000), and binding of MBL or ficolins to the carbohydrate ligands on pathogen surfaces results in activation of a zymogen that is in complex with MBL: MBL-associated serine proteases (MASPs): MASP-1, MASP-2 and MASP-3 (Sekine et al., 2013). All MASPs cleave C4 and C2 to generate the C3 convertase. MBL levels in plasma are extremely varied between individuals, and in some cases, it may not be detectable, however this is not always detrimental (Ishii et al., 2011).

1.2.4 Alternative Pathway

Initiation of the alternative pathway begins when circulating C3 undergoes spontaneous hydrolysis at an internal thioester bond, creating a C3b like molecule denoted as C3(H_2O) (Turner and Moake, 2013). In the presence of a pathogen, C3(H_2O) will bind, in a complex with properdin (Factor P, FP) and factor B (FB) in a Mg^{2+} dependent manner (Bettoni et al., 2016). Serine protease factor D (FD) will cleave FB, creating the initial C3 convertase of the AP, C3bBb (Yuan et al., 2017b). A small peptide, Ba, is released which is thought to play a role in recruitment of immune cells. The initial C3 convertase can then cleave further C3, leading to C3b binding to C3bBb forming the C5 convertase C3bBbC3b, this can also act as an amplification loop of the CP and LP. The C5 convertase can then initiate the terminal pathway, cleaving C5 and inducing the terminal pathway (Thurman and Holers, 2006). The AP is the only pathway of complement that involves a positive regulator. A component of the initial C3 convertase, FP, acts as a cofactor for FB, increasing binding of FB to C3b (Lesher et al., 2013). The C3 and C5 convertases of the AP are stabilised by FP and there is some evidence that FP may also act as a pattern recognition molecule, initiating complement activating at the pathogen surface (Spitzer et al., 2007).

1.2.5 C3

Complement component C3 is vital in all three activation pathways. It keeps the cascade alert through tick-over, is a convergence point for the three pathways and it amplifies the response through downstream mechanisms. It is not only important for immune responses, it also plays roles in tissue regeneration, debris clearance and tumour homeostasis (Ricklin et al., 2016b).

Native C3 is inactive and initiation of CP and LP through pattern recognition molecules and AP activation via tick-over of C3(H_2O) leads to the formation of initial C3 convertases which cleave C3 into C3a and C3b. C3b/C3(H_2O) can directly form

new convertases through interactions with FB, FP and FD, fuelling an amplification loop (Lachmann, 2009). Target surfaces are rapidly opsonised through covalent interactions with C3b, and C3a levels increase, recruiting immune cells to the site of inflammation. Increasing levels of C3b lead to formation of C5 convertases, ultimately driving the terminal pathway and MAC formation (Gadjeva et al., 1998).

C3 is comprised of an α- and a β-chain, held together via a disulphide bond. C3 is comprised of eight macroglobulin (MG) domains and a thioester-containing domain (TED) inserted into the CUB domain which is placed between MG7 and MG8. The C-terminal (C345C) forms a knob-like protrusion. C3b can be degraded into important fragments C3f, iC3b, C3dg and C3c (Figure 1.3), which interact with receptors that mediate phagocytosis and immune adhesion and stimulate adaptive immunity (Nishida et al., 2006). iC3b is formed when C3b is cleaved by complement factor (F)I with factor (F)H as a cofactor (Nilsson et al., 2011), releasing the C3f fragment. iC3b does not share the same activity as C3b, it does not interact with convertases or CR1, but gains reactivity for receptors such as CR2, CR3 and CR4 mediating B cell responses and phagocytosis (Ross et al., 1983).

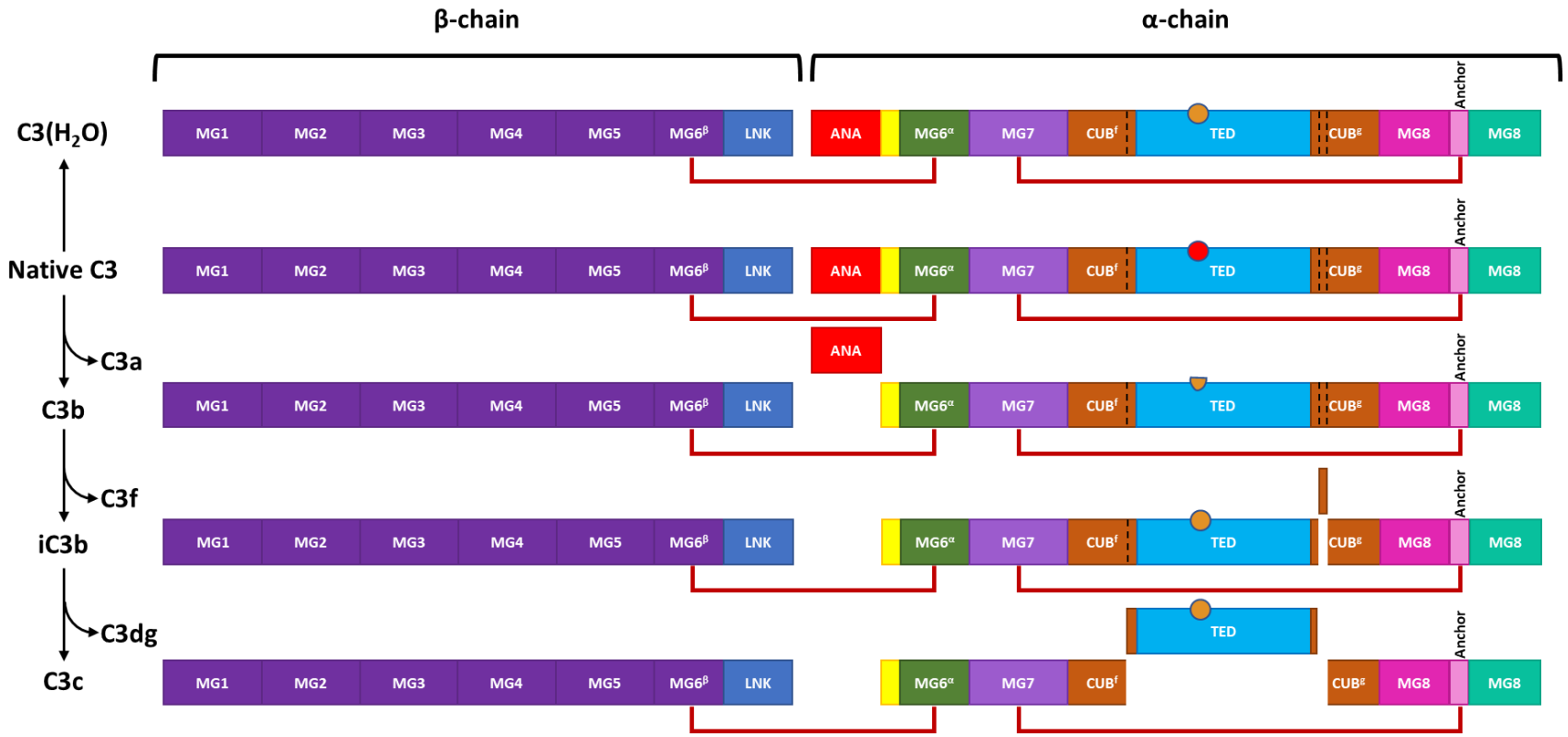


Figure 1.3 Schematic representation of the structure of complement component C3, and the changes it undergoes during activation and regulation.

Figure adapted from (Nishida et al., 2006).

1.2.6 Properdin

Pillemer and colleagues first discovered FP in 1954 (Pillemer et al., 1954), but his findings of FP-dependent complement activation were controversial. As the field of complement became more popular, his theories of FP being able to initiate complement were debated FP being described as a positive regulator of the AP C3 convertase. The literature also suggests that FP may also act as a pattern recognition molecule, and can directly activate complement on surfaces, however this has recently been disputed (Agarwal et al., 2010, Harboe et al., 2017). Properdin is a soluble serum glycoprotein which is formed by the cyclic polymerisation of identical 53 kilodalton (kDa) monomers. It is produced in neutrophil granules and endothelial cells and has a theoretical isoelectric point of more than 9, suggesting that at pH 7, the protein would be highly positively charged and more likely to bind to negatively charged surfaces, potentially increasing the stability of the C3 convertase complex. Properdin is difficult to work with in purified systems, as it aggregates extremely easily. These non-physiological aggregates have been known to activate complement (Cortes et al., 2013). It can be released locally at sites of inflammation and circulates freely at a concentration range of 4-25 µg/mL. Properdin exists physiologically as a dimer, trimer or tetramer in a 26:54:20 ratio respectively, formed by a head-to-tail association (Figure 1.4). Properdin is composed of seven non-identical thrombospondin type-1 repeats (TSR) numbered TSR0-TSR6 (Blatt et al., 2016), each encoded by a separate exon with an elongated structure and large exposed surface area, and each TSR has a different function (Adams and Tucker, 2000). TSR2 and TSR3 do not appear to be necessary for oligomerisation or C3 convertase stabilisation (Schwaeble and Reid, 1999). TSR4 is required for stabilising the C3 convertase. TSR5 is required for binding to C3b and to sulfatides (Perdikoulis et al., 2001). TSR6 is necessary for polymerisation of FP monomers, as is TSR0 (Sun et al., 2004).

The role of FP in complement activation has been controversial, with some evidence that FP may be able to directly activate complement, initiating the formation of the initial C3 convertase on bacterial surfaces. One study observed that FP-treated *Neisseria gonorrheae* directly recruited C3b and the C3 convertase assembled as a result, likely via interacting with lipo-oligosaccharides (LOS) on the surface of the bacteria (Hourcade, 2006). Supporting this theory, a study determined that complement activation could not be induced in FP-deficient sera after treatment with lipopolysaccharides (LPS) and LOS. However, in FP deficient mice LOS could activate complement independently of FP, whereas LPS could not (Kimura et al., 2008).

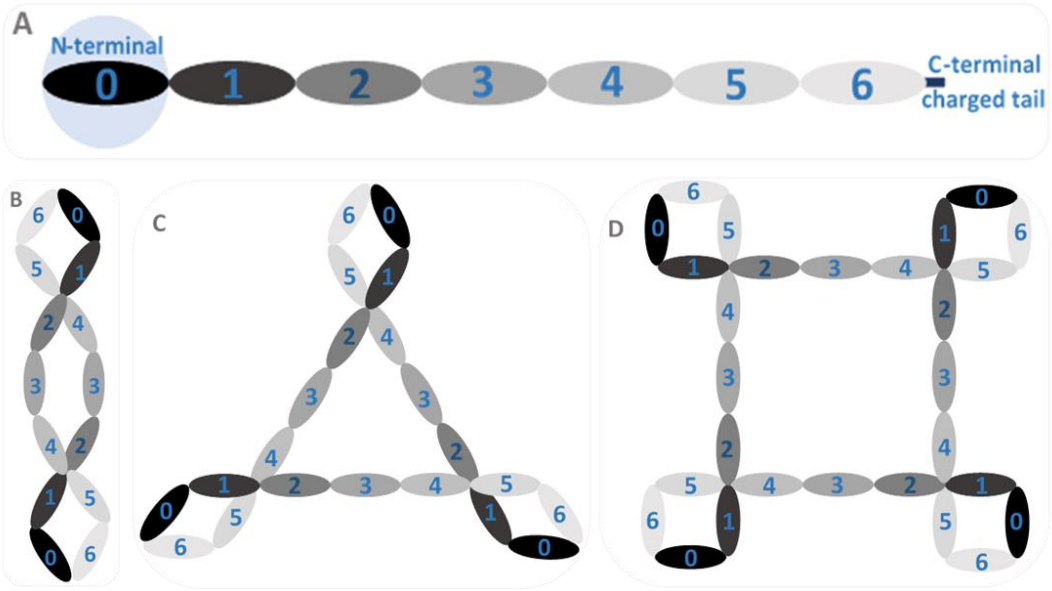


Figure 1.4 Schematic representation of FP structures.

A) Monomeric FP with seven TSR domains, not found in plasma in this state. B) Dimeric FP. C) Trimeric FP. D) Tetrameric FP. The bigger the complex of FP, the higher the activity. D demonstrates ten times more activity than B (Schwaeble and Reid, 1999). Figure adapted from (Blatt et al., 2016).

1.2.7 Alternative Pathway C3 Convertase

The C3 convertase of the AP can have two different forms. Spontaneous hydrolysis of the internal thioester bond of C3 produces a C3b-like molecule known as C3(H2O). This process is also known as “tick-over” and occurs in the blood (Bexborn et al., 2008). This amounts to the formation of the initial fluid phase C3 convertase, which is comprised of C3(H2O), FB(b), and FP. The FB subunit of the complex is most often inhibited by FH on host cells, however in the presence of foreign negatively charged surfaces, such as those of bacterial cell walls, the complex will bind, and AP will be activated (Ferreira et al., 2010). In Gram-negative bacteria, the presence of LPS within the cell wall gives rise to a highly negatively charged membrane. LPS is a well-known activator of the AP of complement (Joiner et al., 1986) and is used in the Shwartzman reaction (Ohta et al., 1985). The strong negative charge of the LPS allows binding of initial C3 convertase which changes the conformation and blocks further hydrolysis, allowing FB and FP to bind. Subsequently, FD can cleave the serine protease FB to Bb, resulting in the active initial C3 convertase. C3 is the substrate for Bb, and the initial complex can find further C3b molecules after cleavage of C3, which leads to the formation of the main C3 convertase of the AP. Properdin is the positive regulator and cofactor of the initial C3 convertase and is vital for this reaction, extending the half-life of the complex 10-fold (Fearon and Austen, 1975a).

1.2.8 Terminal Pathway

The terminal pathway is initiated when C5 is cleaved to C5a and C5b, via activation of all three pathways. The MAC is generated via C5b recruiting the terminal components C6, C7, C8 and several C9 molecules (Serna et al., 2016). MAC forms an irreversible pore with the cell wall of a pathogen or infected host cell and induces lysis, destroying it. C5b binds to C6 which then recruits C7, anchoring the MAC to the surface of the target pathogen. C8 then joins, penetrating the surface to form the assembly precursor for the pore forming C9 molecules. One C9 molecule binds to

the penetrating C6, which allows the polymerisation of multiple C9 molecules, of which ten or more are needed to form the pore (Bayly-Jones et al., 2017). Several MACs are required for lysis of nucleated cell. Ca^{2+} enters through the pore, inducing rapid loss of mitochondrial membrane potentials, initiating acute cell death (Tegla et al., 2011).

C5 is structurally similar to C3 and C4 and it is mainly expressed in hepatocytes, however it has also been observed that macrophages and alveolar epithelial cells can synthesise C5 (DiScipio et al., 1983). C6, C7, C8α, C8β and C9 are all homologous in that they all contain a segment known as the MAC/perforin (MACPF) domain (Slade et al., 2008). C8 consists of three polypeptides: the α-, β- and γ-chains. The α- and β-chains are structurally similar and are linked by a non-covalent bond, and the γ-chain is linked to the α-chain via a disulphide bond. The insertion of MAC into pathogen membranes has been associated with C8α-MACPF which inserts due to a conformational change after binding to C5b-7 at the surface (Bubeck et al., 2011). Formation of the MAC is sequential. C6 binds to the α-chain of C5b via its Cys-rich C-terminal and undergoes a conformational change which allows it to interact with the lipid bilayer (Hu et al., 1981). C7 can then bind, undergoing a similar conformational change, resulting in a high affinity for the lipid membrane, anchoring the C5b-7 complex (Shin et al., 1977). When this complex forms in the fluid phase, it is rapidly inhibited and cannot interact with the membrane (DiScipio et al., 1988). C8β binds to C5b of the C5b-7 complex, resulting in an amphiphilic transformation in both C8α and C8β and C8α can then penetrate the membrane and C9 can bind. The C5b-9 complex is an extremely stable structure, resistant to sodium dodecyl sulfate (Tegla et al., 2011).

1.2.9 Regulation of Complement

Complement is a useful, versatile surveillance system, helping to detect microbial invasion and eliminate unwanted cellular debris. However, uncontrolled complement

activation can be detrimental and has been implicated in many disorders and diseases (Ricklin et al., 2016a). Complement can also undergo spontaneous and continuous low-level activation, therefore a regulatory system must be in place to avoid uncontrolled inflammatory responses (Turner and Moake, 2013). The regulators can be both soluble and surface bound and can affect complement at different stages of activation. CP regulation can be achieved through two main inhibitors, C1 esterase-inhibitor (C1-INH) and C4b-binding protein (C4BP). These two regulators can also inhibit LP activation. C1-INH irreversibly binds to the initiating proteases of both the CP and the LP; C1r and C1s, and MASP-1 and MASP-2 respectively (Wijeyewickrema et al., 2016). C1-INH is also the main inhibitor of the contact pathway of coagulation (Cugno et al., 2009).

The main negative regulator of AP activation is a 155 kDa serum glycoprotein expressed in the liver, FH (Adinolfi et al., 1981). FH can regulate both fluid and solid phase complement activity by accelerating the decay of the initial C3 convertases (Harrison and Lachmann, 1980), or by acting as a recognition molecule and binding to polyanionic markers on host surfaces, such as glycosaminoglycans (GAGs), to prevent inappropriate complement activation (Pangburn et al., 2008). In the absence of FH, the spontaneous fluid phase AP activation becomes unregulated, leading to host damage and consumption of complement components FB and C3 (Schreiber et al., 1978). FH competes with FB to bind to C3b and C3(H_2O), resulting in dissociation of the C3/5 convertase complex (Farries et al., 1990). FH is also a cofactor for factor (F)I, which cleaves and inactivates C3b, resulting in further decay of fluid phase C3 convertases. FH will bind to markers on host cell surfaces along with C3b deposits, inhibiting further C3b deposition on host cells, but allowing AP activation on cells that do not express host markers (Wu et al., 2009).

FI can help to regulate all three pathways by aiding the decay of the C3 convertases through targeted cleavage of C3b and C4b (Masaki et al., 1992). Membrane bound

receptors, sometimes known as clusters of differentiation (CD) can also act as regulators. Membrane cofactor protein (MCP; CD46), complement receptor 1 (CR1; CD35) and decay accelerating factor (DAF; CD55) act in all three pathways, when they converge at C3 (Murray et al., 2000). C3a and C5a can be regulated by carboxypeptidase-N (Campbell et al., 2002), and protectin (CD59) can inhibit MAC formation (Li and Parks, 2018).

1.2.10 Pathophysiological Mechanisms of Complement in Disease

Complement is involved in a variety of mechanisms involving immune surveillance, opsonisation of invading pathogens, homeostasis and mediation of many types of inflammatory responses. The complement system requires a balancing act and, once disrupted, the effects can be observed downstream by looking at the overall response of the immune system (Ricklin et al., 2010). There are many regulatory pathways in place to prevent complement activation occurring uncontrollably, or to stabilise and enhance certain pathways, however when these mechanisms fail, or a deficiency, dysfunction or mutation is present, complement can be excessively activated or may not be as efficient as it should be (Figure 1.5). There is often a pattern observed; complement proteins recognising danger patterns, over-amplification leading to stimulation of downstream responses, and insufficient regulation by negative regulatory mechanisms. This pattern will often lead to a feedback loop of intense, uncontrolled complement activation. A heightened immune response may occur, resulting in host tissues becoming damaged (Ricklin and Lambris, 2013). Non-self surfaces can lead to excessive complement activation through antibody mediated activation of complement through the CP, such as that seen in transplants and with the use of biomaterials, leading to rejection (Nauser et al., 2017). Some deficiencies of complement components can increase susceptibility to infections, often those caused by encapsulated bacteria. Other deficiencies may have no effect at all (Heitzeneder et al., 2012) (Table 1.2).

Table 1.2 Dysfunction of the complement system and the complications that arise.

Often, dysregulation of the complement systems can go undetected, however in some conditions, there can be serious consequences (Ricklin and Lambris, 2013).

Complication	Complement Components	Symptoms and Characteristics
Atypical Haemolytic Uremic Syndrome (aHUS)	FH, CD46, C3 (polymorphisms) FH autoantibodies	Haemolytic anaemia Thrombocytopenia Renal impairment
Dense Deposit Disease (DDD)	C3, FH	Hypertension C3 accumulation in glomerulus Renal impairment
Paroxysmal Nocturnal Heamoglobinuria (PNH)	CD55, CD59	Intravascular haemolysis Platelet activation Thrombosis
Age-Related Macular Degeneration (AMD)	FH, FI, C3, C2, C9, FB	Photoreceptor cell degeneration Loss of vision Disruption of retinal pigment epithelium
Alzheimer's Disease (AD)	C1q, C3, C5	Cognitive dysfunction Psychiatric symptoms
Systemic Lupus Erythematosus (SLE)	C1q autoantibodies, C2, C4, C3	Arthritis Anaemia Mucous membrane ulcers
Rheumatoid Arthritis (RA)	C3, C4, C5, FB	Fatigue Pain Poor appetite and weight loss
Antiphospholipid Syndrome (APS)	C5b-9, C5, C1q	DVT Hypertension Miscarriage or premature birth
Sepsis	MBL, MASPs, C1q, C3, C4, Properdin, C5b-9, FH, FI, FD, FB,	Purpura Thrombocytopenia Respiratory impairment
Antibody Mediated Transplant Rejection	C1q, C4	Fever Fatigue Nausea

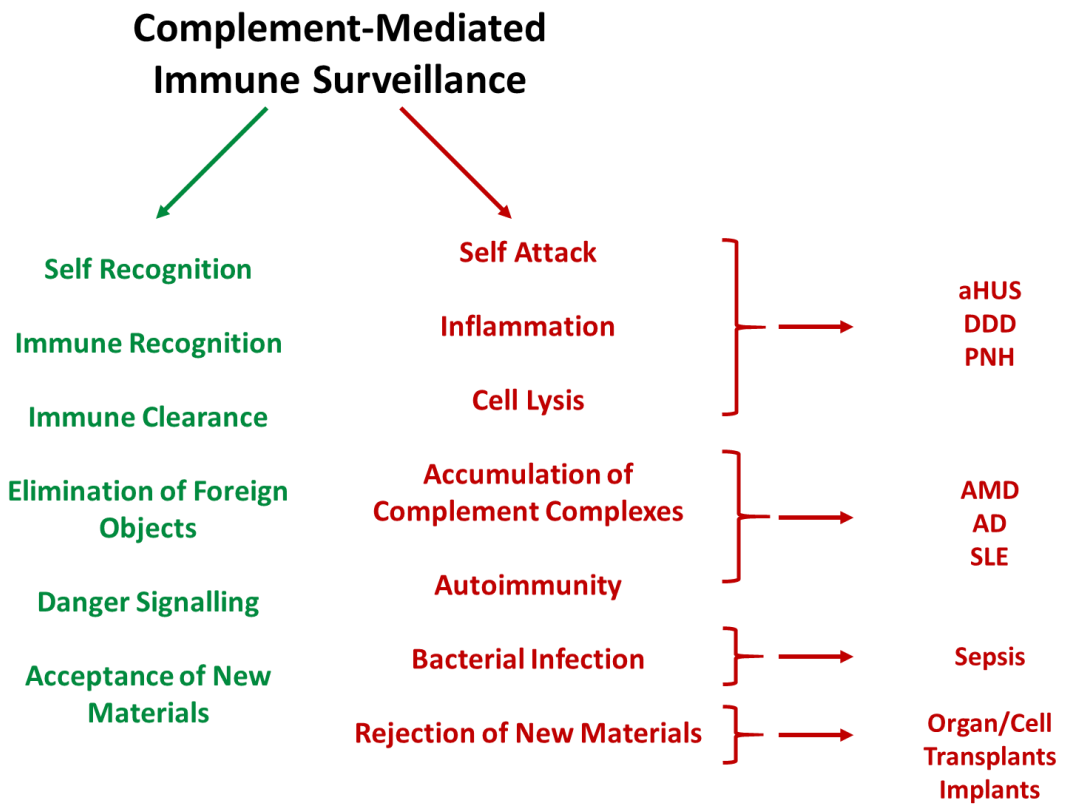


Figure 1.5 Complement is a double-edged sword.

These important processes (green) can become dysregulated and may lead to toxic accumulation of complement complexes, destruction of self-cells or consumption of important regulators (red), leading to serious disease states (red).

1.3 The Haemostatic System

The immune and haemostatic systems are intricately linked, with both processes being involved in cellular and humoral responses with complex catalytic cascades. The complement and blood coagulation cascades are descended from a common ancestry (Krem and Di Cera, 2002) and interactions between the two do exist, however they are yet to be completely defined. These two complex cascades are implicated in thromboinflammatory events; thus, they should not be considered as separate systems (de Bont et al., 2019).

1.3.1 Haemostasis and Thrombosis

The haemostatic system is composed of a variety of highly regulated mechanisms and sequential cascades that have evolved to prevent blood loss when presented with injury and maintain blood in a fluid-state until presented with infection. Activation of the haemostatic system leads to vasoconstriction, platelet activation, the formation of a platelet plug stabilised by a fibrin clot, eventuating in cessation of bleeding at the site of trauma (Bochenek and Schafer, 2019) (Figure 1.6). This is defined by three stages; primary, secondary and tertiary haemostasis. Primary haemostasis involves vasoconstriction, and platelet plug formation induced by aggregation and adhesion processes. Secondary haemostasis comprises of a process known as coagulation, where blood transforms from liquid to a gel-like substance; fibrinogen, a soluble plasma protein, is cleaved into insoluble fibrin forming the scaffold for a blood clot. Coagulation encompasses platelet activation, and two main pathways of thrombin generation created through sequential activation of serine proteases and fibrin clot formation. Tertiary haemostasis is referred to as fibrinolysis, and is the dissolution of the blood clot through degradation of fibrin by the activation of plasminogen (Stassen et al., 2004).

Platelets are activated during vascular injury when they come in to contact with exposed subendothelial proteins. During activation, platelets will change shape, and

begin to express specific receptors allowing them to interact with one another, and with other cells such as neutrophils. They then aggregate to form a plug as the initial haemostatic response (Offermanns, 2006).

The extrinsic pathway is initiated through the activation of factor (F)VII in complex with tissue factor, and the intrinsic pathway (also known as the contact pathway) is initiated by activation of factor (F)XII upon interaction with a surface. The two pathways of activation of coagulation lead to the common pathway which involves activation of factor (F)X, and thrombin generation, necessary for the cleavage of fibrinogen to fibrin, which forms a mesh to hold the clot together (Mann et al., 2003). Activation of coagulation can also lead to initiation of inflammatory pathways including complement and the kallikrein-kinin system (KKS). Dysregulation of coagulation through overwhelming injury or infection or through inefficient regulation can lead to issues such as thrombosis or haemorrhage. Once a clot is formed, it eventually undergoes a degradation process involving a pathway known as fibrinolysis (Bick and Murano, 1994).

Thrombosis is the term used to describe a blood clot, or a thrombus, that has formed inside a blood vessel, creating an obstruction and reducing or preventing the flow of blood. This thrombus can become dislodged and can migrate to other areas of the circulatory system which can have devastating consequences, such as a heart attack or stroke. The pathophysiology of venous and arterial thrombosis differs; venous thrombosis involves a clot made predominantly of fibrin, and arterial thrombosis is primarily a platelet rich clot (Mackman, 2008).

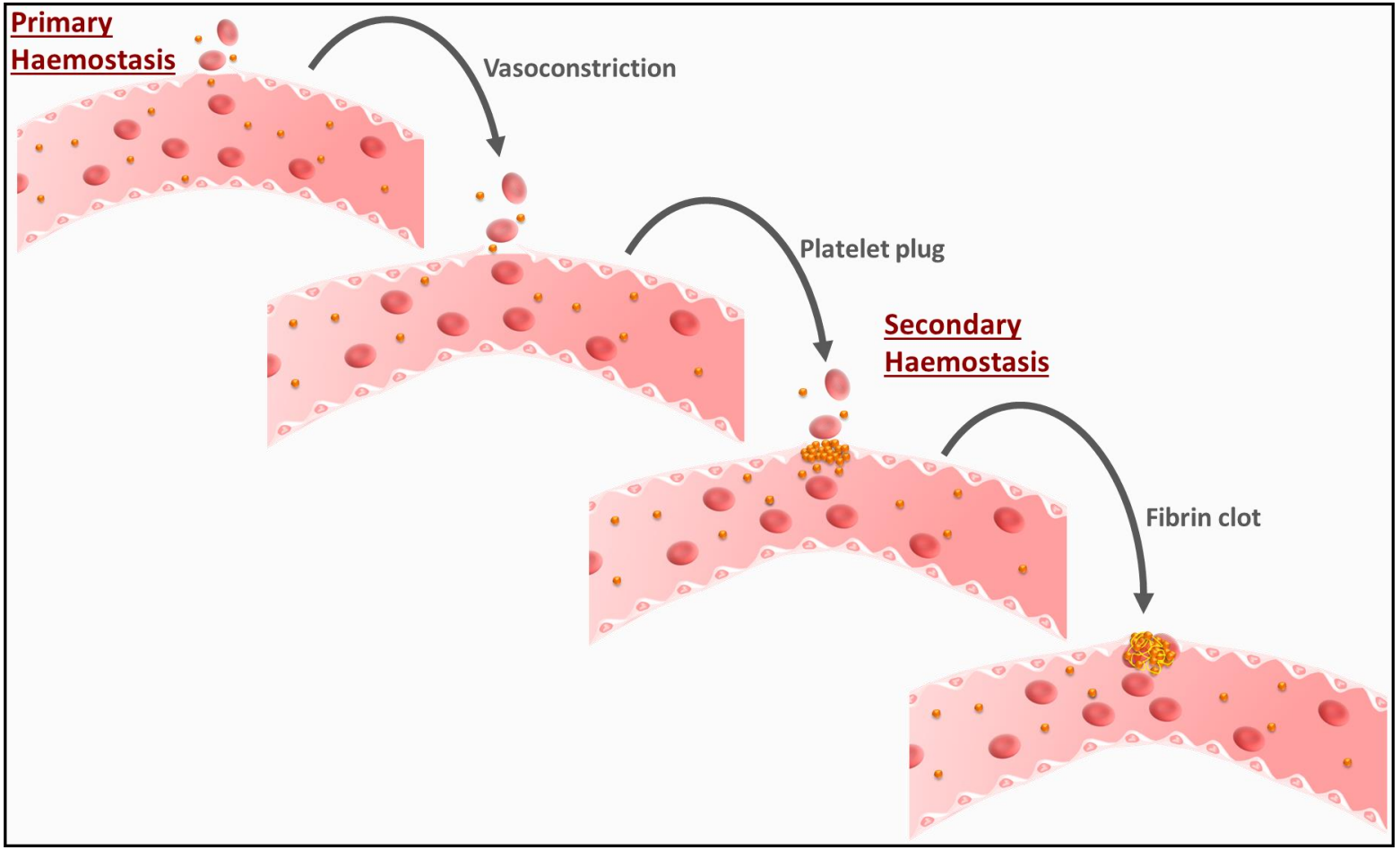


Figure 1.6 Haemostasis prevents blood loss from an injured vessel.

Primary haemostasis involves vasoconstriction and platelet activation leading to the formation of a platelet plug. Secondary haemostasis comprises of activation of the enzymatic coagulation cascade, resulting in thrombin generation and formation of a stable fibrin clot.

1.3.2 Platelets

Thrombopoiesis is the mechanism by which platelets are produced and released by megakaryocytes. Platelets are anucleated cells and are derived from megakaryocytes. Platelet release occurs via two suggested mechanisms. The extension of long platelet precursor extensions called podosomes through the walls of sinusoidal capillaries that run through the bone marrow will lead to the release of proplatelets from the podosomes when under shear stress. These podosomes then mature into platelets (Figure 1.7) (Zuckerfranklin, 1970). It is suggested that megakaryocytes contain specific intracellular territories that are preformed platelets, which are released upon cytoplasmic degradation when influenced by shear stress (Zuckerfranklin and Petursson, 1984). It has also been implied that megakaryocytes can undergo this process to release platelets into the pulmonary vasculature by maturation within lung tissue (Zucker-Franklin and Philipp, 2000).

Platelets adhere to injured vessel walls by several means, under both high and low shear rates. Plasma von Willebrand Factor (vWF) is produced and released by endothelial cells and platelets (Sadler, 1998). vWF contributes to thrombus formation under flow conditions through direct mediation of platelet adhesion to extracellular components, such as collagen exposed upon vascular injury (Ruggeri et al., 1999), and via modulating factor (F)VIII clearance from the plasma through direct binding, thus indirectly aiding thrombin generation (Nogami et al., 2002). Platelets bind to the immobilised vWF through glycoprotein (GP)Ibα and will become tethered at the site of damage. This tethering allows synergistic substrate-receptor interactions i.e. collagen and GPVI, leading to downstream platelet activation signalling (Savage et al., 1998). Platelets become further anchored to the injury site, mediated by the binding of integrins such as αIIbβ3, and their ligands including fibrin(ogen) and fibronectin (Plow et al., 1985). Activated platelets provide a surface for thrombin generation, adding to the formation of a fibrin clot, and they also play an important

role in wound healing and possibly aid in controlling bacterial dissemination (Sjobring et al., 2002).

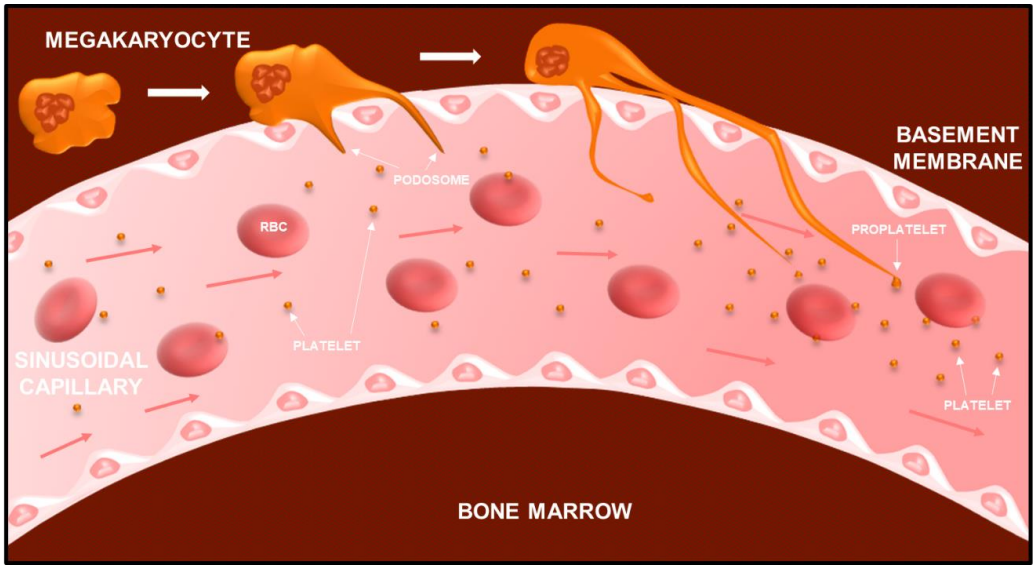


Figure 1.7 Megakaryocytes infiltrate the blood stream to produce platelets.

Megakaryocytes from the bone marrow protrude podosomes into capillaries. The shear stress causes the formation of proplatelets which then get released and mature into platelets.

1.3.3 Classical Coagulation Cascade

1.1.1.0 Common Pathway and Fibrin Formation

The intrinsic and extrinsic activation pathways both lead to a common pathway which begins when FX becomes activated (FXa) by one of two forms of tenase (Figure 1.8). The extrinsic pathway leads to formation of a ternary complex which comprises of a combination of tissue factor (TF), factor (F)VII and Ca^{2+} that assembles on a phospholipid surface (Bom and Bertina, 1990). Alternative substrates of this ternary complex include factor (F)VII (Neuenschwander et al., 1993) and factor (F)IX (Komiyama et al., 1990). The intrinsic pathway converges with the common pathway when the tenase complex of FVIII, activated factor IX (FIXa), phospholipids and Ca^{2+}

activates FX (Autin et al., 2005). Factor Xa can then form a complex with its cofactor, factor (F)V and cleave prothrombin into thrombin, which in turn, in the presence of Ca^{2+} ions, can cleave the soluble fibrinogen into insoluble fibrin (Kane et al., 1980). Thrombin can also cleave FVII (Radcliffe and Nemerson, 1975), cofactors FV and FVIII (Camire and Bos, 2009), and FXIII (Greenberg et al., 1985). Current literature states that there is a thrombin feedback loop, wherein thrombin can also cleave factors from the intrinsic pathway, such as factor (F)XI (Oliver et al., 1999), to amplify coagulation, however this is still debated (Pedicord et al., 2007). Once insoluble fibrin is formed, the monomers aggregate to form a lateral fibrin mesh, which is cross-linked by factor (F)XIIIa (Pisano et al., 1968), aiding in the formation of a stable clot.

1.3.3.1 Fibrinogen and Fibrin Crosslinking

Fibrinogen is a large (340 kDa) hexameric homodimer which plays a vital role in haemostasis and inflammation. It is mainly secreted from the liver in a constitutive manner and this can be upregulated during inflammatory events. Fibrinogen consists of two each of three polypeptide chains: Aα, Bβ and γ (Zhang and Redman, 1992). A variety of physiologically relevant variants exist, including the elongated γ' (arising due to splice variation), which mostly circulates with γA. γA-γ' comprises 8-15% of total fibrinogen in healthy individuals (Kattula et al., 2017). During haemostasis, soluble fibrinogen is proteolytically cleaved to insoluble fibrin by thrombin. Thrombin cleaves fibrinopeptides A and B from the N-termini of Aα- and Bβ-chains respectively, exposing "knobs" which insert into binding pockets in the globular C-terminal domains of the γ- and β- chains, resulting in protofibril formation (Pechik et al., 2006). The protofibrils bind in a half-staggered manner, creating a mesh-like structure which is strengthened via crosslinking by the activated transglutaminase FXIIIa (Lord, 2011). Factor XIII circulates in its inactive form (FXIII-A_2B_2). It is a heterotetrameric zymogen comprised of two A subunits and two B subunits, and it circulates in complex with fibrinogen at a plasma concentration of approximately 70 nM (14-28 µg/mL). An

activation peptide blocks the catalytic site on FXIII-A_2B_2. Thrombin cleaves this activation peptide, and upon binding of Ca^{2+}, the A and B subunits dissociate (a process that is enhanced by the presence of fibrin) resulting in formation of activated FXIII (FXIIIa) (Katona et al., 2014). Factor XIIIa forms a binary complex with fibrin through a thioester bond and catalyses the transfer of an acyl group to the acyl acceptor amine. Factor XIIIa is then released and the fibrin is crosslinked. Factor XIII deficiency manifests as a bleeding diathesis and can be congenital or acquired and can often lead to bleeding within the central nervous system, which can be life-threatening (Ariens et al., 2002).

1.3.3.2 Extrinsic Pathway

The extrinsic pathway is initiated at a site of trauma on the vascular wall where blood becomes exposed to the extrinsic protein, TF. TF is an integral membrane protein, which is usually only expressed on the outer surface of blood vessels. The mechanism of the activation of FVII to its activated form (FVIIa) has long been speculated. Factor VII largely circulates in its zymogen form, but a small amount FVIIa is also present. This low-level of FVIIa may serve as a catalyst in the activation of FVII when in a complex with TF. It has also been noted that low levels of factor (F)IX correlate to lower levels of circulating FVIIa, thus suggesting that FIXa, may be a key activator of the TF pathway by causing low level FVII activation (Wildgoose et al., 1992). TF is a cellular receptor and a cofactor for FVII, when FVII is complexed with TF it becomes automatically activated in a Ca^{2+} dependent manner (Nemerson, 1966). The TF and activated FVII complex (TF-FVIIa) is then able to cleave more FVII, creating a positive amplification loop, forming high amounts of TF-FVIIa. This complex, in conjunction with Ca^{2+} ions and phospholipids, is a ternary extrinsic catalytic complex which activates FX following extrinsic pathway activation, leading to the common pathway (Bom and Bertina, 1990) (Figure 1.8).

1.3.3.3 Intrinsic Pathway

The intrinsic pathway is initiated through exposure of negatively charged surfaces resulting in what is known as contact activation. Prekallikrein (PK) and factor (F)XII become activated in a reciprocal manner. This process is enhanced in the presence of artificial and physiological anionic surfaces (Ivanov et al., 2019), producing activated FXII(a) and kallikrein (PKa). An efficient amplification loop is observed whereby FXIIa generates PKa, and PKa generates FXIIa. Factor XIIa can then cleave and activate FXI, activated FXI (FXIa) can then cleave FIX, resulting in formation of the intrinsic tenase complex. Factor XII is much more efficient in the presence of high-molecular weight kininogen (HK) (Renne et al., 2012). The majority of FXI and PK both circulate in non-active complexes with HK, which leads to Zn^{2+} dependent surface binding, in conjunction with FXIIa, causing activation of PK into PKa, and FXI into FXIa (Mohammed et al., 2018). Kallikrein can then cleave further FXII, or can cleave HK to release the vasoactive peptide, bradykinin (BK) (Renne et al., 2012), and recently FIX has been identified as an alternative substrate for PKa (Visser et al., 2020, Noubouossie et al., 2020). Factor XI has also been shown to cleave FX (Matafonov et al., 2013a), FVIII and FV (Whelihan et al., 2010) independently of FIX. Zn^{2+} is an important cofactor for sufficient contact activation. It is required for surface binding of HK, and for FXII activation. HK requires Zn^{2+} to bind to polyanionic surfaces, which allows the colocalisation of the substrates PK and FXI, with FXIIa (Griffin and Cochrane, 1976). Evidence suggests that a conformational change is induced by the presence of Zn^{2+}, increasing the susceptibility of FXII to gain proteolytic activity (Schousboe, 1993).

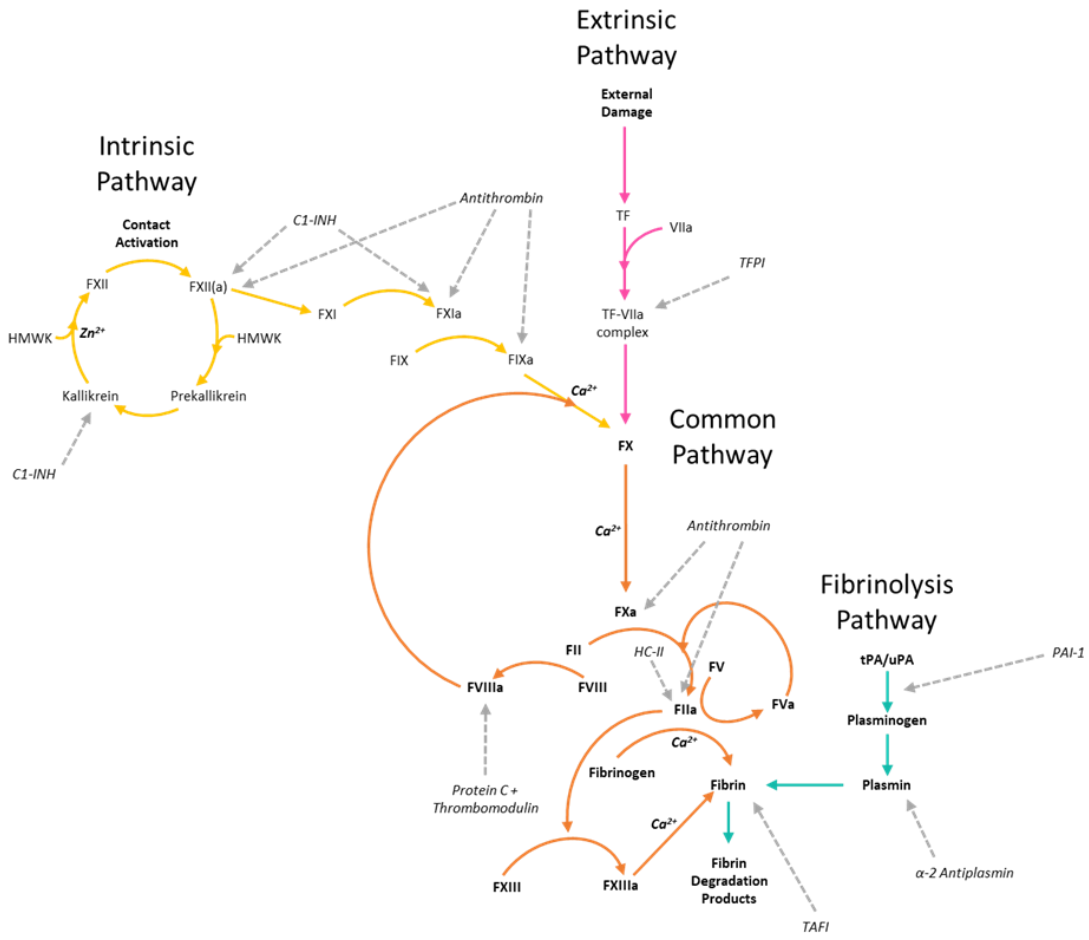


Figure 1.8 The coagulation cascade.

A complex, sequential cascade of enzymatic reactions resulting in the formation of a stable fibrin clot. Inhibitors are indicated using a grey, dashed arrow.

1.3.3.4 Coagulation Factor XII

FXII, also known as Hageman Factor, is an 80 kDa polypeptide. It is comprised of a non-catalytic heavy chain and a trypsin-like light chain (Tans and Rosing, 1987) and is primarily expressed by hepatocytes (Gordon et al., 1990). Factor XII circulates in the plasma at a concentration of around 375 nM (30 µg/mL) (Reddigari et al., 1993). Factor XII is activated upon binding to negatively charged surfaces, and it has been debated whether the activation of FXII should be classed as proteolytic and non-proteolytic. Factor XII gains proteolytic activity in the presence of polyanions,

suggesting that single chain FXII does not need to be cleaved, and that a conformational change is sufficient to induce contact activation of coagulation. Factor XII binds to polyanions via the heavy chain, which exposes the active site on FXII. Single chain FXII can generate PKa from PK creating a surface-dependent amplification loop wherein PKa reciprocally cleaves FXII (Ivanov et al., 2017a). Upon proteolytic cleavage of FXII by PKa, αFXIIa is formed which comprises of a heavy chain and a light chain held together by a single disulfide bond. de Maat & Maas hypothesised that this activation of FXII also leads to the exposure of a FXI binding site, allowing αFXIIa to induce coagulation through FXI cleavage and activation (de Maat and Maas, 2016) and that PKa can then cleave αFXIIa to produce the heavy chain of FXII, and βFXIIa (Revak et al., 1977). βFXIIa is composed of the serine protease domain detached from the heavy chain (Tans and Rosing, 1987) and displays limited anionic binding properties and negligible amidolytic activity towards FXI (Revak et al., 1978).

The heavy chain of FXII is necessary for surface interactions and consists of fibronectin (Fib)-I and -II domains, two endothelial growth factor (EGF)-like domains, a kringle domain and a proline-rich region (Stavrou and Schmaier, 2010). Fib-II comprises of the collagen-binding site, and potentially mediates the binding of FXII to artificial surfaces (Yamada, 1983), with anionic binding sites being mapped to Fib-II (Clarke et al., 1989) and Fib-I (Pixley et al., 1987). Fib-II also facilitates binding to FXI (Citarella et al., 1998). Zn^{2+} binding sites have been identified on Fib-II and potentially on the first EGF-like domain, suggesting a role for Zn^{2+} in anion-binding interactions and FXII activation (Rojkjaer and Schousboe, 1997). It is speculated that the EGF domains may mediate cellular responses in endothelial cells and neutrophils through FXII/receptor interactions, however this is still debated. The kringle domain precedes the proline rich region, and though the functions of these domains are not

yet certain, it appears they play a role in surface binding, thus supporting exposure of the active site (Stavrou and Schmaier, 2010).

Natural activators of FXII include a variety of negatively charged surfaces, including exposed phosphatidylserine (PS) (Yang et al., 2017), polyphosphates (polyP) released from platelets (Mitchell et al., 2016) and misfolded protein aggregates (Maas et al., 2008). Activated platelets expose PS (Schoenwaelder et al., 2009) and release polyP upon activation by collagen, adenosine diphosphate (ADP) and thrombin (Muller et al., 2009). This interaction mediates both venous and arterial thrombosis in murine models (Smith et al., 2012). Heparin is a naturally occurring GAG exclusively released from mast cells upon activation by IgE/antigen interactions. Heparin synthesised and released by mast cells can activate FXII selectively facilitating activation of the KKS leading to BK release with the absence of coagulation (Oschatz et al., 2011). The mechanism behind this remains unclear, but a similar process is observed in the presence of misfolded protein aggregates as seen in Alzheimer's Disease and amyloidosis (Maas et al., 2008).

1.3.3.5 Coagulation Factor XI

FXI is a dimer (160 kDa) consisting of two identical monomers (80 kDa each) connected by a disulfide bond and circulates at a concentration of approximately 30 nM (5 µg/mL). Each monomer is comprised of four apple domains (A1, A2, A3, A4) and a catalytic domain (Papagrigoriou et al., 2006), with 58% sequence homology to plasma PK (Fujikawa et al., 1986). Factor XI circulates as a dimer, via a disulphide bridge linking the A4 domains, with salt bridges contributing to dimer stability (Zucker et al., 2009). A3 and A2 are also involved in dimerization, however the mechanisms behind this are not clear (Cheng et al., 2003).

Factor XI is activated to FXIa by αFXIIa (Colman, 1984) and mediates thrombin generation through two pathways. The main substrate for FXIa is FIX, which cleaves

FX and initiates the common pathway of coagulation (Gailani et al., 2014). Factor XIa also modulates the “thrombin feedback loop” in the presence of negatively charged surfaces (Naito and Fujikawa, 1991b). During the initial phase of coagulation small amounts of thrombin generated through intrinsic pathway activation activate further FXI and this process is greatly enhanced by the presence of negatively charged surfaces, such as PS on activated platelet surfaces and GAGs. The physiological relevance of this has, however, been disputed. Deficiency of FXI predisposes a mild to moderate bleeding diathesis known as Haemophilia C suggesting that FXI is not an essential component of haemostasis but is part of the amplification process of thrombin generation (Emsley et al., 2010). Factor XI autoactivation is a phenomenon observed *in vitro*, yet the physiologic relevance is still being debated. Anion binding site (ABS)1 and ABS2 play an important role in autoactivation, as a negative surface is required (Geng et al., 2013a).

FXI can bind to anions through clusters of basic amino acids, found on A3 (Ho et al., 1998) and the catalytic domain (Yang et al., 2009) via ABS1 and 2 respectively. These sites can bind to polyanions including heparin, polyP and nucleic acids. ABS1 and ABS2 both bind to heparin, a cofactor necessary for the inhibition of FXIa by antithrombin (AT)III (Olson et al., 2004).

HK is a 120 kDa plasma protein that circulates in complex with FXI and PK. In purified assays, HK increases the cleavage of FXI by FXIIa when a surface is present. During autoactivation of FXI, the presence of HK appears to inhibit the reaction, suggesting a regulatory role for HK (Ivanov et al., 2017b). The A2 domain is required for HK binding to FXI (Renne et al., 2002), though A1 and A4 may also contribute to the formation of this complex (Mohammed et al., 2018).

Activation of FXI to FXIa is reported to occur via the action of FXIIa, thrombin, or through autoactivation whereby one FXI(a) subunit cleaves the other (Mohammed et

al., 2018). Factor XIa is used to describe the form of activated FXI with both subunits cleaved. In most circumstances, a species known as ½-FXIa is first formed, where only one of the subunits is cleaved, however ½-FXIa acts in a similar manner to FXIa and is still able to cleave FIX (Smith et al., 2008). Factor XIIa cleaves FXI through two mechanisms: a *trans*-mechanism wherein FXIIa binds to one subunit and cleaves the other (Wu et al., 2008), and by a *cis*-mechanism where FXIIa cleaves the subunit it is bound to (Geng et al., 2013b). Factor XII binds to FXI via two interactions: binding to a region on the A4 domain, and a substrate recognition site on FXI (Baglia et al., 1993).

1.3.3.6 Plasma Kallikrein-Kinin System

As stated above, activation of FXII by polyanions including sulfatides and nucleic acids, can lead to the release of a short-lived vasoactive peptide known as BK, via PKa. HK cleavage by PKa, that is generated by FXIIa, results in the release of BK, from HK. BK interacts with two different subtypes of G-protein coupled receptors (GPCR) expressed on the surfaces of cells in many tissues known as B_1R and B_2R receptors. These interactions lead to specific intracellular Ca^{2+} fluctuations, which influence different signalling mechanisms. B_1R expression can be induced and upregulated as a result of tissue injury and during inflammatory events, in the presence of cytokines and endotoxins, however, B_2R is constitutive (Higashida et al., 1986). BK interaction with B_2R during inflammatory events increases vascular permeability and can lead to endothelial leakage causing oedema. This may be implicated in non-inflammatory oedema mechanisms such as hereditary angioedema. The effects of BK include inflammation, swelling and the sensation of pain (Terzuoli et al., 2018).

1.3.4 Cell-Based Model of Haemostasis

A “cascade” or “waterfall” coagulation model was proposed by two separate groups in 1964 (Davie and Ratnoff, 1964, Macfarlane, 1964), where coagulation factors were

identified to contribute towards the activation of prothrombin. These coagulation factors become known as proenzymes (or zymogens) and procofactors that would, in a defined order, sequentially cleave and result in the activation of a zymogen. The classical model is important in understanding the role of coagulation. This model has also allowed for the development of laboratory tests including activated partial thromboplastin time (aPTT) (Poller, 1980) and prothrombin time (PT) (Loeliger and Vanhalemvisser, 1975), which can determine discrepancies in the intrinsic and extrinsic pathways, respectively. Understanding of the coagulation cascade has enabled the study of specific interactions/kinetics of individual reactions within the cascade, and purification of separate components has led to thorough examination of the individual enzymes and their cofactors. Unidentified mechanisms are still being revealed, suggesting further crosstalk and potential compensatory mechanisms. However, many questions remain unanswered, particularly for *in vivo* coagulation, which led to the development of a newer model, the cell-based model of coagulation. This model defines the intrinsic and extrinsic pathways as interdependent and describes the necessity of cells to initiate, propagate and amplify the classical cascade through negatively charged surfaces and expression of specific receptors, which localise zymogen activation at the cell surface (Hoffman and Monroe, 2001).

1.3.4.1 Initiation

The integral membrane protein, TF, is the primary initiator of coagulation. It is expressed by a variety of cell types under inflammatory conditions, including monocytes and endothelial cells found in the blood and interior vasculature (Witkowski et al., 2016), but also, by some extravascular cells under normal conditions. TF remains localised to the cell membrane and is a cell-surface receptor for FVII(a) (Martin et al., 1995), but may also be expressed in vesicle form separate from the cell membrane itself (Giesen et al., 1999). Although it has been shown that TF is not expressed or stored in circulating platelets, it has been suggested that

during thrombus formation these TF containing vesicles may bind to the platelet surface (Giesen et al., 1999).

Haemostasis during injury is initiated when the vessel wall is ruptured, and TF expressed on the surface of extravascular cells is exposed to blood. This leads to the rapid and tight binding of plasma protein FVII to TF leading to the activation of FVII to FVIIa, and the formation of the extrinsic tenase complex TF/FVIIa (Bom and Bertina, 1990). This complex cleaves FX and FIX, which mediates the activation of small amounts of prothrombin to thrombin, allowing the activation of FV at the cell surface (Monkovic and Tracy, 1990a). However, plasma FXa will be rapidly inhibited by several plasma inhibitors including tissue factor pathway inhibitor (TFPI) and antithrombin III (ATIII). At the cell surface FXa complexes with FVa to form a prothrombinase complex, leading to further thrombin formation ultimately initiating an amplification loop. This thrombin formation plays an important role in the next amplification phase of coagulation (Monroe et al., 1996).

1.3.4.2 Amplification

The formation of the platelet plug plays an important role in the amplification stage. Platelets are exposed to the extravascular cells and matrix components including collagen and matrix metalloproteases. Platelets bind to matrix proteins and will become further activated in a TF rich environment. The TF expressed on the extravascular cells will support further platelet adhesion and enhance platelet activation as more thrombin is generated (Diaz-Ricart et al., 2000), which in turn leads to activation of FV, FVIII and FXI, through thrombin activity at the platelet surface (Monroe et al., 1996). Thrombin is an important platelet activator via the protease-activated receptors (PAR) (Hung et al., 1992). Once activated, α-granules containing FV are secreted from the platelets and FV is released at the platelet surface where it becomes activated by FXa or thrombin (Monkovic and Tracy, 1990b, Schuijt et al., 2013). Thrombin can also bind to other, non-PAR receptors and will

remain active at the platelet surface, activating other procoagulant proteins. Factor VIII in complex with vWF will bind to platelets, and thrombin will cleave FVIII allowing it to be released from the complex (Hultin, 1985). Factor VIIIa will remain at the platelet surface, as will FVa, and the extensive amplification of thrombin generation can begin (Hoffman and Monroe, 2001).

1.3.4.3 Propagation

Once components of the tenase and prothrombinase complexes are bound at the platelet surface, the extensive propagation of thrombin generation is initiated. Several coagulation factors bind with high affinity to the platelet surface, including FIX(a), FX(a) (Yang and Walsh, 2005) and FXI(a) (Yang et al., 2009), all of which contribute to thrombin generation *in vitro*. Factor IXa is not rapidly inhibited and can migrate from its site of activation to bind to FVIIIa, forming the intrinsic tenase complex, FVIIIa/FIXa. Factor X is subsequently cleaved and activated, and this can immediately form a complex with its cofactor, FVa bound at the platelet surface, which can cleave prothrombin. Factor XI can also bind to activated platelets with high affinity, and can be activated by thrombin, leading to direct activation of FIX by FXIa at the platelet surface amplifying the formation of the tenase complexes. These mechanisms all contribute to the surge in thrombin generation required to form a fibrin clot substantial enough for the cessation of bleeding (Hoffman and Monroe, 2001).

1.3.5 Fibrinolysis

Fibrinolysis is the enzymatic process of fibrin degradation, which results in fibrin degradation products (FDPs), necessary to control clot formation and prevent total occlusion of a vessel. Plasminogen is a circulating zymogen, which is activated at the surface of the clot to plasmin by both tissue plasminogen activator (tPA), released from endothelial cells (Hoylaerts et al., 1982), and urokinase plasminogen activator (uPA) (Bugge et al., 1996). tPA cleavage of plasminogen is enhanced 1000-fold when

the cofactor fibrin is present and therefore only occurs in locations of fibrin formation. However, uPA mainly acts in extravascular locations and does not require fibrin (Bugge et al., 1996). There are many inhibitors of fibrinolysis, the most important are the serine protease inhibitors (SERPINs) plasminogen activator inhibitor (PAI)-1 and α2-antiplasmin (A2AP) (Schneider and Nesheim, 2004). SERPINs are a large family of **SER**ine **P**rotease **IN**hibitors, which bind covalently to activated enzymes via the active site, resulting in a permanent conformational change in the enzyme inactivating it. Through the process of fibrinolysis, C-terminal lysine residues will slowly become exposed on fibrin facilitating co-localisation of plasminogen and tPA. As the clot is degraded, more lysines become available, allowing rapid clearance of the fibrin clot (Hudson, 2017). Aside from the SERPINS, fibrinolysis is regulated when thrombin is in complex with thrombomodulin, resulting in the activation of thrombin activated fibrinolysis inhibitor (TAFI), which cleaves fibrin at the C-terminal lysine and arginine residues, preventing binding of plasminogen, blocking fibrinolysis (Bajzar et al., 1995).

1.3.6 Regulation of Coagulation

Coagulation must be tightly controlled to ensure that haemorrhage or thrombosis do not occur. Prothrombotic regulators ensure haemostasis is triggered in response to vascular damage. These regulators include tissue factor, platelet receptors, plasma proteins and even ions including Ca^{2+} and Zn^{2+}. This of course needs to be tightly regulated at each stage by a variety of inhibitory mechanisms to ensure controlled haemostasis and prevention of thrombosis. When these regulatory pathways become dysfunctional, bleeding and clotting problems can occur.

1.3.6.1 Cofactors and Positive Regulation of Coagulation

To reach sufficient thrombin generation to ensure fibrin clot formation, components of the coagulation cascade must interact with one another via a series of complexes and positive feedback loops. Zn^{2+} is an important component of the intrinsic pathway,

necessary for HK surface binding and FXII(a) activity (Vu et al., 2013). Ca^{2+} is required at several stages of the coagulation cascade and is secreted by platelets upon activation. Phospholipids are exposed on activated platelets that provide a surface upon which coagulation complexes form and promote thrombin generation (Mikaelsson, 1991). Ca^{2+} is required for several stages of coagulation to occur in specific interactions including FIX(a), FX(a) and FII(a) (Komiyama et al., 1990). Phospholipids play a large role in haemostasis, acting as a surface for the extrinsic tenase complex, for FXIa activation of FIX and for prothrombinase activity (Morrissey et al., 2010). HK is also a cofactor, playing an important role in the intrinsic pathway. Factor XI and PK both circulate in plasma in complex with HK, which facilitates surface binding and co-localisation of FXII with both substrates (Revak et al., 1977).

1.3.6.2 Inhibitors of Coagulation and Fibrinolysis

Inhibitors act at multiple phases of the coagulation cascade and they can be referred to as the natural anticoagulant system. Most inhibitors are not selective and can often inhibit more than one coagulation protein. An example of these non-specific interactions is AT, the primary inhibitor of thrombin (FIIa). AT can also inhibit FIXa, FXa, FXIa and FXIIa. Heparin released from mast cells, or injected, can enhance the inhibition by AT. TFPI is an extrinsic pathway inhibitor that is expressed by endothelial cells that acts upon the TF-FVIIa complex (Maroney and Mast, 2008) and FXa (Peraramelli et al., 2012).

The protein C pathway works in the propagation phase of coagulation. When thrombin binds to thrombomodulin on the intact endothelium it is able to activate protein C, a serine protease which results in the down-regulation of thrombin and FXa generation via its action on the cleavage of FVa and FVIIIa respectively, rendering them inactive as cofactors for the prothrombinase and tenase complexes (Esmon, 2003). Essential components of the protein C anticoagulant pathway include thrombin, thrombomodulin, protein C, protein S and the endothelial cell protein C

receptor (EPCR) (Dahlback and Villoutreix, 2005). Protein C binding to EPCR augments the activation of protein C by the thrombin-thrombomodulin complex, enhancing the downregulation of coagulation (Mohan Rao et al., 2014). Activated protein C (APC) also binds to the EPCR effecting cellular signalling in turn downregulating cytokine production, and reducing leukocyte-endothelial cell adhesion, also serving as a regulator of inflammation. Deficiency in protein C displays a microthrombosis diathesis, leading to purpura fulminans, and recurring venous thromboembolism (VTE) or DIC (Goldenberg and Manco-Johnson, 2008).

SERPINs play a large role in control of coagulation as it is majorly supported by serine proteases. SERPINs are often influenced by the presence of GAGs such as heparin (Vandeerlin and Tollefsen, 1991), which tend to increase their inhibitory activity through a template mechanism (Sheehan et al., 1994). The negatively charged GAGs bind to a highly positively charged cluster on the SERPIN, leading to a conformational change and substantially accelerates the inhibitory action of the SERPIN on some, not all, serine proteases (Pike et al., 2005). The protease cleaves a peptide bond within the reactive centre loop (RCL) of the SERPIN, leading to covalent binding and deformation of the enzyme as it is trapped. The mechanism is known as a “suicide substrate” mechanism, whereby the SERPIN is also trapped, and the SERPIN-enzyme complex is cleared via various receptors which recognise epitopes that are revealed upon the formation of the covalent bond (Huntington et al., 2000). SERPINs are susceptible to mutations, where they can self-inactivate through insertion of their own RCL into the A β-sheet, or through aggregation and interactions with the RCL of other molecules (Pike et al., 2005). AT is an extremely important SERPIN in anticoagulation. AT inhibits a variety of serine proteases, including FIXa (Kurachi et al., 1976), FXIa (Scott and Colman, 1989) and FXIIa (Stead et al., 1976), PKa and plasmin (Highsmith and Rosenberg, 1974), however more specifically thrombin (Rosenberg and Damus, 1973) and FXa (Buchanan et al., 1985). Heparin

cofactor (HC)-II mRNA rapidly inhibits thrombin when in complex with GAGs such as heparin and dermatan sulfate (Salem and Thompson, 1987) and other polyanions including polyP released from platelets (Church et al., 1988). C1-INH is a major regulator of the intrinsic pathway of coagulation, inhibiting FXIIa, FXIa and PKa, thus inhibiting the KKS and regulating BK formation. Inhibition of other coagulation proteases by C1-INH has been demonstrated, including thrombin, plasmin and tPA. The inhibition of FXIa by C1-INH is greatly increased by GAGs, however this is not the case with FXIIa or PKa (Wuillemin et al., 1996).

PAI-1 and A2AP are crucial regulators of fibrinolysis. Plasmin is inhibited by A2AP much slower when bound to fibrin to allow controlled fibrinolysis, however A2AP is a potent inhibitor of plasmin when not bound to fibrin. PAI-1 inhibits uPA and tPA in solution, however fibrin also modulates these interactions. PAI-2 and PAI-3 and α_2-macroglobulin also inhibit plasmin (Longstaff and Kolev, 2015). Fibrinolysis can also be inhibited through fibrin degradation by TAFIa, which cleaves the c-terminal lysines on fibrin, limiting critical colocalization of plasminogen and tPA by regulating plasminogen binding to fibrin (Silva et al., 2012).

1.3.7 Coagulation Disorders

Abnormal bleeding, and abnormal coagulation can arise from disorders of platelets, the coagulation systems or blood vessels and can be acquired or hereditary. Hereditary disorders include haemophilia, von Willebrand disease (vWD) and other congenital deficiencies caused by clotting factor gene deficiencies or mutations. Acquired disorders include those with a thrombotic phenotype: thrombotic thrombocytopenic purpura and haemolytic anaemia (Triplett, 2000), as well as those that cause bleeding i.e. the development of autoantibodies which act as inhibitors to coagulation factors including FVIII (Yousphi et al., 2019) and FXIII (Souri et al., 2015).

1.4 Crosstalk between the Immune System and Coagulation System

1.4.1 Complement and Coagulation

The complement and coagulation systems are both serine protease cascades that play a central role in thromboinflammation. Both systems interact and are descended from a common ancestor, however the mechanisms between the two are yet to be fully understood. There are similarities in structure and function of proteases from the two systems and it has been demonstrated that coagulation enzymes can initiate complement (Huber-Lang et al., 2006a), and that complement can initiate coagulation and lead to fibrin formation (Gulla et al., 2010). The main inhibitor of the intrinsic pathway and the classical and lectin pathways is C1-INH, as it is the main SERPIN that regulates FXIIa, FXIa, PKa, C1 and MASP activity. Interactions between the two systems were first brought to attention when blood withdrawal without the addition of anticoagulants strongly initiates complement (Mollnes et al., 1988).

1.4.2 Complement and Platelets

During thrombotic events, clot formation and inflammation are intertwined. During injury or infection, platelets play a huge role and when allowed to become unregulated, they can aggravate inflammatory response by release of proinflammatory compounds and recruitment of immune cells. Most research has focussed on the fluid phase interactions of the complement and coagulation systems, however both cascades are activated when in close proximity to cellular surfaces, particularly pathogenic surfaces. It has been demonstrated that the C3a receptor (C3aR) is expressed by platelets, and binding of its corresponding ligand, C3a results in the activation of platelets, defined by the expression of the fibrinogen receptor, GPIIb/IIIa (Sauter et al., 2018).

Many host cells, such as neutrophils (Martin et al., 1997) and endothelial cells (Wu et al., 2016), express complement anaphylatoxin receptors C3aR and C5aR. Both receptors are GPCRs expressed mainly by lymphoid and myeloid cells (Sun and Ye, 2012).

1.4.3 FXII and Innate Immunity

FXII has been shown to modulate pathways outside of contact activation of the intrinsic pathway. *In vitro,* FXIIa can activate complement and can mediate fibrinolysis. Factor XII can interact with pathogens, binding to the surfaces of bacteria, fungi and viruses, and can also become active in the presence of polyP released from bacteria, with *Escherichia coli* and *Salmonella* derived polyP displaying higher potency (Muller et al., 2009). Factor XII can also become active during neutrophil activation, as neutrophils release negatively charged neutrophil extracellular traps (NETs) composed of deoxyribonucleic acid (DNA) and histones, which play a role as an anionic surface for FXII activation (Noubouossie et al., 2017). As previously mentioned, FXII activation leads to the initiation of the inflammatory KKS and BK formation, leading to increased vascular permeability and can also influence leukocyte activity through BK interactions with B_1R (Araujo et al., 2001). Factor XII can influence leukocyte intracellular signalling via uPAR-integrin interactions (Stavrou et al., 2018) and purified FXIIa has been shown to promote neutrophil degranulation (Wachtfogel et al., 1986). Factor XIIa can activate complement via the C1 complex of the classical pathway (Figure 1.9), however whether this can lead to the activation of the complement cascade is still uncertain (Renne et al., 2012).

1.4.4 FXI and Innate Immunity

FXI(a) is best known for its role in coagulation, however there is increasing evidence that it plays a role in the innate immune system. Direct binding of FXI to the neutrophil membrane surface has been demonstrated (Henderson et al., 1994). It has also been observed that the catalytic domain of FXI(a) is important for this interaction with

polymorphonuclear (PMN) leukocytes, and that this is enhanced by the presence of HK and zinc. It was also established that FXIa could partially prevent chemotaxis of PMN leukocytes, initiated by N-formylmethionyl-leucyl-phenylalanine (fMLP) and IL-8, two potent chemoattractants (Itakura et al., 2011). Factor XI has been shown to have a protective role in pneumonia-derived sepsis, where FXI knockout ($^{-/-}$) mice showed increased mortality compared to FXII$^{-/-}$ and wild type (WT) mice, when challenged with two different strains of bacteria, and different severities of infection. It was also determined that this was independent of FXII as the FXII$^{-/-}$ mice were not hypersusceptible to the challenges (Stroo et al., 2017). Stroo et al. also observed that phagocytosis by neutrophils was dramatically reduced, leading to increased severity of infection, as the bacteria could disseminate further. In contrast, inhibition of FXI using a monoclonal antibody, significantly improved survival rates during polymicrobial sepsis induced by cecal ligation and puncture (CLP), which was associated with improved platelet count and reduced coagulopathy, as well as reduced TNF-α and IL-6 plasma concentrations (Tucker et al., 2012).

There is some evidence that FXI may play a role in mediating complement, through cleavage of C3 and C5, generating C3a and C5a, however this is not very potent, and the physiological relevance remains questionable (Amara et al., 2010).

1.4.5 Plasma Kallikrein-Kinin System and Innate Immunity

The KKS can interact with the complement system via activation of zymogens, or through interactions with common receptors. Simultaneous uncontrolled activation of the KKS and complement system can lead to pathophysiological vascular permeability and lead to oedema, thus there has been some characterisation of these interactions. The KKS comprises of three essential proteins: FXII, PK and HK. During an inflammatory event, vascular endothelial cells become procoagulant platforms necessary for the KKS activation and generation of the vasoactive peptide BK (Renne et al., 2012), and they also express surface receptors, including GPCRs gC1qR,

C3aR and C5aR. These three receptors are best known for their interactions with the complement ligands, C1q, C3a and C5a respectively. gC1qR can bind with high affinity to the light chain of HK (Herwald et al., 1996) and can bind to FXII, both in a zinc dependent manner and it has been suggested that this binding is one activation mechanism of the KKS (Joseph et al., 2004). As HK binds to the receptor, PK is pulled down and FXII colocalises, leading to reciprocal activation of PK and FXII and to HK cleavage by PKa, releasing BK. Inactive soluble MAC complexes can bind to endothelial cells and can upregulate the expression of adhesion molecules and TF with procoagulant activity (Tedesco et al., 1997), and can also lead to vascular leakage, which is most often associated with KKS (Bossi et al., 2004).

Kallikrein can independently activate neutrophils during early coagulation leading to the release of neutrophil elastase, neutrophil aggregation and chemotaxis, suggesting an important role for PKa in innate immunity (Wachtfogel et al., 1983). It has also been demonstrated that PKa can cleave complement component C3 in plasma with functional consequences, with C3b being formed and opsonisation of cells observed, and PKa-induced complement activation is regulated by FH (Irmscher et al., 2018) (Figure 1.9).

1.4.6 FXIII and Complement

FXIIIa is a transglutaminase that plays a vital role in the final stages of haemostasis by crosslinking fibrin protofibrils. Factor XIIIa can also crosslink other plasma proteins into the network of the clot, including vWF (Hada et al., 1986), C3 (Richardson et al., 2013) and PAI-2 (Kimura and Aoki, 1986). Not only can FXIII influence complement proteins, MASP-1 from the LP can in fact activate FXIII in plasma (Krarup et al., 2008).

1.4.7 Fibrinolysis and Innate Immunity

Complement activation is regulated in plasma by fibrinolysis components. In plasma and serum, plasmin can cleave C3 and C5, to produce active products (Foley et al., 2016), but it can also degrade C3b into several fragments including iC3b, C3d and C3g. Plasmin can also cleave iC3b further to generate C3c-like, C3d-like and C3dg-like fragments, in a similar way to FI. The cleavage of iC3b appears to be more efficient than the proteolysis of C3 and C3b, suggesting that plasmin may be a physiological regulator of iC3b (Foley et al., 2015).

Plasmin can promote migration of immune cells into tissues through necessary proteolysis of the physiological barrier, the extracellular matrix (ECM), including components such as laminin, thrombospondin and even extravascular fibrin deposits (Das et al., 2010). Plasmin indirectly leads to the degradation of collagen via activation of matrix metalloproteinases (MMPs) (Lijnen, 2001). Plasmin(ogen) can also influence intracellular signalling events in macrophages and neutrophils, which mediates migration and adhesion (Syrovets and Simmet, 2004, Pluskota et al., 2008).

1.4.8 Thrombin and Inflammation

Thrombin is a trypsin-like serine protease, best known for its role in haemostasis, the conversion of fibrinogen to fibrin. Thrombin is generated through cleavage of the zymogen prothrombin by the FVa-FXa prothrombinase complex and by MASP-2. Thrombin generated through the coagulation cascade can activate C3 and C5 of the complement system, suggesting a bypass mechanism, initiating complement leading to C5 generation independent of C3 generation (Amara et al., 2010). The physiological relevance has been debated with one group observing that *in vivo*-generated thrombin does not activate complement in a baboon model of sepsis (Keshari et al., 2017b).

Thrombin can also mediate inflammatory responses via cleavages of PARs, expressed on endothelial cells, leukocytes and platelets. PARs that have been activated then mediate a downstream response through intracellular signalling by binding G-proteins (Coughlin, 2000), leading to the release of serine/threonine kinases that can phosphorylate C3b, ultimately blocking FI inhibition of the C3 convertase (Markiewski et al., 2008) (Figure 1.9).

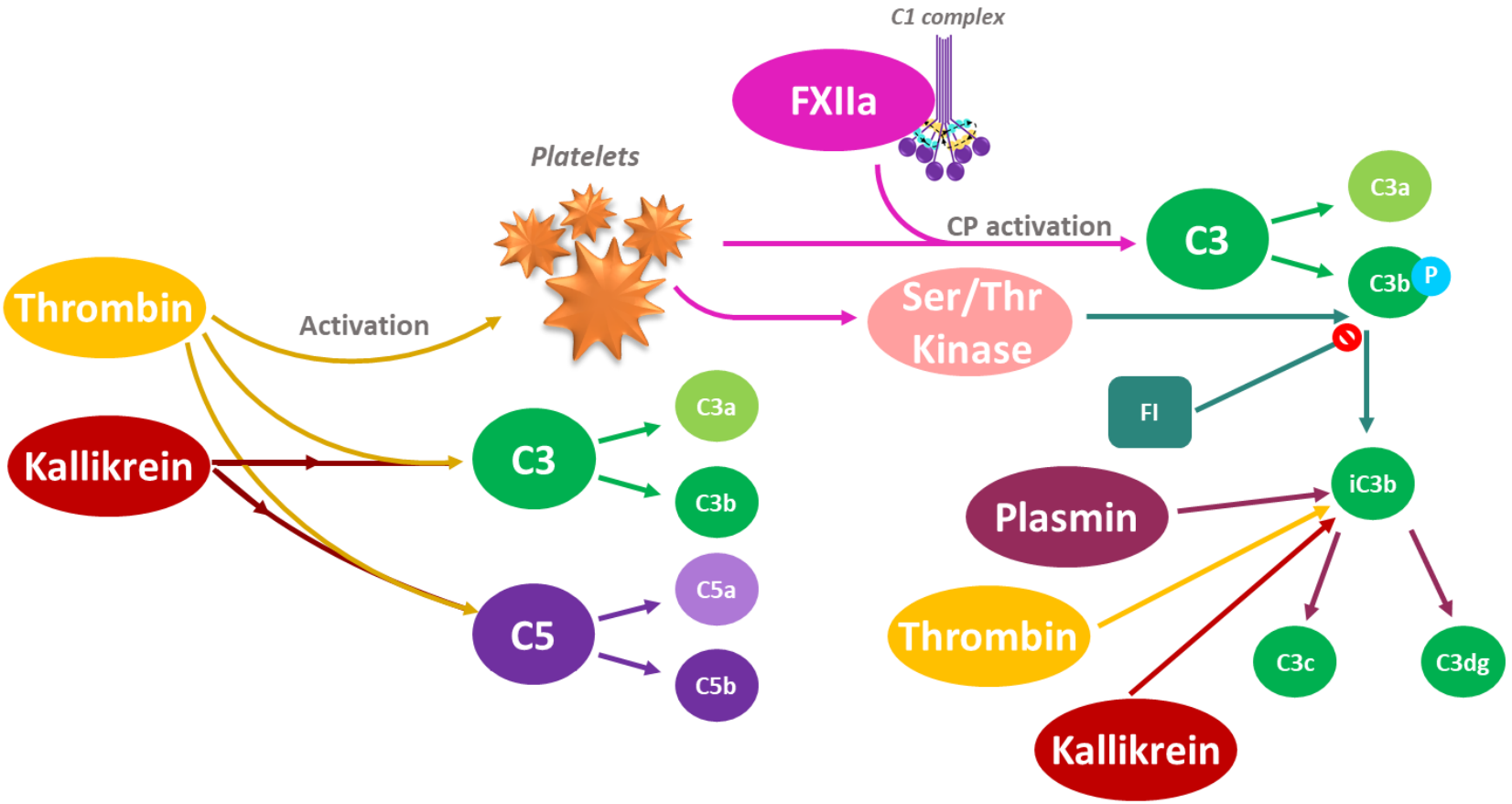


Figure 1.9 Complement-coagulation crosstalk.

Thrombin can induce complement through activation of platelets, leading to phosphorylation (P) of C3b by platelet a derived release serine/threonine (Ser/Ther) kinase, blocking its cleavage by factor I (FI). Thrombin and kallikrein can cleave both C3 and C5, generating functional products C3b and C3a. C1 and FXIIa also interact, and FXIIa can activate C1 to induce complement activation via the classical pathway (CP). Plasmin, thrombin and kallikrein can all degrade iC3b into fragments C3c and C3dg. Figure adapted from (Markiewski et al., 2008).

1.5 Sepsis

1.5.1 What is Sepsis and how is it Diagnosed?

Proliferation of harmful bacteria and release of their toxins within the body, or on the surface of the skin, can lead to infection which is targeted by the immune defence systems and coagulation components. The immune system aims to opsonise and destroy pathogens while the haemostatic systems prevents dissemination of pathogens. These complex defence systems can, however, become uncontrolled and what was a simple infection can often become septic. Sepsis is most commonly caused by bacterial infection and is often fatal. The mechanisms behind sepsis are not yet fully understood, however it appears to involve inappropriate activity and regulation of inflammatory reactions and coagulation leading to thrombotic events and ultimately organ dysfunction. Treatment is dependent on the type of infection and requires urgent treatment, and if not managed promptly, will lead to septic shock. The ever-evolving antimicrobial resistance (AMR) of bacteria and viruses makes it difficult to treat and is a serious factor of mortality (Figueiredo Costa, 2008).

Sepsis can affect anyone, however patients classed as vulnerable include the elderly, hospitalised patients, pregnant women and neonates, as well as people with immune compromising diseases and those undergoing immunosuppressant treatment. Sepsis is emergent and can often go misdiagnosed as the flu or a chest infection. Symptoms of sepsis include fever, shivering, breathing difficulties, increased heart rate, low urine output, purpura, pain and discomfort and a weak pulse. It is important that sepsis is always suspected, and that the symptoms are not underestimated in order to diagnose correctly (Mayr et al., 2014). Some biomarkers are used, such as procalcitonin (greater specificity than other cytokines), to confirm early diagnosis which allows appropriate and time efficient treatment (Vijayan et al., 2017). Once sepsis is diagnosed, it is then possible to identify the causative pathogen, so a tailored treatment plan can be actioned i.e. a wound, must be scouted to control the

dissemination of pathogens and toxins. It is important to maintain fluid levels in sepsis, to ensure appropriate blood pressure to maintain tissue perfusion and organ health and patients must be closely monitored to ensure that the course of treatment is appropriate (Hotchkiss et al., 2016). Sepsis can be severe and has a mortality rate ranging from 30-50% (Vijayan et al., 2017). It may lead to a devastating, and most often fatal, complication known as disseminated intravascular coagulation (DIC) (Iba et al., 2019) (Figure 1.10).

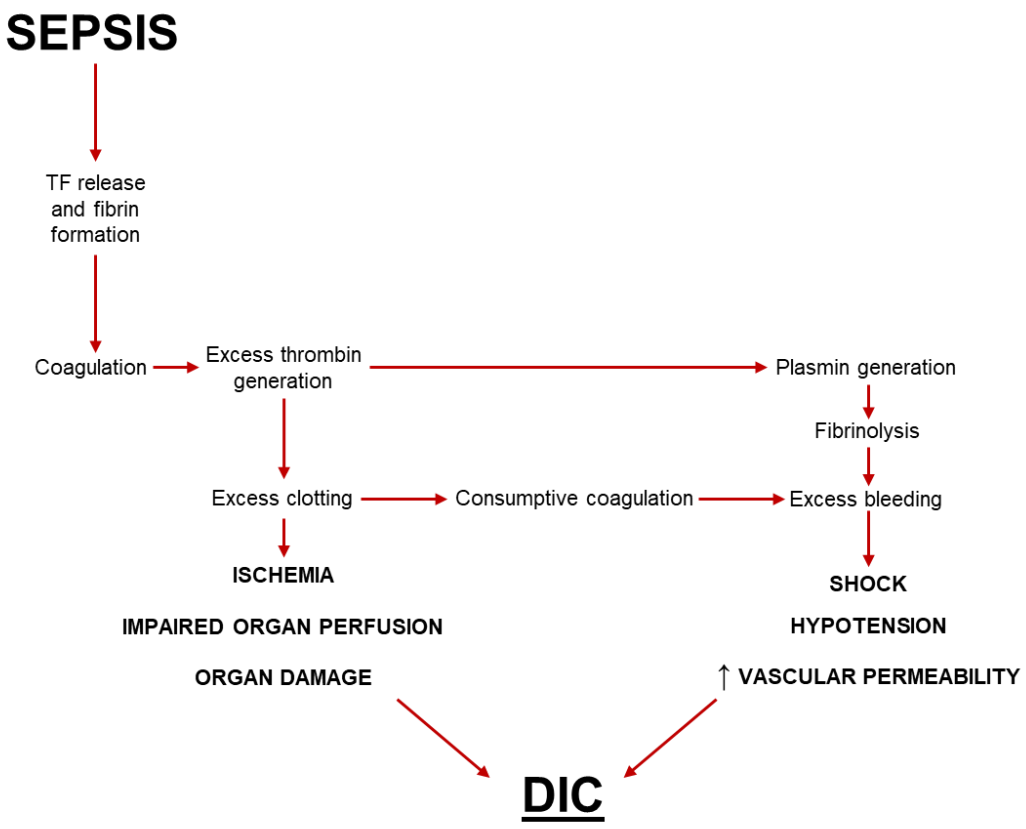


Figure 1.10 DIC arises from sepsis through a hyperactive coagulation cascade.

1.5.2 Disseminated Intravascular Coagulation

Disseminated intravascular coagulation is a complication which arises from several conditions including sepsis, trauma, obstetric complications, cancer, and organ diseases, with infectious diseases being the most common cause (Venugopal, 2014). It is characterised by hyperactivity of the body's immune and coagulation systems, which become dysregulated (Levi and Ten Cate, 1999). In the initial stages of DIC, an overwhelming inflammatory host response can be observed leading to the overexpression of inflammatory mediators, and dysregulation of complement activation (Semeraro et al., 2010). TF is released from inflammatory endothelial cells leading to thrombin generation and further inflammation (Petros et al., 2012). Endothelial cell dysfunction caused by a heightened inflammatory response leads to dysregulation of both coagulation and fibrinolysis through increased levels of PAI-1. This increase in PAI-1 levels can be used as a predictor for DIC mortality (Madoiwa, 2015). Microthrombi are generated in the small blood vessels and are more resistant to fibrinolysis and the clotting factors used to form these thrombi are consumed leading to haemorrhagic events. This vicious cycle is often fatal (Abraham, 2000). DIC is classed as non-overt or overt. In non-overt DIC, some thrombosis occurs, and haemostatic tests will show abnormalities, with increased levels of D-dimer and thrombin-antithrombin (TAT) complexes. Non-overt DIC still shows some control of inflammation and thrombin generation and is classed as compensated. However, if the stimulus remains active, and DIC is exacerbated, coagulation and inflammatory responses become hyperactive and inhibitors are overwhelmed leading to uncompensated, or overt, DIC. Thrombin generation is highly dysregulated, and thrombosis is prevalent, promoting organ dysfunction. Haemostatic factors such as proteins and platelets are consumed and haemorrhage manifests (Lee and Song, 2010).

1.5.2.1 Diagnosing DIC

Scoring systems are in place for the diagnosis of DIC. Laboratory tests are performed, including platelet count and prothrombin time analysis, and measurement of biomarker levels such as D-dimer and thrombin-antithrombin (TAT) complexes. Patients are monitored and several important clinical and pathophysiological events are associated with DIC:

1. Inflammatory response is activated and involves multiple cell types, which causes extensive damage to microvascular endothelium
2. Vasodilation generating leaky endothelial barriers which leads to shock
3. Coagulation and fibrinolysis excessively activated
4. Microthrombosis caused by dysregulated thrombin generation away from the source of injury or infection, leading to multiple organ failure
5. Thrombocytopenia and depletion of coagulation factors manifesting in excessive bleeding and causing haemorrhage

"DIC is an acquired syndrome characterized by the intravascular activation of coagulation with loss of localisation arising from different causes. It can originate from and cause damage to the microvasculature, which is sufficiently severe, and can produce organ dysfunction."

(Taylor et al., 2001)

1.5.2.2 Treatment/Scoring

There are three sets of guidelines for DIC diagnosis: the British Committee in Standards for Haematology (BCSH), International Society on Thrombosis and Haemostasis (ISTH) guidelines and the Japanese Association for Acute Medicine (JAAM) scoring system. These guidelines are all similar, however their recommendations for treatment differ slightly. This has been coordinated by the Scientific and Standardisation Committee (SSC), a subcommittee of the ISTH, into a

generalised report (Taylor et al., 2001). DIC is characterised into several subtypes: bleeding, organ failure, massive bleeding and non-symptomatic depending on the scores of hypercoagulation and hyperfibrinolysis. To efficiently manage DIC, it is vital to treat the underlying disorder. Once this is rectified, coagulopathy will also resolve. However, it is important to be aware that supportive treatment may still be required to ensure that the coagulopathy does not become uncontrollable while the underlying cause is still being treated. DIC consumes coagulation proteins and platelets, therefore it is logical to treat a patient to replenish these. Blood transfusions, platelet concentrates, and fresh frozen plasma therapies can be used, and are often overloaded, to replenish lost coagulation components during the coagulopathy, though guidelines advise that these treatments should only be given when a patient is actively haemorrhaging, and not to go by laboratory test results alone. Heparin can be given if extensive activation of coagulation is evident, and it should not be given to any patient with a bleeding phenotype. In critically ill patients with non-bleeding DIC, prophylactic doses of unfractionated heparin of low molecular weight heparin (LMWH) is recommended to reduce the risk of deep vein thrombosis and pulmonary embolism (Levi et al., 2009). It has been suggested that administration of recombinant thrombomodulin or antithrombin may be considered to treat certain types of DIC (Hayakawa, 2018). Antifibrinolytic agents may be used to treat those with a hyperfibrinolytic phenotype, often seen in patients with severe trauma and leukaemia (Wada et al., 2014).

1.5.3 Complement in Sepsis and DIC

Theories behind complement and its involvement in sepsis have been contradictory. Deficiency of C3 has been shown to increase sepsis-associated mortality rates in animal models (Quezado et al., 1994), yet C5a inhibition revealed a protective mechanism (Ward, 2008). These studies show the importance of the various

functions of complement components that may help to understand the implications of complement activation during different stages of sepsis.

Complement targets pathogens in three ways: opsonisation leading to phagocytosis, lysis of certain pathogens and mediation of downstream inflammatory events (Lambris et al., 2008). C3b is vital for efficient opsonisation, to prevent dissemination of pathogens. When opsonisation is compromised it contributes to an increased susceptibility to infections, including *Staphylococcus pneumoniae* (Walport, 2001). Lysis of a pathogen occurs as a consequence of MAC formation and insertion into the cell wall, forming pores. Due to the increased risk of infection from *Neisseria* bacteria observed in MAC component deficiencies (Ross and Densen, 1984), inhibition of MAC formation could be detrimental in sepsis.

Complement activation is directly involved in the modulation of several downstream inflammatory events via generation of anaphylatoxins C3a and C5a including increased vascular permeability and chemotaxis. Recruitment of immune cells such as monocytes and neutrophils to the site of infection is a critical process and is coordinated by the anaphylatoxins released via complement activation. C3a and C5a also mediate the systemic release of cytokines via complement receptors expressed on cell surfaces (Markiewski and Lambris, 2007).

Toxins on the surface of Gram-negative bacteria, or toxins released from Gram-positive bacteria can activate immune cells and induce inflammatory mechanisms. In some cases, pathogens can evade immune surveillance, which may lead to host tissue damage and unregulated inflammation, as the immune system that has been activated by toxins tries to target the evading pathogen (Cohen, 2002). During the host response to infection, cells are activated and release a host of cytokines including TNF-α and IL-1 which mediate a variety of cellular responses. Cell adhesion

molecules are upregulated, which allow immune cell migration into tissues to search for the source of infection (Dinarello, 1997).

When the inflammatory response becomes uncontrolled and systemic, it is referred to as systemic inflammatory response syndrome (SIRS), and is clinically defined using several criteria: body temperature, heart rate, respiratory rate, arterial blood gases and leukocyte count (Bone et al., 1992). SIRS is the exacerbation of steady immune responses due to the lack of destruction of pathogens, creating a toxic environment, leading to host damage including multiple organ dysfunction (MOD) and immunosuppression due to consumption (Sriskandan and Altmann, 2008) (Figure 1.11).

Targeting inflammatory mediators may not seem logical as it may impair the hosts ability to fight off pathogens (Eichacker et al., 2002). However, if coordinated with antibiotic therapies and timed correctly it could be a useful therapeutic approach.

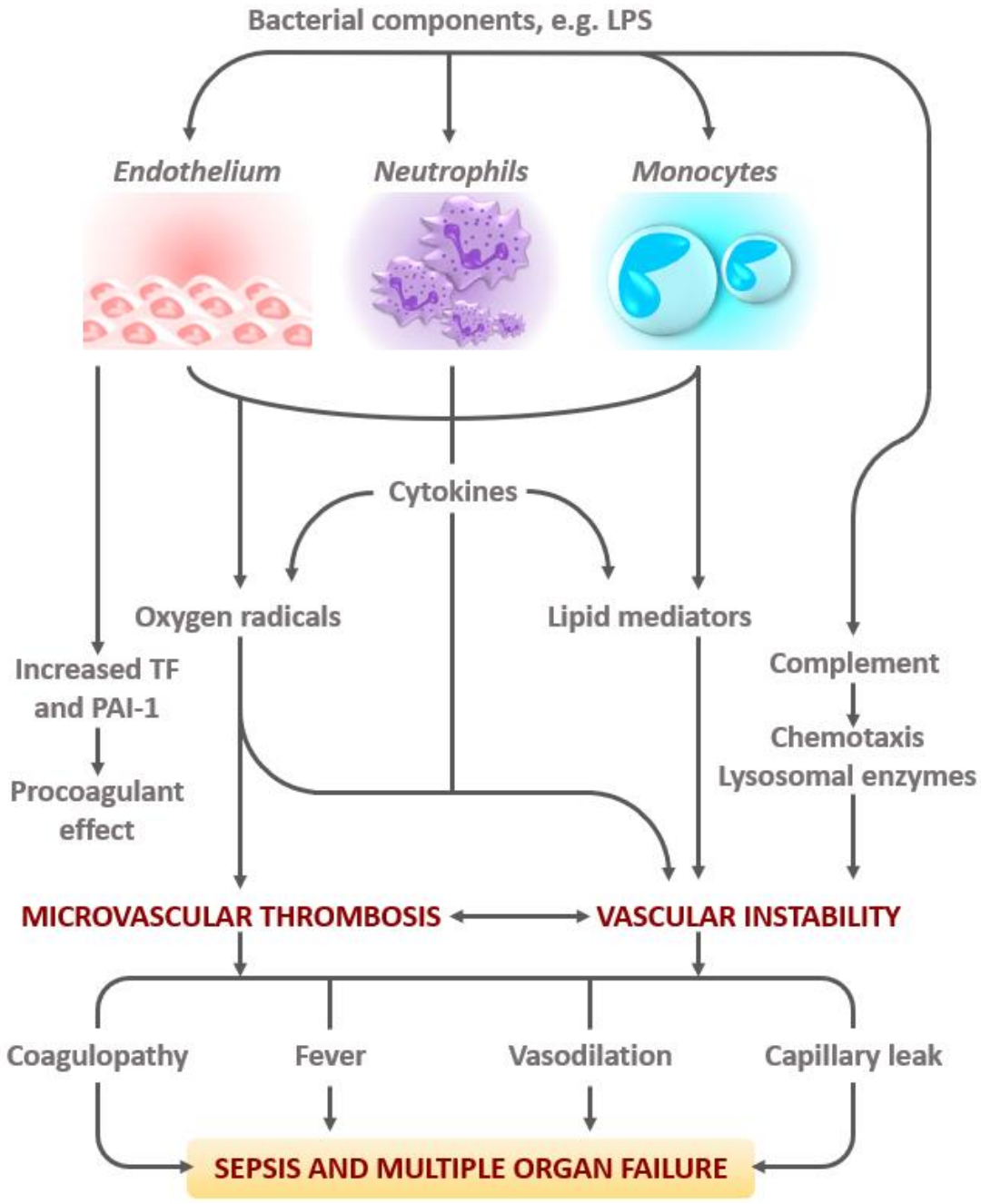


Figure 1.11 Pathogenesis of septic shock.

Bacterial components and toxins such as LPS modulate several inflammatory pathways, manifesting in microvascular thrombosis and impaired vascular tone, ultimately leading to multiple organ failure (Cohen, 2002).

1.6 Purpose of the Study

1.6.1 Aims

Complement and coagulation are closely intertwined, and inhibition of the complement pathway is the primary interest of this PhD to discover a novel therapeutic target for the treatment of DIC, which is predominantly recognised as a coagulation disorder. As it has been shown previously that properdin knockout murine models demonstrate protection from the development of DIC, it seems sensible to begin with the C3 convertase of the alternative pathway of coagulation. Therefore, the first key aim of the study was to validate if it was possible to inhibit the C3 convertase pathway by employing small molecules. The second key aim of this project was to characterise intercommunication between coagulation and complement. As it is known that these two systems can become synergistically dysregulated, there is work outstanding to identify a link to further comprehend these mechanisms. The aim here was to identify and attempt to characterise unknown interactions between the complement and coagulation pathways.

1.6.2 Hypotheses

Crosstalk between complement and coagulation exists, and both can be implicated in thromboinflammatory conditions. Complement plays a vital role in the progression of such disorders, and may be a useful therapeutic target, however the intercommunication between complement and coagulation remains undefined. It has also been highlighted that properdin plays a role in thrombus formation in DIC. From this, several hypotheses have been formed for this thesis:

1. The formation of the C3 convertase complex of the alternative pathway of complement can be inhibited using small molecules.
2. Components of the alternative pathway of complement interact with components of the coagulation cascade.
3. Excessive concentrations of FP modulate clot formation, clot lysis and thrombin generation.

Chapter 2 Materials and Methods

2.1 Materials

Zymogens prothrombin, FIX, FX, FXI and FXII were purchased from Haematologic Technologies Inc., Essex, VT. PK, fibrinogen, plasminogen, FVII, FXIa was obtained from Enzyme Research Laboratories, Swansea. C1-esterase inhibitor and BSA fraction V was obtained from Sigma-Aldrich Corporation (Merck Group) St Louis, MO.

Dextran sulfate sodium salt from *Leuconostoc* sp. (500 kDa) sulfatides from bovine brain, heparin sodium salt was supplied by Sigma-Aldrich Corporation (Merck Group) St Louis, MO. Polyphosphates (long- and short-chain) were gifted by Dr James Morrissey, University of Michigan.

Human complement proteins C3b, FB and FP and Zymosan A were sourced from Complement Technologies Incorporated, Tyler, TX.

Polycarboxylate high capacity (PCH) sensor chips were supplied by Molecular Devices, LLC, San Jose, CA. Amine coupling reagents were obtained from GE Healthcare, Chicago, IL. Slide-a-Lyzer® MINI dialysis device (MWCO 7000 Da), Life Technologies, Grand Island, NY.

Chromogenic substrates S-2288 and S-2765 were obtained from Chromogenix (Instrumentation Laboratory), Bedford, MA. Phospholipids were obtained from Avanti Polar Lipids, Alabaster, AL.

4-12% Bis-Tris Gels, 4x sample buffer, 10x LDS reducing buffer, 20x MES running buffer, PVDF membrane and filter paper were all supplied by Thermo Fisher Life Technologies, Grand Island, NY. Precision Plus Protein™ Dual Colour Standard was obtained from Bio-Rad, Hercules, CA. InstantBlue™ Protein Stain was from Expedeon Ltd., Cambridge, UK.

PTT-automate 5 silica activator (PTT-a) and Technoplastin-His were purchased from Stago, Theale, UK.

C5a ELISA kit was sourced from Abcam (Cambridge, UK). WIESLAB® Complement System Screen was obtained from Euro Diagnostica (Malmö, Sweden).

Rabbit anti-human C9b/C9 HRP-conjugated polyclonal antibody was purchased from BioSS Antibodies, Woburn, MA. Sheep anti-human complement C5 polyclonal antibody was obtained from Thermo Fisher Scientific, Waltham MA.

All buffer reagents (Table 2.1) were supplied by Sigma-Aldrich Corporation (Merck Group) St Louis, MO, unless otherwise stated.

Table 2.1 List of buffers.

Buffer	**Reagents**
HBS	10 mM HEPES, 150 mM NaCl
TBST	50 mM Tris base, 100 mM NaCl, 0.05% (v/v) Tween®20
RBT	10 mM HEPES, 150 mM NaCl, 0.05% (v/v) Tween®20
RBTM	10 mM HEPES, 150 mM NaCl, 2 mM $MgCl_2$, 0.05% (v/v) Tween®20
RBTM++	10 mM HEPES, 150 mM NaCl, 2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 40 µM $ZnCl_2$, 0.05% (v/v) Tween®20
Transfer Buffer	25 mM Tris base, 192 mM glycine, 20% (v/v) methanol
Blocking Buffer	TBST + 5% (w/v) BSA

2.2 Methods

2.2.1 SPR

SPR was performed using the Molecular Devices Pioneer biosensor platform at a temperature of 22°C. Running buffer (RB) contained 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 2 mM $MgCl_2$ and 0.05% (v/v) Tween®20 (RBTM) unless otherwise stated. A COOHV chip was used for protein-protein binding assays and a COOH5 chip was used for small molecule-protein binding assays.

$$R_{MAX} = \frac{M_W \text{ analyte}}{M_W \text{ ligand}} \times R_L \times S_m$$

R_{MAX} = Maximum response
M_W = Molecular weight
R_L = Amount of ligand immobilised
S_m = Stoichiometry

Figure 2.1 R_{MAX} equation to determine levels of immobilisation for SPR.

Sensor chips were installed according to manufacturer's instructions and primed with degassed water. Each chip was preconditioned using 0.1% SDS, 10 mM HCl, 50 mM NaOH and normalised with 100% DMSO to ensure the dips were aligned and the flow channels (FC) were stable. Level of protein immobilisation was first calculated using the Rmax equation (Figure 2.1). Proteins were immobilised to FC1 and FC3 using a standard amine coupling method and using the unmodified FC2 as the reference channel with no protein immobilised. The amine coupling method consisted of treating the FCs with a cocktail of 200 µL 50 mM N-hydroxysuccinimide (NHS) and 100 mM 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) injected at a flow rate of 25 µL/min to prime for immobilisation. Up to 0.05 mg/mL protein was injected

at a flow rate of 5 µL/min and once target amount of protein was immobilised, free amine groups on the FCs were blocked by injecting 100 µL of 1 M ethanolamine at a flow rate of 20 µL/min. Level of immobilised protein was determined as response units (RU), by observing the change in the angle of incidence of the light refracted through the prism versus time (seconds).

The sensor surface matrix was preconditioned for regeneration with 60 µL 1 M, 2 M and 3 M NaCl injected at 30 µL/min, with 3 injects of 60 µL RB in between each concentration at the same flow rate. Loops were then purged to ensure all salt had been washed off the surface. The chip was then primed three times with RB prior to kinetic binding analysis.

Analytes were dialysed into running buffer prior to using in assays to prevent spikes from changes in refractive index. Buffer exchange was performed using 0.5 mL Thermo Scientific™ Slide-A-Lyzer™ MINI Dialysis Devices (Thermo Scientific™, Waltham, MA) at 4°C with stirring, buffer was changed after for 4 hours and samples were dialysed for a further 16 hours. Analyte concentration was determined using a Nanodrop1000 (Thermo Scientific™, Waltham, MA). To first determine if a binding interaction is present, a manual inject was performed. This allows manual concentration scouting to determine the optimal conditions for each assay. This optimisation step also allows the development of the best regeneration cocktail for that assay. OneStep® assays were performed to determine the binding kinetics using Taylor dispersion to create a concentration gradient through the capillary tube prior to entering the FC. This allows a single injection of analyte, rather than a manual titration, producing a single binding curve which does not need to reach steady state to obtain kinetic data, reducing assay time and experimental variability. The calculation of K_D was performed using the Qdat data analysis software package (Pall FortéBio, San Jose, CA), which is specific for Molecular Devices Pioneer data sets. Binding response curves were processed using either the kinetics tab to produce a

simple k_a/k_d model, or the point studies tab, adjusting parameters to improve the fit and discount refractive index changes due to sensitivity to buffer change and flow rates. Refractive index and molecular weight are taken into consideration, and retention and aggregation taken into account depending on the shape and fit of the curve. Response curves from FC2 were subtracted from FC1 and FC3. Buffer blanks were averaged and subtracted from the binding curves. The assay was run in triplicate and the standard error of the mean was calculated using GraphPad Prism v8 software.

2.2.2 Normal Pooled Serum and Normal Pooled Plasma Preparation

Ethical approval has been granted for this study by the University Faculty Research Ethics Committee (HSLTLM12045). After healthy individuals had consented (Appendix 1 & 2), whole blood was collected from a healthy volunteer through venepuncture. A 2 mL discard was included to limit the amount of activated clotting and complement factors in the sample.

Serum preparation involved the collection of blood into a syringe which was immediately dispensed into gold topped serum separator tubes (SST) containing silicone particles to induce clotting. The tube was inverted 8 times and the blood allowed to clot for 45 minutes. The tubes were centrifuged for 15 minutes at 2400 RCF at 21°C (Eppendorf Centrifuge 5810/5810 R, Eppendorf, Hamburg) and the serum was pooled (6 donors), aliquoted, snap-frozen using liquid nitrogen and stored at -80°C for future use. Samples were thawed slowly at 37°C in a water bath, gently swirling until half thawed and kept on ice thereafter.

Preparation of platelet-poor normal pooled plasma (NPP) required blood to be taken into BD Plus plastic vacutainers™ containing a 10% (v/v) of 0.109 M sodium citrate followed by centrifugation at 2000 RCF at 21°C for 5 minutes (Eppendorf Centrifuge 5810/5810 R, Eppendorf, Hamburg). Then ¾ of the plasma was removed, being

careful not to disturb the buffy coat, and was centrifuged at 10,000 RCF at 21°C for 10 minutes (Eppendorf Centrifuge Minispin plus, Eppendorf, Hamburg) to remove any residual cellular materials. NPP was prepared by pooling plasma from 27 donors. NPP was aliquoted, snap-frozen using liquid nitrogen and stored at -80°C for future use. Samples were thawed before use at 37°C until completely thawed and were kept at 21°C thereafter.

2.2.3 ELISAs

2.2.3.1 Development of a Functional ELISA to Detect Complement Activation

I attempted to develop and optimise an ELISA to determine MAC formation for the purpose of characterisation of effect of small molecules on complement activation. The principle of the assay was to activate the complement pathway in serum using zymosan A; followed by performing an ELISA on this plate to detect the solid phase MAC (see section 2.2.3.2 below), or separately, by transferring the complement activated serum to a 96-well plate coated with anti-C5 antibody to capture the fluid phase MAC and detecting with an anti-C9 antibody.

2.2.3.2 MAC ELISA

Wells of a Costar 3590 flat-bottomed polystyrene 96-well plate (Corning Inc, Corning, NY) were coated with 100 µL of the prepared zymosan A and incubated for 16 hours at 20°C. The fluid was removed, and the wells washed with 10 mM phosphate buffer, 2.7 mM KCl, 137 mM NACl, 0.1% (v/v) Tween®20, pH 7.4 (PBST). Wells were blocked with PBST with 1% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) for 1 hour at 37°C, and then washed three times with PBST. Serum was diluted 1 in 2 in 5 µM sodium barbital, 0.5 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1% (w/v) bovine gelatin, pH 7.5 (VBG++) with 0.05% (v/v) Tween®20 and 100 µL added to each well. The plate was incubated for 30 minutes at 37°C. The plate was moved to ice and 10 µL of 220 mM EDTA was added to a final concentration of 20 mM to stop complement

activation. The serum was aspirated from the plate and pooled for use in the MAC sandwich ELISA.

Anti-C5 polyclonal antibody (Abcam, Cambridge, UK) was used to capture the MAC and was diluted to 10 µg/mL in 100 mM Na_2CO_3 pH 9.6. Wells were coated using 100 µL of diluted antibody for 16 hours at 4°C. The plate was washed 3 times using HiPBST and then blocked using 350 µL PBSC pH 7.4 for 1 hour at 37°C. The plate was washed 3 times using HiPBST. Samples from the complement activation assay were titrated (four-fold serial dilution starting at 1/20 from the original sample) into PBSCT and 100 µL was added to each well and incubated for 2.5 hours at room temperature. The plate was washed 3 times with HiPBST. The detection antibody used was an HRP-conjugated anti-C9 antibody (Bioss Antibodies, Boston, MA), and was titrated into PBSCT (two-fold serial dilution starting at 1/500). 100 µL of detection antibody was added into each well and incubated for 1 hour at room temperature with gentle shaking. The supernatant was discarded, and the plate washed 3 times with HiPBST. Wells were incubated with 100 µL 0.4 mg/ml o-Phenylenediamene (OPD) (Sigma-Aldrich, St. Louis, MO) substrate diluted in sodium citrate buffer pH 5 (48.5 mL 0.1 M citric acid, 51.5 mL 0.2 M dibasic sodium phosphate dehydrate, pH 5 then add 40 µL 30% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO) for 30 minutes at room temperature, covered to avoid light, and the reaction was stopped with 75 µL 2.5 M sulfuric acid and readings were taken at 492 nm.

2.2.3.3 Abcam C5a ELISA

The C5a ELISA kit was sourced to determine overall complement activation by measuring C5a levels in serum and plasma (Appendix 3). The C5a standard was prepared immediately before use by briefly centrifuging and adding 400 µL of Assay Diluent and mixing thoroughly, to make a stock of 50 ng/mL. The reconstituted standard was stored thereafter at -20°C. The standard was diluted to 2000 pg/mL, which was then used to create a two-fold serial dilution to a concentration of 62.5

pg/mL. Eight standards were made in total, with the final standard consisting of buffer only. Serum or plasma were used and were diluted 40-400-fold. All materials in this assay were equilibrated to room temperature before use. 100 µL of each standard and sample was added to each well. The plate was covered and incubated for 2.5 hours at 21°C, or overnight at 4°C with gentle shaking. The wells were then aspirated and washed four times with 300 µL 1 X Wash Buffer, ensuring wells were emptied by tapping gently on clean paper towels. 100 µL of previously prepared biotinylated human C5a Detection Antibody was added to each well and the plate was incubated for 1 hour at 21°C with gentle shaking. The wash step was repeated as previous, followed by the addition of 100 µL of 1 X HRP-Streptavidin solution to each well. The plate was incubated at 21°C for 30 minutes, in the dark, with gentle shaking. 50 µL of Stop Solution was added to each well and the plate was immediately read at 450 nm using a plate reader.

2.2.3.4 WIESLAB® Complement System Screen

An ELISA was sourced to assess the complement functional activity of all three pathways of complement (Appendix 4). This was used to determine the effects of the small molecules on complement activation, to ensure only rhe AP was targeted. This ELISA was purchased from Euro Diagnostica (Sweden). Strips of wells were coated with specific activators of each pathway of complement, and complement activation was assessed by detecting solid phase MAC formation. Serum was collected as before and kept in an ice bath before analysis. Negative and positive serum controls were provided. The positive control was reconstituted by adding 200 µL distilled water (dH_2O) and was left to stand for 5 minutes, followed by gentle mixing until fully dissolved. The positive control could be stored for up to 4 hours at 2-8°C and could be frozen at -70°C and thawed once. Small molecules were added to serum at 100 µM, with a final concentration of 1% (v/v) DMSO, including a 1% (v/v) DMSO only control. All serum samples, including controls, were diluted in diluents containing

specific blocking agents to ensure activation of the pathway intended for analysis. CP and MBL pathway tests required serum to be diluted 1 in 101 and the AP test required serum to be diluted 1 in 18. All serum samples can be left for up to 1 hour at 20°C, and the sample for MBL was required to be incubated at 20°C for 15 minutes before analysis. 100 µL of each diluted sample was added to each well and incubated for 1 hour at 37°C with a lid. Wells were aspirated and washed three times with 300 µL of washing solution provided, ensuring no wash solution was left after the final wash step. 100 µL of the conjugate provided was added to each well and incubated at 20°C for 30 minutes. The wells were aspirated and washed three times, as before. 100 µL of the substrate solution provided was added to each well and was incubated at 20°C for 30 minutes. The absorbance was read at 405 nm on a BioTek microplate reader. Each sample was performed in duplicate and the mean was calculated. 100% activation was defined by normalising to the 1% (v/v) DMSO control. Data was analysed using GraphPad Prism v8.

2.2.4 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Samples were prepared to a final volume of 30 µL, to a concentration which would allow 1-2 µg of the protein of interest to be loaded into the wells. Samples contained 7.5 µL pf NuPAGE LDS sample buffer (4x), 3 µL of sample reducing buffer (10x), sample and the volume was made up to 30 µL using HBS. The samples were gently mixed and heated at 70°C for 10 minutes, mixed and quickly centrifuged. 5 µL of Precision Plus Protein™ Dual Colour Standards was loaded for reference of molecular weights, and 20 µL or 15 µL of each sample was loaded into the well of either 10 well or 15 well gels respectively. Gels were run at 200 V for 52 minutes. Once the run was completed, gels were washed in MilliQ water for 15 minutes with gentle shaking followed by staining using InstantBlue™ Protein stain for 15 minutes with gentle shaking, or overnight if better sensitivity was required. Once stained, the gel was washed in MilliQ water for 15 minutes with gentle shaking before imaging

using Syngene G:BOX Chemi and GeneSys software. Bands that required further investigation were sent to the University of Leeds Mass Spectrometry Facility for protein identification.

2.2.5 Western Blot

SDS-PAGE was performed as described in (2.2.3), without staining. The gel was soaked in transfer buffer for 10 minutes. Polyvinylidene difluoride (PVDF) membrane was carefully cut to size and prepared by soaking in methanol for 15 seconds, MilliQ water for 2 minutes and transfer buffer for 2 minutes. Filters and sponges were soaked in transfer buffer until quenched. The gel was trimmed and was carefully placed in the transfer cassette between sponge, filter and the membrane, ensuring it stays wet with transfer buffer and no bubbles were visible. The cassette was clamped and placed in the tank, with a flea to circulate the buffer, an ice block and chilled transfer buffer. The transfer was run at 100 V for 1 hour. The membrane was removed and washed in Tris buffered saline with 0.05% (v/v) Tween®20 (TBST) for 10 minutes on a shaker. The membrane was blocked using 5% (w/v) skimmed milk powder in TBST for 1 hour at 20°C. Primary antibody was diluted accordingly into blocking buffer and incubated for 1 hour at 21°C. The primary antibody was decanted, and the membrane was incubated with the secondary conjugated HRP-antibody diluted in blocking buffer for 1 hour at 21°C. The membrane was washed quickly five times with TBST, and then left to wash for 1 hour at 21°C in TBST with gentle shaking. 2 mL of SuperSignal™ West Pico PLUS Chemiluminescent Substrate was added to the membrane, incubated for 2 minutes, and the blot was imaged using Syngene G:BOX Chemi and GeneSys software.

2.2.5.1 C9 Western Blot

100 µL 1 µg/mL LPS or zymosan A was coated on a plate as previously described. The plate was blocked with 100 µL PBS containing 1% (w/v) BSA for 1 hour at 37°C. The plate was washed three times with PBST. 100 µL serum was added to the wells

and was incubated for 1 hour at 37°C. To stop the reaction, 10 µl ice cold 220 mM EDTA was added to each well to a final concentration of 20 mM and the plate was incubated on ice. The serum was then carefully diluted (avoiding the zymosan A coat) to 1:10 in gel sample buffer and was prepared for non-reducing) SDS-PAGE and Western blot as previously described.

2.2.6 Chromogenic Assays in a Purified System

All chromogenic assays were performed using half-volume clear flat bottom polystyrene 96-well plates (Fisher Scientific Ltd.) and 500 µM Chromogenix S-2288 chromogenic substrate with a running buffer of HEPES buffered saline pH 7.4 with 1% (w/v) PEG_{8000} (HBS [10 mM HEPES, 150 mM NaCl]-P) unless otherwise stated. All experiments were run in triplicate on one plate and were exported from Microsoft Excel into GraphPad Prism v8 for analysis. Readings were taken using a PowerWave HT Microplate Spectrophotometer (BioTek, UK) at 37˚C at 405 nm every 12 seconds for 360 minutes unless otherwise stated.

2.2.6.1 Autoactivation of FXI

30 nM FXI was incubated with 0.6 µg/mL dextran sulfate 500 kDa (DXS_{500kDa}). The generation of FXIa formed was subsequently determined by monitoring cleavage of S-2288. The assay was also performed in the presence of a titration of FP (5-25 µg/mL).

2.2.6.2 Michaelis-Menten Kinetics of FXIa

3 nM FXIa was incubated with and without 0.6 µg/mL DXS_{500kDa}, in the presence and absence of 25 µg/mL FP. Catalytic activity of FXIa was determined using a 2-fold serial dilution of S-2288 (0.2 - 3.0 mM). Michaelis-Menten kinetic analysis was performed using GraphPad Prism v8.

2.2.6.3 Inhibition Assay of FXIa by DXS_{500kDa} and C1-INH

The assay was performed using HBS with 1% (v/v) PEG. Reactions were incubated for 120 minutes prior to adding 500 µM S-2288, and the assay was monitored for 90 minutes.

3 nM FXIa was incubated with a titration of DXS_{500kDa} (0-10000 ng/mL) in the presence and absence a titration of C1-INH (0-180 nM).

Reactions of 3 nM FXIa, 2500 ng/mL DXS_{500kDa}, 25 µg/mL FP were incubated in the presence and absence of a titration of C1-INH (0-180 nM). Reactions of 3 nM FXIa, 19.5 ng/mL DXS_{500kDa} and 25 µg/mL FP were incubated with and without 45 nM of C1-INH (0-180 nM). Reduction of FXIa catalytic activity was determined by measuring the cleavage of S-2288.

2.2.6.4 Inhibition Assay of FXIa by DXS_{500kDa} and HSS

The assay was performed using HBS with 1% (v/v) PEG. Reactions of 3 nM FXIa, 100 ng/mL HSS and 45 nM C1-INH in the presence and absence of 25 µg/mL FP were incubated for 120 minutes prior to adding 500 µM S-2288, then the assay was run for 90 minutes. Reduction of FXIa catalytic activity was determined by measuring the cleavage of S-2288.

2.2.6.5 Preparation of Phospholipids

Phospholipids (PL) were prepared as previously described (Ahnström et al., 2011). PL were supplied in chloroform and micelles were formed using 20% (v/v) di-oleic phosphatidylethanolamine, 20% (v/v) di-oleic phosphatidylserine and 60% (v/v) di-oleic phosphatidylcholine. They were dried using a continuous stream of nitrogen gas and were then resuspended in HBS and extruded through a membrane (1 µm pore size).

2.2.6.6 FIX cleavage by FXIa

Reactions were performed using HBS with 1% (w/v) PEG and 1.5 mM $CaCl_2$. 25 µg/mL FP, 1 mM S-2765 and 3 µM FIX were added to the wells, HBS replaced certain components for controls. 125 pM FXIa was then added to start the reaction and FIX activation was recorded every 12 seconds as the change in absorbance over 5 hours.

2.2.6.7 FX cleavage by FXIa

Reactions were performed using HBS with 1% (w/v) PEG and 1.5 mM $CaCl_2$. 25 µg/mL FP, 350 µM S-2765, 10 µM PL and 30 nM FX were added to the wells, HBS replaced certain components for controls. 30 pM FXIa was then added to start the reaction and FX activation was recorded every 12 seconds as the change in absorbance over 6 hours.

2.2.6.8 FXII Activation of FXI by PTT Automate

A half volume 96 well plate was blocked with 1% (w/v) PEG in HBS with 0.01% (v/v) Tween®20 for 1 hour at 37°C in order to minimise any contact activation of FXII when in close proximity to the well surface. Reactions were performed using HBS with 1% (w/v) PEG and 10 µM $ZnCl_2$. 25 µg/mL FP, 30 nM HK, 30 nM FXI, 500 µM S-2288 and 1% (w/v) PTT automate were added to the wells, HBS replaced certain components for controls. 2 nM FXII was then added to start the reaction and FXI activation was recorded every 12 seconds as the change in absorbance over 6 hours.

2.2.6.9 FXII Activation of FXI by Sulfatides

A half volume 96 well plate was blocked with 1% (w/v) PEG in HBS with 0.01% (v/v) Tween®20 for 1 hour at 37°C in order to minimise any contact activation of FXII when in close proximity to the well surface. Reactions were performed using HBS with 1% (w/v) PEG and 10 µM $ZnCl_2$. A titration of FP (1-25 µg/mL), 30 nM HK, 30 nM FXI, 500 µM S-2288 and 50 µg/mL sulfatides from bovine brain were added to the wells, HBS replaced certain components for controls. 2 nM FXII was then added to start the

reaction and FXI activation was recorded every 12 seconds as the change in absorbance over 3 hours.

2.2.7 Purified Turbidity and Lysis

All assays were run using a half volume plate as previously mentioned. Fibrinogen from human plasma (Fib3; Enzyme Research Laboratories, Swansea) was first purified to eliminate plasma protein contaminants by the use of IF1 (fibrinogen mAb; Kamiya Biomedical Company, Seattle, WA) affinity chromatography as previously described (Takebe et al., 1995) followed by analysis by 10% SDS-PAGE to confirm the purity of the protein and the absence of degradation. 50 µL reactions of 0.5 mg/mL IF1-purified fibrinogen, 0.5 µM plasminogen, 6.25 ng/mL tPA and 0.1 U/mL thrombin were incubated for 2 hours at 37°C and absorbance readings were recorded at 340 nm every 12 seconds for 2 hours. Assays were run with final concentrations of BSA, $CaCl_2$ and $MgCl_2$ at 1% (w/v), 7.5 mM and 3.75 mM respectively. Complement components were added to determine if there was an effect on clot structure, clot formation or clot lysis.

2.2.8 Plasma Turbidity and Lysis

All assays were run using a half volume plate as previously mentioned. 50 µL reactions of 25% NPP, thrombin (titrated 0.001-0.1 U/mL), 7.5 mM $CaCl_2$, 100 ng/mL tPA and FP (titrated 0-25 µg/mL). Reactions were incubated for 2 hours at 37°C and absorbance readings were recorded at 340 nm every 12 seconds for 2 hours.

2.2.9 Thrombin Generation

Thrombin generation was performed using NPP using Fluoroscan Ascent FL plate reader (Thermo Labsystem, Helsinki, Finland) and Thrombinoscope software (Synapse BV, Maastricht, the Netherlands). Before and after each use, the plate reader is primed to remove any air and cleaned. Reactions were incubated on a 96-well round bottom plate. 120 µL reactions containing 60 µL NPP, 20 µL FP, 20 µL

prewarmed agonist/calibrator and 20 µL fluorescent substrate containing CaCl2 (FluCa) were incubated at 37°C for 60 minutes. The experiment was initiated by the automatic addition of FluCa to the plate by the plate reader. The amount of thrombin generated was determined by the cleavage of the substrate by thrombin. FP was titrated two-fold (0-25 µg/mL), and thrombin generation was initiated through the extrinsic pathway using PPP agonist, or via the intrinsic pathway using 1% (v/v) aPTT agonist. Thrombin generation is measured by calculating the first derivative and algorithms are used to correct for α2-macroglobulin inhibition of thrombin via the Thrombinoscope software. Results are normalised against the data from the thrombin calibrator (α2-macroglobulin-thrombin complex) data sets.

2.2.10 Coagulometer

Clotting times in NPP were evaluated with a two-fold titration of FP (0-25 µg/mL) to determine the effect of FP on time to clot formation. Assays were performed in duplicate and were activated via the intrinsic pathway using aPTT reagent, or through extrinsic activation (prothrombin time [PT]) using Technoplastin-HIS (thromboplastin and $CaCl_2$). Clotting times were recorded using STart benchtop coagulometer (Stago, UK).

The aPTT test required an incubation of 45 µL NPP with 5 µL FP/HBS and 50 µL prewarmed aPTT at 37°C for 180 seconds. 50 µL pre-warmed 25 mM $CaCl_2$ was then added to the mix, initiating the recording of clotting time. PT required an incubation of 67.5 µL NPP with 7.5 µL FP/HBS at 37°C for 60 seconds. 75 µL pre-warmed Technoplastin-HIS was then added to the mix, initiating the recording of clotting time. Data were then analysed using GraphPad Prism v8.

2.2.11 Data Analyses

All graphs were produced and analysed using GraphPad Prism v8 (La Jolla, CA).

SPR was analysed in Qdat (specific for Pioneer data sets) and data then were exported to Microsoft Excel. Graphs were produced using GraphPad Prism v8. Data are represented as mean ± SEM unless otherwise stated.

SDS-PAGE and Western blot were imaged using Syngene G:BOX Chemi and GeneSys software. Band analysis was performed using Fiji.

Turbidity and turbidity & lysis assays were analysed as previously described (Longstaff, 2017).

2.2.11.1 Statistical Analyses

Data were first processed using Microsoft Excel and were exported to GraphPad Prism v8 where graphs were produced, and statistical analyses were performed ad hoc. Statistical significance was represented as $p \leq 0.05$. Continuous parametric data were shown as mean ± standard error of the mean and were analysed using one-way ANOVA, followed by Tukey's multiple comparisons test.

Chapter 3 Small Molecule Inhibition of the Alternative Pathway C3 Convertase

3.1 Introduction

The complement cascade is complex. A variety of diseases occur due to complement dysregulation, therefore there is evidence that therapeutic intervention of complement activation is of importance. There are currently a host of inhibitors in clinical trials aimed at different components of the complement cascade (Table.3.1).

The main complement therapeutic used today is a monoclonal antibody which targets C5a, known as eculizumab (Soliris, Alexion). Eculizumab is used in the treatment of paroxysmal nocturnal haemoglobinuria (PNH), a rare haemolytic disorder caused by the lack of complement regulatory proteins on the surface of mature blood cells (Medof et al., 1984, Rollins and Sims, 1990). This culminates in unregulated complement activation leading to haemolytic anaemia induced by MAC (Mastellos et al., 2014), platelet activation (Wiedmer et al., 1993) and thrombosis (Hugel et al., 1999).

Eculizumab treatment of thrombotic microangiopathies (TMA) secondary to disorders such as atypical haemolytic uremic syndrome (aHUS) (Wijnsma et al., 2019) and DIC (Abe et al., 2017) has been successful. aHUS is caused by AP dysregulation, via mutations in FH and related proteins, thus the C3 convertase is not inhibited at the host surface, often resulting in reduced kidney function, haemolysis and thrombotic complications (Noris et al., 2010). As stated in table 3.1 there are biosimilar mAbs, including ravulizumab which are also available clinically.

Table 3.1 Complement therapeutics currently in clinical trials or available in the clinic.

Adapted from (Mastellos et al., 2019). Disorders highlighted in italics are being treated in the clinic with the corresponding drug. Trial status can be searched using NCT numbers via www.clinicaltrials.gov or using the ISRCTN registry found at www.isrctn.com.

Drug (Target)	Disorders	Drug class	Trial identifier
C1-INH (Cetor) (inhibition of C1r/s, MASPs/other serine proteases)	Sepsis/MOD, Kidney I/R	Protein	NCT01766414, NCT02134314
C1-INH (Berinert) (inhibition of C1r/s, MASPs/other serine proteases)	Kidney Tx/AMTR	Protein	NCT01134510
C1-INH (Cinryze) (inhibition of C1r/s, MASPs/other serine proteases)	Kidney Tx/AMTR	Protein	NCT02547220
IFX-1/CaCP29 (blocks C5a binding to C5aR1)	Sepsis/MOD, Inflammatory skin diseases	mAb	NCT02246595, NCT03487276
Mirococept (APT070) (inhibition of C3/C5 convertases)	Kidney I/R	Protein	ISRCTN49958194
TP10/CDX-1135 (soluble complement receptor 1) (inhibition of C3/C5 convertases)	Myocardial infarction	Protein	NCT00082121
Eculizumab (Soliris) (inhibition of C5 activation)	Kidney Tx/AMTR, Positive cross match (anti-human leukocyte antigen)	mAb	NCT01567085, NCT01399593, NCT00670774, NCT03518203, NCT00935883, NCT01892345

	kidney Tx, *PNH, aHUS,* transplant associated TMA, AMD, gMG, *RNO*		
Ravulizumab/ALX1210 (Ultomiris) (inhibition of C5 activation, same epitope as eculizumab)	*PNH*, aHUS	mAb	NCT03131219
SKY59/RO7112689 (inhibition of C5 activation, different epitope to eculizumab)	PNH	mAb	NCT03157635
Tesidolumab/LFG316, (inhibition of C5 activation, different epitopes to eculizumab)	PNH, AMD	mAb	NCT02534909, NCT01527500
Pozelimab/REGN3918 (inhibition of C5 activation, different epitopes to eculizumab)	PNH	mAb	NCT03115996
ABP959 (inhibition of C5 activation, biosimilar of eculizumab)	PNH	mAb	NCT03818607
SB12 (inhibition of C5 activation, biosimilar of eculizumab)	PNH	mAb	NCT03722329
Coversin/OmCI (inhibition of C5 activation)	PNH, aHUS	Protein	NCT03427060, NCT03829449, NCT03829449
Zilucoplan/RA101495 (allosteric inhibition of C5 activation)	PNH	Peptide macrocycle	NCT03078582
Cemdisiran/ALN-CC5 (inhibition of hepatic expression of C5)	PNH	RNAi	NCT02352493

Zimura/avacincaptad pegol (inhibition of C5 expression)	AMD	RNA aptamer	NCT02686658, NCT03362190
AMY-101 (inhibition of C3 activation)	Periodontal disease, PNH, C3G, AMD	Non-PEGylated peptide	NCT03694444, NCT03500549, NCT03226678, NCT03453619, NCT03525613
APL-2	Autoimmune haemolytic anaemias	PEGylated peptide	NCT03226678
LNP023 (FB: inhibition of AP C3 convertase)	PNH, C3G	SM	NCT03439839, NCT03832114
IONIS-FB-L_{Rx} (FB: inhibition of AP C3 convertase)	AMD	ASO	NCT03815825
ACH-4471/ACH-0144471 (FD: inhibition of AP C3 convertase)	PNH, C3G	SM	NCT03053102, NCT03472885, NCT03459443
Sutimlimab/BIV009/TNT009 (C1s: inhibition of CP)	Autoimmune haemolytic anaemias,	mAb	NCT03347422, NCT03347396
Avacopan/CCX168 (C5aR1 antagonist)	aHUS, anti-neutrophil cytoplasmic antibody-associated vasculitis, C3G	SM	NCT02464891, NCT02994927, NCT03301467
IPH5401 (inhibition of C5aR1 signalling)	Cancer	mAb	NCT03665129
OMS721 (MASP2: LP inhibition)	Transplant associated TMA, IgA nephropathy, C3G	mAb	NCT02222545, NCT03608033, NCT02682407, NCT02682407
CLG561 (FP: inhibition of AP amplification)	AMD	mAb	NCT02515942
GEN1029 (DR5: Enhancement of CDC against $DR5^+$ tumours)	Cancer	Hexabody	NCT03576131

C1-INH (berinert®, cinryze®) is clinically available for the prophylactic treatment of hereditary angioedema (HAE) (Riedl et al., 2016, Cocchio and Marzella, 2009) a genetic condition where the patients are deficient in or have abnormal function of C1-INH and suffer from painful swelling in their extremities due to dysregulation of the KKS and fibrinolytic system and complement system (Schmaier, 2019). Studies are underway into the efficacy of this drug to reduce the effects of AMR in kidney transplant patients (Tatapudi and Montgomery, 2019). During AMR, the CP can be activated via C1 complex activation upon binding to donor specific antibodies (DSA) that have recognised the foreign human leukocyte antigens (HLA) on the surface of the organ. This activation of complement leads to inflammation mediated by the generation of anaphylatoxins C3a and C5a, as well as vascular injury facilitated by MAC generation. Therefore, inhibition of this pathway is of great interest to aid in successful organ transplantation (Stegall et al., 2012).

Complement pathways have been a therapeutic target choice for many years, however the AP is of particular interest as it is the only pathway to involve a positive regulator, FP. Targeting the AP C3 convertase could influence the pathophysiology of many inflammatory states and in this instance the interaction between FP and C3b/C3(H2O) would be the target of interest. It has been shown previously that FP knockout murine models do not appear to develop DIC during the localised Shwartzman reaction in the cremaster muscle (Ali, 2005). This suggests that FP is playing a vital role in the development of thrombi as a result of DIC, however it has not been determined whether this is due to the decrease in complement activation, if FP is playing a structural role in thrombus formation, or if FP is playing a vital role in another pathway. This study was initiated by exploring the binding characteristics of the AP C3 convertase using surface plasmon resonance (SPR).

3.2 Surface Plasmon Resonance

3.2.1 FP Binds to C3b with High Affinity

Initially the interaction between C3b and FP was investigated, to ensure a binding interaction could occur between these proteins. C3b was immobilised to an RU of 85.90, on FC1.

After chip installation and preconditioning with 0.1% SDS, 10 mM HCl, 50 mM NaOH, C3b was immobilised at the sensor surface using standard amine coupling (Chapter 2 Materials and Methods 2.2.1) on FC1 with the unmodified FC2 (blocked with 1 M ethanolamine) as the reference channel. Once the ligand was immobilised the sensor-chip was primed three times and the matrix was preconditioned with 1, 2 and 3 M NaCl, and with 1, 2 and 3 mM NaOH to prepare the sensor-chip ready for subsequent regeneration. The assay was run using a running buffer of 10 mM HEPES with 0.05% (v/v) Tween®20 (RBT), 2 mM $MgCl_2$ (RBTM), 1.5 mM $CaCl_2$ and 0.04 mM $ZnCl_2$ (RBTM++). All analytes were dialysed into RBTM++. $MgCl_2$ was included in the buffer as the solid phase binding interaction of the AP C3 convertase is dependent on $MgCl_2$ (Pryzdial and Isenman, 1986).

FP was injected using a OneStep® 100% of loop inject at a flow rate of 30 µL/min, with a dissociation period of 300 seconds (Figure 3.1). The chip was regenerated using a fast injection (60 µL/min) of 5 µL regeneration cocktail of 1 M NaCl, 3 mM NaOH with a dissociation time of 30 seconds.

Analysis in Qdat revealed that FP displayed extremely high affinity to immobilised C3b with a K_D value of 2.24×10^{-20} M. This extremely high affinity suggests an ill-fitting within the Qdat analysis software; it is much more likely that the affinity would lie in the nM - pM range. This may be due to the dissociation time not being sufficient to calculate an accurate K_D. It has been previously demonstrated using SPR that FP binds to immobilised C3b with a K_D of 22 nM (van den Bos et al., 2019).

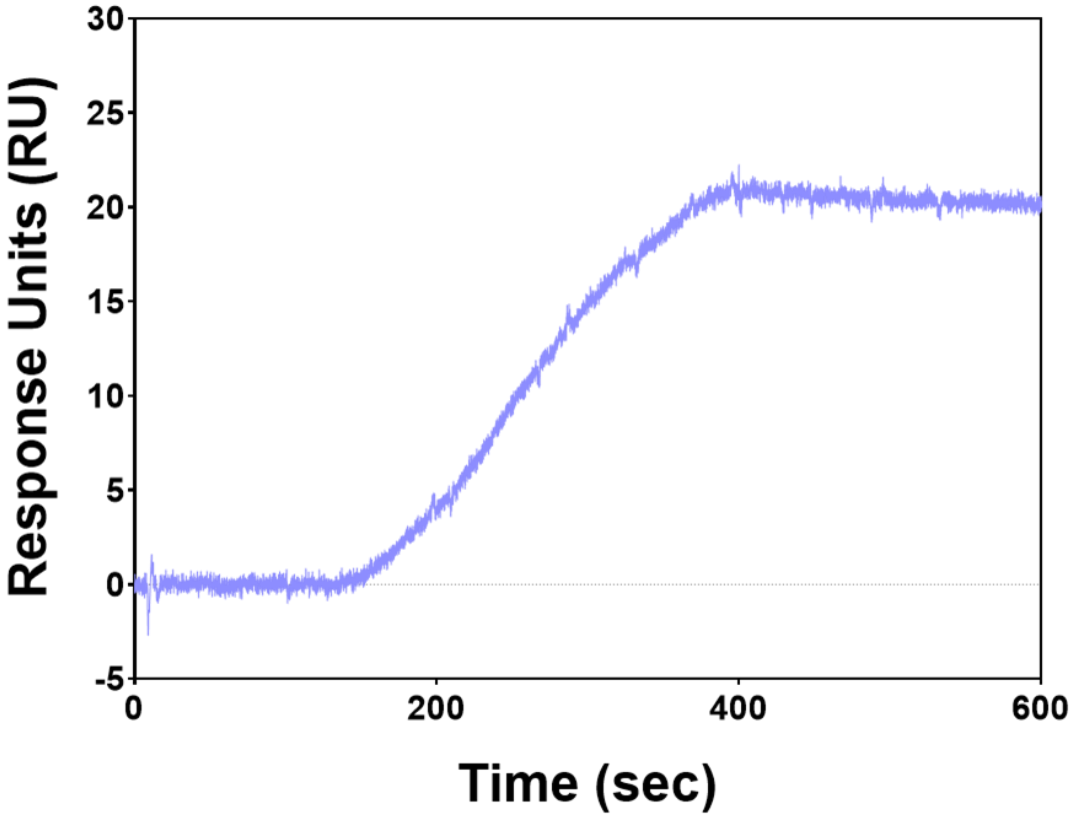


Figure 3.1 Properdin binds to immobilised C3b.

25 nM FP was injected over the sensor surface with C3b immobilised to 85.90 RU.

3.2.2 FP Increases the Affinity of FB and C3b

After determining the high binding affinity ($2.24x10^{-20}$ M) of C3b and FP, the affinity of FB for this complex was explored. First, the affinity between FB and C3b was investigated followed by the interaction of FB with an immobilised complex of C3b and FP.

A new chip was installed and preconditioned with 0.1% SDS, 10 mM HCl, 50 mM NaOH. C3b was immobilised to the sensor-chip surface on FC1 and FC3, with the blank FC2 as the reference channel which was blocked with 1 M ethanolamine. Once the ligand was immobilised the sensor-chip was primed three times and the matrix was preconditioned with 1, 2 and 3 M NaCl followed by 1, 2 and 3 mM NaOH to prepare the matrix of the sensor-chip for regeneration. The assay was run using RBTM with the analytes dialysed into the same buffer.

750 nM FB was first injected using a OneStep® 100% of loop inject at a flow rate of 30 µL/min, with a dissociation period of 200 seconds (Figure 3.2). The sensor-chip was regenerated using a fast injection (100 µL/min) of 10 µL regeneration cocktail of 1 M NaCl, 16.67 mM NaOH, 16.67 mM glycine with a dissociation time of 100 seconds, followed by another fast injection of 30 µL of the previous regeneration cocktail at a flow rate of 100 µL/min with a dissociation time of 100 seconds.

The C3b coated sensor-chip was subsequently treated with a slow injection of 30µL 750 nM FP, at a flow rate of 0.5 µL/min with no dissociation period, creating a complex of C3b and FP at the sensor surface.

A new assay was started to retrieve the correct baseline. 750 nM FB was injected via a OneStep® 100% of loop inject at a flow rate of 30 µL/min, with a dissociation period of 100 seconds over the C3b and FP surface. The sensor-chip was further regenerated with 10 µL of the previous regeneration cocktail at a flow rate of 150 µL/min with a dissociation time of 60 seconds, followed by 30 µL of regeneration

cocktail at a flow rate of 150 µL/min with a dissociation time of 300 seconds (Figure 3.2). To ensure the C3b/FP complex as not disturbed, the baseline was taken at the beginning of the assay before the start of the inject of FB, and was checked at the end of the assay, after regeneration: the response returned to baseline, thus the complex remained at the sensor surface.

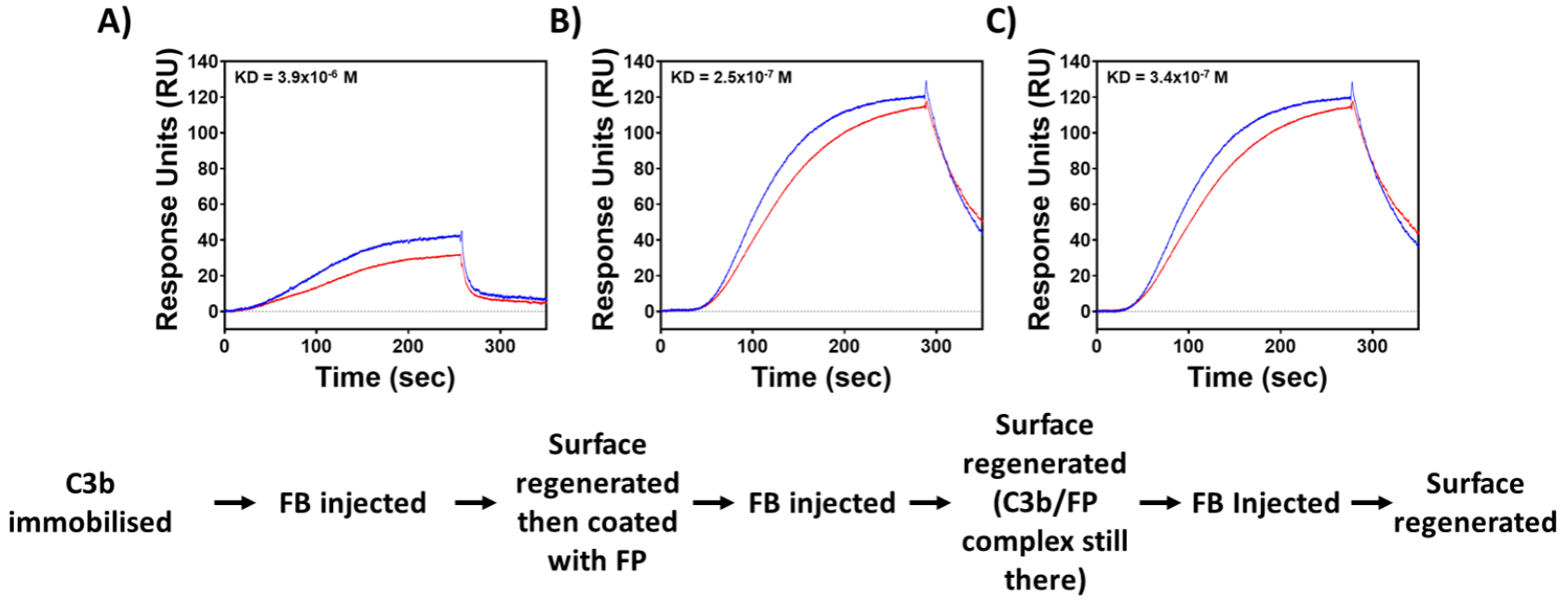


Figure 3.2 FP enhances the binding interaction between FB and C3b in the alternative pathway C3 convertase determined by SPR.

C3b was immobilised to both FC1 (blue 743.8 RU) and FC3 (red 1933.4 RU). A) Sensorgram representing FB only binding to C3b. The sensor-chip was then subject to an inject of FP to create a C3b/FP complex at the sensor-chip surface. B) Sensorgram demonstrating an inject of FB over the C3b/FP complex. The chip was regenerated back to baseline, not disturbing the C3b/FP complex. C) FB was injected again, with this sensorgram representing the binding of FB to the C3b/FP complex.

The literature states that FP stabilises the AP C3 convertase complex ten-fold (Fearon and Austen, 1975b). This binding data supports this concept where FP clearly stabilises the C3bBb complex (Figure 3.2), as is demonstrated by the decrease in the value for the KD, which represents a higher affinity binding of FB to the complex. The KD of FB binding to C3b in the absence of FP is $3.9x10^{-6}$. In the presence of FP this affinity is increased by one order of magnitude to $2.5x10^{-7}$ and after regeneration this affinity did not change, demonstrating the high affinity interaction between FP and C3b.

3.2.3 Initial Screen of Small Molecule Interactions with the C3 Convertase of the Alternative Pathway

The AP is dysregulated in several disorders including PNH, aHUS and AMD (Yuan et al., 2017a, Geerlings et al., 2017) with drugs in clinical trials for each clinical indication (Table 3.1). There is evidence that AP dysregulation also plays an important role in the pathology of DIC. The data suggest that FP is not necessary for C3b and FB to bind, though it does accelerate complex formation. To target the interaction of C3b and FP may dampen the AP of complement, without complete suppression.

These data suggest that targeting the interaction between FP and the C3 convertase complex may be beneficial as a therapeutic target for DIC.

A small molecule drug has a low molecular weight (900 Da or less). They are useful in regulating biological targets including enzymes, receptors and cofactors to modify pathological mechanisms in diseases. Many pharmaceutical therapeutics available today are small molecules and are often delivered orally but can also be delivered subcutaneously and intravenously. The low molecular weight of compounds allows the possibility to cross cell membranes under the correct conditions and therefore may be able to reach targets within a cell if necessary (AstraZeneca, 2020).

There are limitations to the use of small molecules as therapeutics. Small molecules can be selective, binding to their target with relatively high affinity thus effects may be difficult to reverse. Due to their size, only a limited number of modifications can be made to ensure they are selective, and they most often target a “groove” to ensure tight interactions with several points of the small molecule. They may continue to modify target mechanisms when unnecessary, often leading to unwanted side effects (Gurevich and Gurevich, 2014).

Three plates containing 2 µL of 10 mM compounds were supplied by the School of Chemistry, University of Leeds. These were screened using SPR, to determine the effect they had on the C3 convertase complex. The assay for this initial small molecule screen consisted of C3b immobilisation to the sensor surface, and a mixture of 50 nM FB and 10 nM FP as the negative control and was run using RBTM with 5% (v/v) DMSO (RBTDM). Microcalibrations were performed: 4% (v/v) and 6% (v/v) DMSO to account for refractive index changes caused by DMSO, and a bulk standard of 3% (w/v) sucrose was used. Unless otherwise stated, all data for the compound screening will be represented as % binding, which has been normalised to the first positive control. Compounds have been named according to the order on the initial data sets to ensure anonymity of the compounds, and they have been named as SM (small molecule) followed by a number, starting from 1. Standard curves were generated using the positive controls and the chip degradation over time was corrected for using the linear equation $y = (mx) + c$, where y = corrected response and x = cycle number.

A COOHV chip was installed and C3b was immobilised to FC1 (2492 RU) and the unmodified FC2 was used as the reference channel. The matrix was preconditioned with 1, 2 and 3 M NaCl and 1, 2 and 3 mM NaOH and primed three times as previously described. The positive controls (10 nM FP with 50 nM FB) were injected periodically, every 8 injects. The plates containing compounds were prepared to final

concentrations of 150 µM compounds, 10 nM FP, 50 nM FB, and 5% (v/v) DMSO in RBTM. Fast injects of 60 µL of sample were performed at a flow rate of 30 µL/min with a dissociation of 120 seconds (Figure 3.3 & 3.4). The chip was regenerated after each inject with 6 µL of 1 M NaCl, 16.67 mM NaOH, 16.67 mM glycine regeneration cocktail at a flow rate of 60 µL/min with a dissociation time of 30 seconds. The system was purged before running the next inject.

Forty-one compounds were chosen to take forward to the secondary screening process (Table 3.2).

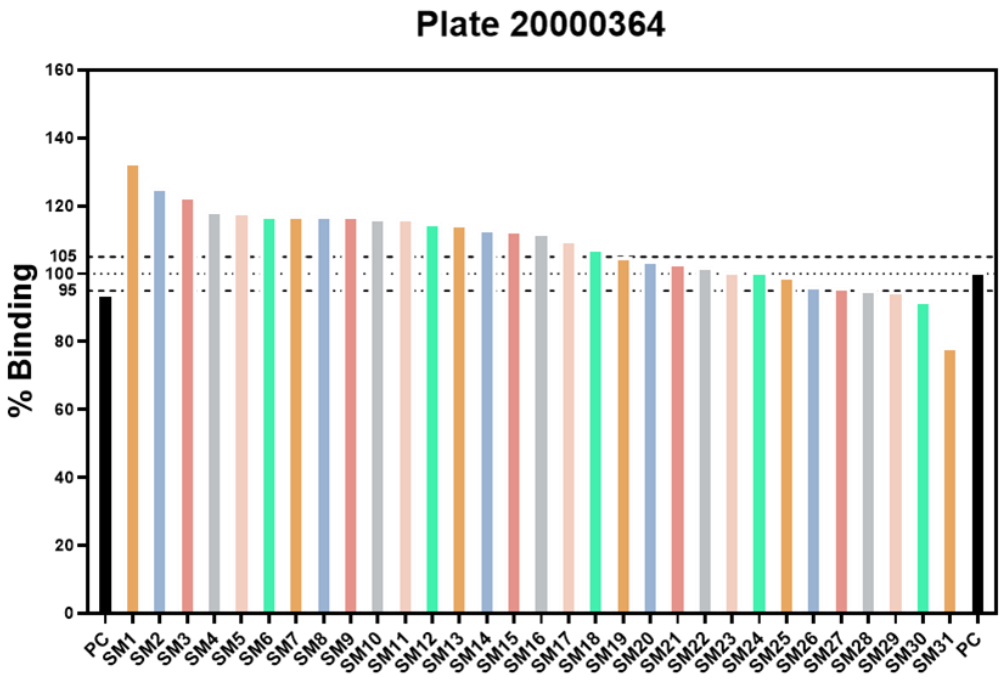


Figure 3.3 Plate 1 of the initial small molecule screen using SPR.

150 µM small molecules (SM) in the presence of 10 nM FP and 50 nM FB were injected over C3b immobilised to 2492 RU on FC1. Positive controls (PC) of 10 nM FP with 50 nM FB are represented using black bars with the coloured bars indicating presence of 150 µM small molecules. The first and last positive controls of the assay are shown to determine if any sensor-chip degradation over time. *Cyclic positive controls omitted for simplicity.*

For the next two plates a new COOHV chip was installed and C3b was immobilised to FC1 (2991 RU) and FC3 (2228 RU), and the unmodified FC2 was used as the reference channel. The matrix was preconditioned with 1, 2 and 3 M NaCl and 1, 2 and 3 mM NaOH and primed three times as previously described. The assay was run the same as the previous assay.

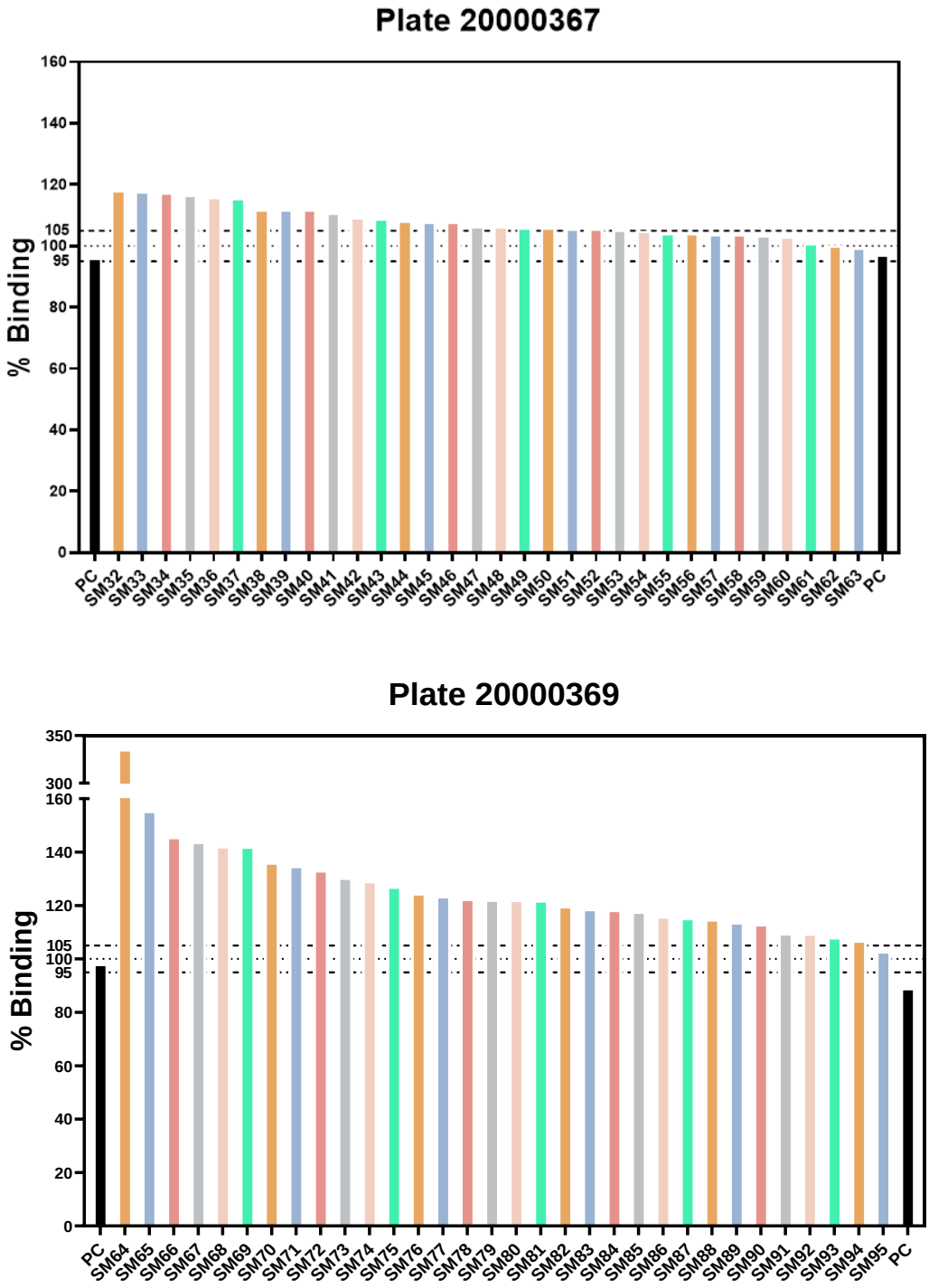


Figure 3.4 Plates 2 and 3 of the initial small molecule screens using SPR.

150 µM small molecules (SM) in the presence of 10 nM FP and 50 nM FB were injected over C3b immobilised to 2991 RU on FC1 and to 2228 RU on FC3. Positive controls (PC) of 10 nM FP with 50 nM FB are represented using black bars and coloured bars indicating presence of 150 µM small molecules. The first and last positive controls of the assay are shown to determine if any sensor-chip degradation over time. Injects involving SM were averaged (n=2 [FC1 and FC3]). *Cyclic positive controls omitted for simplicity.*

Table 3.2 Modulation of C3b/FP/FB interaction by small molecules.

41 small molecules of interest were chosen to carry forward for the secondary screening. Molecules were chosen depending on their percentage binding. Green represents binding > 105% and red represents binding < 95%. Orange represents the compounds chosen as negative controls.

Compound	% binding
SM31	75
SM32	146
SM33	152
SM34	119
SM35	146
SM36	142
SM39	125
SM40	127
SM42	110
SM43	96
SM44	107
SM45	121
SM47	130
SM48	128
SM49	117
SM50	110
SM51	106
SM52	127
SM53	91
SM54	89
SM55	92

Compound	% binding
SM56	102
SM57	98
SM58	108
SM59	94
SM60	90
SM61	92
SM62	86
SM63	82
SM64	295
SM65	156
SM66	140
SM67	147
SM68	142
SM69	152
SM70	135
SM71	131
SM72	129
SM73	131
SM75	114
SM94	125

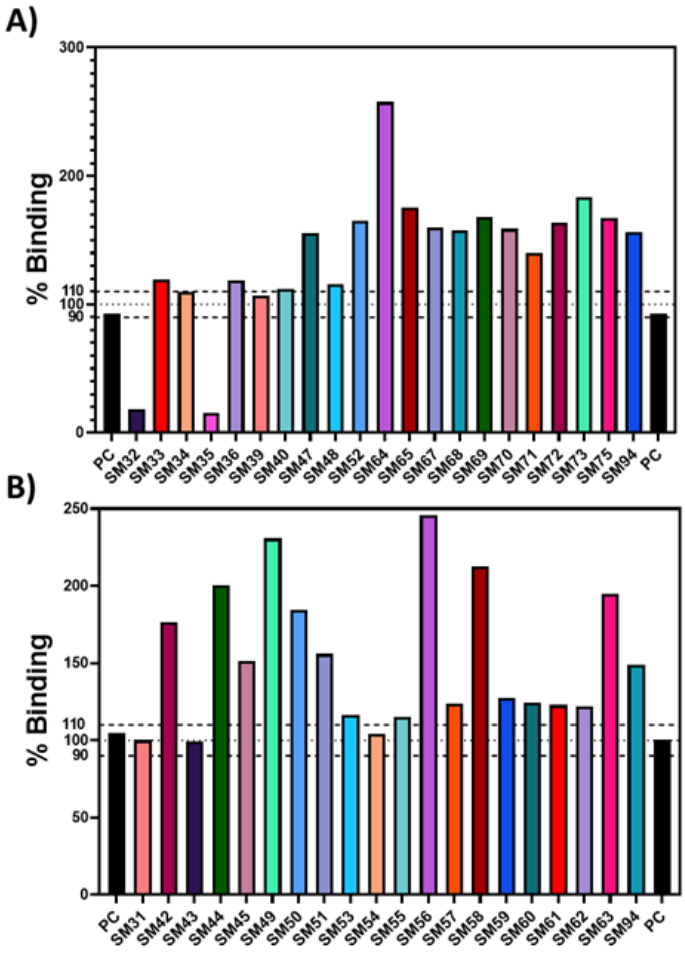
3.2.4 Secondary Screen of the chosen Small Molecules

A second affirmation screen using SPR is useful to determine whether the interactions of the small molecules can be confirmed.

Forty-one compounds were taken forward depending on how they interacted with the complex binding. Compounds were selected depending on the relative disruption or enhancement effect they had on the binding of the complex – i.e. compounds that decreased or increased the binding by 5% or more; and three were chosen as negative controls (did not affect the binding) (Table 3.2). The effect compounds exerted on the C3 convertase complex was determined again using SPR. Two more plates containing the compounds of interest were supplied by the School of Chemistry, University of Leeds. The assay for this second stage of small molecule screen consisted of C3b immobilisation to the sensor surface, and a mixture of 50 nM FB and 10 nM FP (optimised concentrations to achieve maximal binding of the complex) as the reference control and was run using RBTM with 5% (v/v) DMSO (RBTDM). Microcalibrations were performed, using 4% (v/v) and 6% (v/v) DMSO to account for refractive index changes caused by DMSO, and a bulk standard of 3% (w/v) sucrose was used.

The assay was performed as two separate runs, using the previous sensor-chip with C3b immobilised to FC1 (2991 RU) and FC3 (2228 RU), and the unmodified FC2 as the reference channel. The positive controls of 50 nM FB and 10 nM FP were injected periodically, every 6 injects. The concentration for these controls was optimised prior to running the experiment to ensure the response reached the calculated Rmax. The plates containing compounds were prepared to final concentrations of 150 µM compounds, 10 nM FP, 50 nM FB, and 5% (v/v) DMSO in RBTM. Fast injects of 60 µL of sample were performed at a flow rate of 30 µL/min with a dissociation of 120 seconds (Figure 3.5). The sensor-chip was regenerated after each inject with 6 µL of 1 M NaCl, 16.67 mM NaOH, 16.67 mM glycine regeneration cocktail at a flow rate of

60 µL/min with a dissociation time of 60 seconds. The system was purged before running the next inject.



Analyte	% Binding	Analyte	% Binding
PC	93.22	SM65	175.47
SM32	18.33	SM67	159.72
SM33	119.62	SM68	157.70
SM34	109.59	SM69	168.14
SM35	15.45	SM70	158.84
SM36	118.98	SM71	140.21
SM39	106.83	SM72	163.39
SM40	111.86	SM73	183.58
SM47	155.46	SM75	167.02
SM48	115.75	SM94	156.16
SM52	165.30	PC	93.19
SM64	257.68		

Analyte	% Binding	Analyte	% Binding
PC	104.41	SM55	115.17
SM31	100.22	SM56	245.71
SM42	176.70	SM57	123.58
SM43	99.22	SM58	212.51
SM44	200.30	SM59	127.71
SM45	151.18	SM60	124.35
SM49	230.76	SM61	122.81
SM50	184.44	SM62	122.24
SM51	155.98	SM63	194.84
SM53	116.37	SM94	149.08
SM54	104.18	PC	100.49

Figure 3.5 Plates 1 and 2 of the secondary small molecule screens using SPR.

150 µM Small molecules (SM) in the presence of 10 nM FP and 50 nM FB were injected over C3b immobilised to 2991 RU on FC1 and to 2228 RU on FC3. Positive controls (PC) of 10 nM FP with 50 nM FB are represented using black bars and coloured bars indicating presence of 150 µM small molecules. The first and last positive controls of the assay are shown to determine any chip degradation over time. Injects involving SM were averaged (n=2 [FC1 and FC3]). *Cyclic positive controls omitted for simplicity.* Pink shading represents < 95% binding, orange shading represents < 5% effect on binding, green represents > 105% binding.

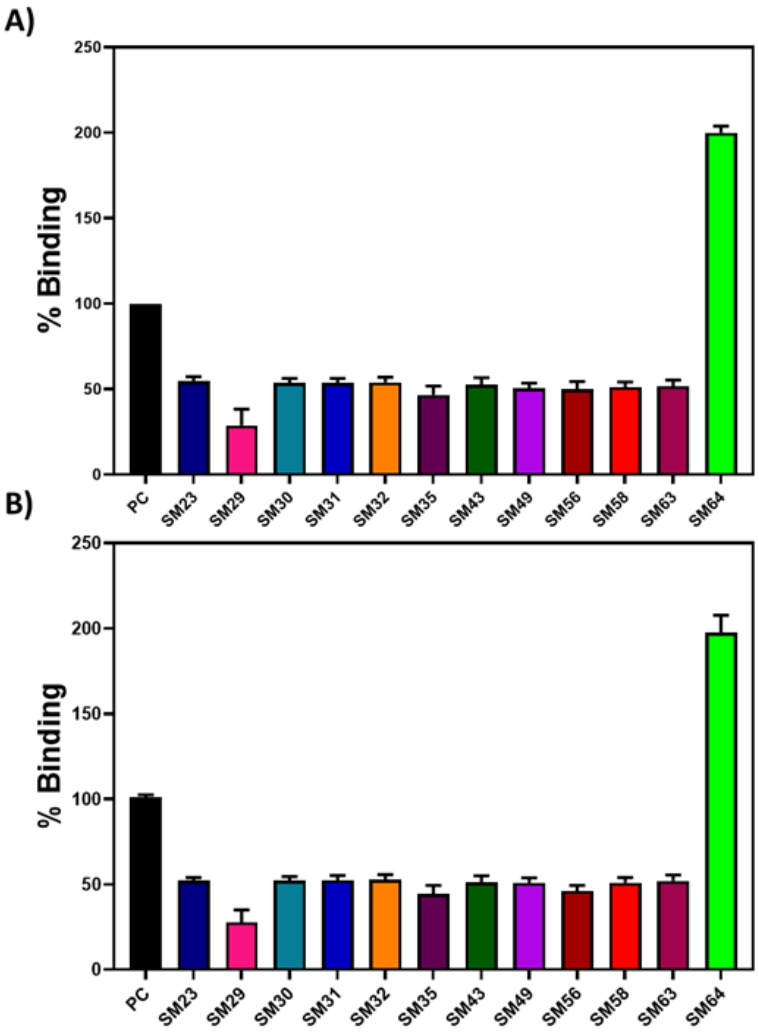
From the combined results from Figure 3.3 and 3.4, twelve compounds were chosen: SM23, SM29, SM30 and SM31 from Figure 3.3, SM35, SM43, SM49, SM56, SM58, SM63 and SM64 from Figure 3.4. These were carried through to the next stages of screening. Before each run, assays were optimised to determine the amount of protein needed in the positive controls and to ensure the regeneration conditions were suitable and did not damage the chip by loss of ligand. The relative binding the compounds exerted on the C3 convertase complex appeared to be variable, therefore optimisation was key to ensure highest level of accuracy. The next stage was to determine the effect of these 12 small molecules on the interactions of the C3 convertase when FP was immobilised to the sensor-chip surface. It has been documented that both FP and C3b may be responsible for pathogen binding of the complex, and it was investigated if there was a difference in complex stability depending on the orientation of the complex; the compounds may interact differently under different circumstances.

The compounds were selected on the basis that they had effects on the interactions in both the preliminary and secondary screens. With some of the compounds, the interactions differed between the two runs, in that in one enhancement of the complex was observed whilst inhibition was observed in another. Therefore, these compounds were explored in subsequent assays.

3.2.5 Investigation into the Orientation of the C3 Convertase Complex

FP was subsequently immobilised instead of C3b, to determine whether the compounds behave differently in a different permutation of the complex. The main role of the C3 convertase is to allow opsonisation of pathogens by C3b, induced by the binding of the C3 convertase to the surface of the target (Pangburn et al., 1980). It has been stated however that FP may play a role in C3 convertase deposition via binding to PAMPs (Spitzer et al., 2007, Kemper et al., 2010).

A new COOHV chip was installed and FP was immobilised on FC1 (588 RU) and FC3 (309 RU), with FC2 as the reference channel. The matrix was preconditioned with 1, 2 and 3 M NaCl and 1, 2, and 3 mM NaOH and primed three times as previously described. The system was primed three times before the assay began, and the positive controls were injected periodically, every 2 cycles which is more often than previously to ensure sufficient controls were used. The plates containing compounds were prepared to final concentrations of 100 µM compounds (lower than previous runs to conserve the compounds), 30 nM C3b, 30 nM FB, and 5% (v/v) DMSO in RBTM. Fast injects of 80 µL of sample were performed at a flow rate of 30 µL/min with a dissociation of 300 seconds (Figure 3.6). The chip was regenerated after each inject with 6 µL of 1.5 M NaCl, 5 mM NaOH regeneration cocktail at a flow rate of 30 µL/min with a dissociation time of 30 seconds. The system was purged before running the next inject.



Analyte	% Binding	SEM
PC	100.00	0.01
SM23	54.70	2.93
SM29	28.73	3.55
SM30	53.60	3.19
SM31	53.59	3.07
SM32	53.96	2.66
SM35	46.59	2.65
SM43	52.67	2.49
SM49	50.36	5.22
SM56	49.88	4.56
SM58	51.16	3.96
SM63	51.72	9.54
SM64	199.95	3.82

Analyte	% Binding	SEM
PC	101.28	1.28
SM23	52.21	1.87
SM29	27.83	7.12
SM30	52.22	2.38
SM31	52.47	2.73
SM32	52.65	3.13
SM35	44.17	5.17
SM43	51.09	3.84
SM49	50.95	2.90
SM56	46.13	3.21
SM58	50.92	3.15
SM63	51.97	3.49
SM64	197.42	10.25

Figure 3.6 The C3 convertase interactions can be both inhibited and enhanced by small molecules.

FP was immobilised to FC1 and FC3 to 587.5 RU (A) and 306.6 RU (B) respectively. 100 µM small molecules (SM) were injected in the presence of 30 nM C3b and 30 nM FB. Positive controls (PC) of 30 nM C3b with 30 nM FB are represented using black bars. *Cyclic positive controls omitted for simplicity.* Pink shading represents < 95% binding, green represents > 105% binding.

Immobilisation of FP did not affect the binding interactions of the chosen small molecules. All compounds selected for this screening interaction inhibited the interaction between the analytes FB and C3b with FP except for one, SM64, which enhanced complex formation, increasing the maximum response, suggesting more FB and C3b could bind to immobilised FP. This enhanced binding may be explained by the compound exhibiting a conformational change on FP, which increases the binding capacity towards FB and C3b, or the increased response may be due to the compound itself binding to FP.

The effect of the compounds was consistent for both FC1 and FC3 (Figure 3.6), suggesting that the amount of FP immobilised did not affect the interaction.

The permutation of the complex may affect compound binding. When proteins bind to surfaces, in this case the amine coupling of FP to the dextran chip, it can cause conformational changes in proteins, which must be considered in later experiments.

3.2.6 Small Molecules Binding to C3b and FP

To investigate which protein the compounds were targeting, a OneStep® assay was performed using the 12 compounds of interest and both C3b and FP immobilised on two separate flow cells on the same chip.

After COOH5 chip installation and preconditioning with 0.1% SDS, 10 mM HCl, 50 mM NaOH, C3b and FP were immobilised at the sensor surface on FC1 (16145.47 RU) and FC3 (10907.98 RU) respectively, with the treated blank FC2 as the reference channel. Once the ligand was immobilised the sensor-chip was primed three times and the matrix was preconditioned with 1, 2 and 3 M NaCl, and with 1, 2 and 3 mM NaOH to prepare the proteins for regeneration. The assay was run using RBTDM. 100 µM of compound was injected using a OneStep® 100% of loop inject at a flow rate of 30 µL/min, with a dissociation period of 200 seconds. The chip was regenerated using a fast injection of 6 µL RBTDM at a flow rate of 30 µL/min with a dissociation time of 30 seconds (Figure 3.7). Data were collected at a frequency of 10 Hz. Four bulk standard cycles of 3% (w/v) sucrose were performed to correct for the diffusion coefficient (m^2/s) of the compounds, and microcalibrations were performed using 4% and 6% (v/v) DMSO in RBTM to correct for refractive index changes caused by DMSO concentration fluctuations. These calibration steps were corrected for when data were exported to Qdat.

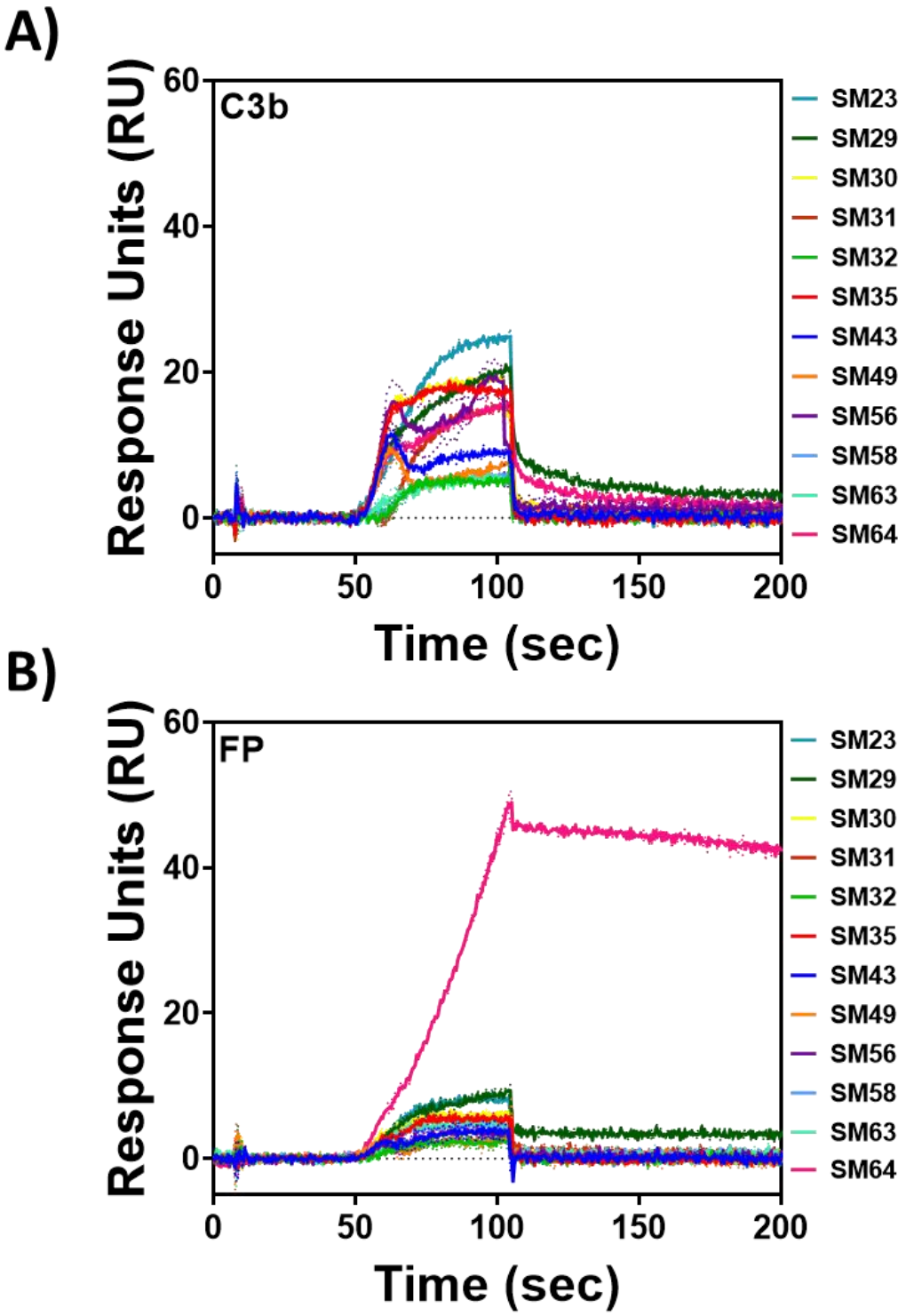


Figure 3.7 SPR determines that the final 12 small molecules bind to immobilised C3b and FP.

100 µM small molecules (SM) were injected over C3b and FP. A) FC3 – C3b immobilised to 16145.47 RU. B) FC3 – FP immobilised to 10907.98 RU. Binding is represented as change in refractive index, or response units, over time (sec). Data represent triplicates run in one assay, mean ± SEM (indicated by a dotted line).

Table 3.3 KD values of 100 µM small molecules binding to C3b and FP via a OneStep® 100% loop inject.

Compound	KD (M)	KD (M)
	C3b	**FP**
SM23	$4x10^{-2}$	$2.2x10^{-3}$
SM29	$1.8x10^{-7}$	$4.6x10^{-11}$
SM30	$4.3x10^{-5}$	$2.3x10^{-5}$
SM31	$7.4x10^{-2}$	$1.8\ x10^{-4}$
SM32	$3x10^{-3}$	$3.5x10^{-5}$
SM35	$4.4x10^{5}$	$2.5x10^{-5}$
SM43	$2x10^{-5}$	$2.3x10^{-5}$
SM49	$1.8x10^{-5}$	$2.5x10^{-6}$
SM56	$2.5x10^{-5}$	$2.4x10^{-5}$
SM58	$7x10^{-3}$	$1.6x10^{-4}$
SM63	$8x10^{-3}$	$6.6x10^{-4}$
SM64	$4.6x10^{-7}$	$4.8x10^{-6}$

All compounds bound to both C3b and FP with varying affinities ranging between 46 pM (SM29 binding to C3b) to 74 mM (SM31 and FP) (Table 3.3). SM64 bound to C3b with an affinity of 460 nM. For FP, the KD was 4.8 µM. The binding curves were very different for both C3b and FP with SM64. This compound shows a slower on and off rate with FP when compared to C3b yet has a higher affinity for C3b. The high RU of the compound binding to FP and the higher KD value suggest that this compound may be binding promiscuously to FP. This could be a result of the charge of FP being highly positive at pH 7.4, if the compound (SM64) is negatively charged; the structure of the compound is unknown at this point. This also supports the theory that the "enhanced" binding interaction of C3b and FB to immobilised FP (Figure 3.6) may have a contribution arising from compound binding.

Promiscuity of small molecules can become an issue, as they can be non-selective, creating issues in further characterisation methods.

3.2.7 The Effect of Small Molecules on Separate Components of the AP C3 Convertase

The next stage of the investigation was to determine how the molecules can interact with the different aspects of the complex. C3b was immobilised to the sensor surface, and FP, FB, and FB+FP were the analytes in the presence and absence of the small molecules.

After chip installation and preconditioning with 0.1% SDS, 10 mM HCl, 50 mM NaOH, C3b was immobilised at the sensor surface on FC1 and FC3 to 664 RU and 1222 RU respectively with the treated blank FC2 as the reference channel. Once the ligand was immobilised the sensor-chip was primed three times and the matrix was preconditioned with 1, 2 and 3 M NaCl, and with 1, 2 and 3 mM NaOH to prepare the proteins for regeneration. The assay was run using RBTDM. Optimisations were performed prior to the experiment to determine the following concentrations; 10 nM FP/80 nM FB/10 nM FP + 80 nM FB in the presence and absence of 100 µM compound was injected using a fast, fixed concentration inject at a flow rate of 35 µL/min, with a dissociation period of 300 seconds. The concentration of the proteins was optimised prior to the run to ensure the response reached Rmax. The chip was regenerated using a fast injection of 5 µL regeneration cocktail (2.5 mM NaOH, 1 M NaCl) at a flow rate of 100 µL/min with a dissociation time of 20 seconds (Figure 3.8). Data were collected at a frequency of 10 Hz. Three bulk standard cycles of 3% (w/v) sucrose were performed to correct for the diffusion coefficient (m^2/s) of the compounds, and microcalibrations were performed using 4% and 6% (v/v) DMSO in RBTM to correct for refractive index changes caused by DMSO concentration fluctuations. These calibration steps were corrected for when data were exported to Qdat.

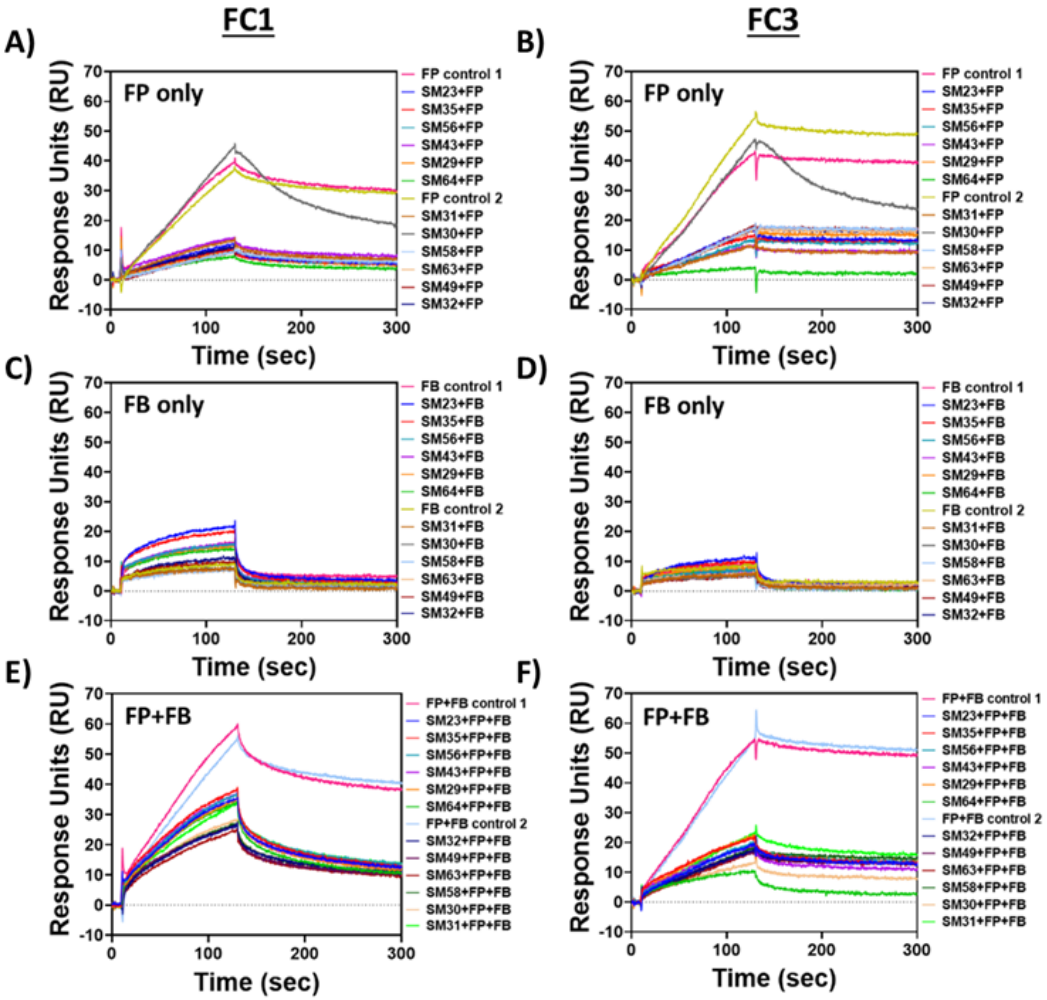


Figure 3.8 The effect of small molecule interactions on the binding of defined components of the AP C3 convertase.

C3b was immobilised on FC1 (664 RU) and FC3 (1222 RU). 10 nM FP (A&B), 80 nM FB (C&D) and 10 nM FP + 80 nM FB (E&F) were injected in the presence and absence of 100 µM small molecules (SM). Assays were run as two separate assays due to limitations of running buffer volumes. SM23, SM35, SM56, SM43, SM29 and SM64 should be compared to control 1 as they were performed during the same assay. SM32, SM49, SM63, SM58, SM30 and SM31 should be compared to control 2 as they were performed during the same assay.

The investigation of the interactions between the individual components of the complex allows the elucidation of the mechanisms behind the compound interactions. There are some discrepancies in the data between FC1 and FC3 which may be explained by the difference in the amount of C3b immobilised. The higher immobilisation, could mean more “crowding” of the protein at the sensor surface, restricting interactions between the ligand and the analytes. The effects of the compounds on the interactions of the individual components follow similar trends on both FC1 and FC3.

All small molecules investigated demonstrated an inhibitory effect on FP binding to C3b, except for SM30 which only affected the off rate (Figure 3.8A & B). The faster off rate demonstrated on both FC1 and FC3 suggests using compound SM30 indicates that the FP/C3b complex is still able to form, however it is less stable, and FP dissociates much faster when the compound is present. In contrast to earlier data, SM64 appears to have the largest inhibitory effect on FC3. During the course of these experiments it was found that FP formed non-physiological aggregates during dialysis, thus a slow centrifugation step was performed to pull the aggregates to the bottom, and the supernatant was used in experiment. This may be the cause of the different effects seen here, where SM64 now appears inhibitory.

When FB only was the analyte over the immobilised C3b, the signal of binding was relatively low (around 30% calculated Rmax). When FB was injected in the presence of 100 µM compound there was no enhancement or inhibition of FB binding to C3b, however SM23 and SM35 appeared to have an enhancing effect on FB binding to C3b, with the RU being higher (Figure 3.8C & D).

The whole C3 convertase complex C3b/FP/FB was partially inhibited in the presence of all compounds, including SM64, that had the largest inhibitory effect on FC3 (Figure 3.8E & F).

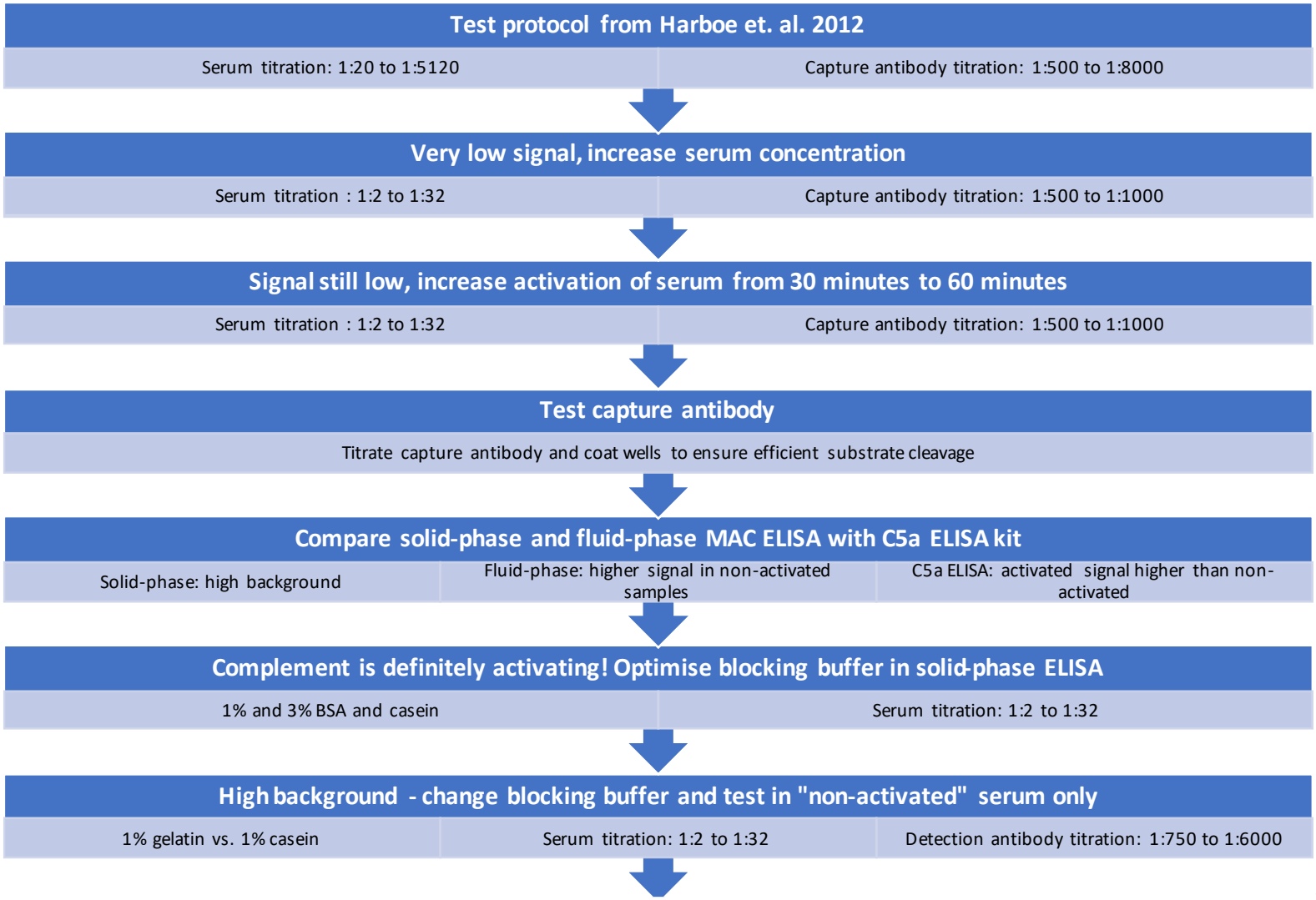
The next step of the screening process was to optimise a more physiologically relevant method of analysis, using human serum. SPR by nature utilises purified proteins to study affinities between ligand and analyte, whilst useful to explore the individual effects of the compounds, this does not reflect the overall effect in a biological system. Of course, whilst high affinity interactions detected by SPR can suggest a physiologically relevant interaction, this must be tested in more physiologically relevant conditions to consider any off-target effects, and non-specific binding interactions with the myriad other proteins present in a biological setting.

3.3 ELISA

3.3.1 MAC ELISA

In order to study complement and complement-targeted compounds in a physiological system, an ELISA utilising human serum was employed. Complement activation is most often explored using serum, rather than plasma. Complement activity is measured by detecting the formation of terminal complement components, such as C5a or MAC formation.

The initial aim was to develop an *in-house* MAC sandwich ELISA using protocols adapted from Harboe *et al.* (2012). Complement was activated by incubating serum diluted 1:1 in veronal buffer with gelatin, $CaCl_2$ and $MgCl_2$ (VBG++) with 0.05% (v/v) Tween®20 on an “activation” plate which was coated with the well-known inflammatory molecule, activated zymosan A, prepared from cell walls of *Saccharomyces cerevisiae* (Harboe et al., 2012). The “activated” serum was then aliquoted, diluted further and incubated on a plate coated with anti-C5 capture antibody, and the MAC complex was subsequently detected with the anti-C9 antibody. In depth optimisation steps were performed (Figure 3.9), however it was impossible to decrease the background signal, and in some cases the non-activated serum gave signal higher than the activated serum. It was decided that a line must be drawn under this ELISA optimisation, therefore a different approach was taken and a commercial C5a ELISA kit was purchased.



See Page 110 for legend.

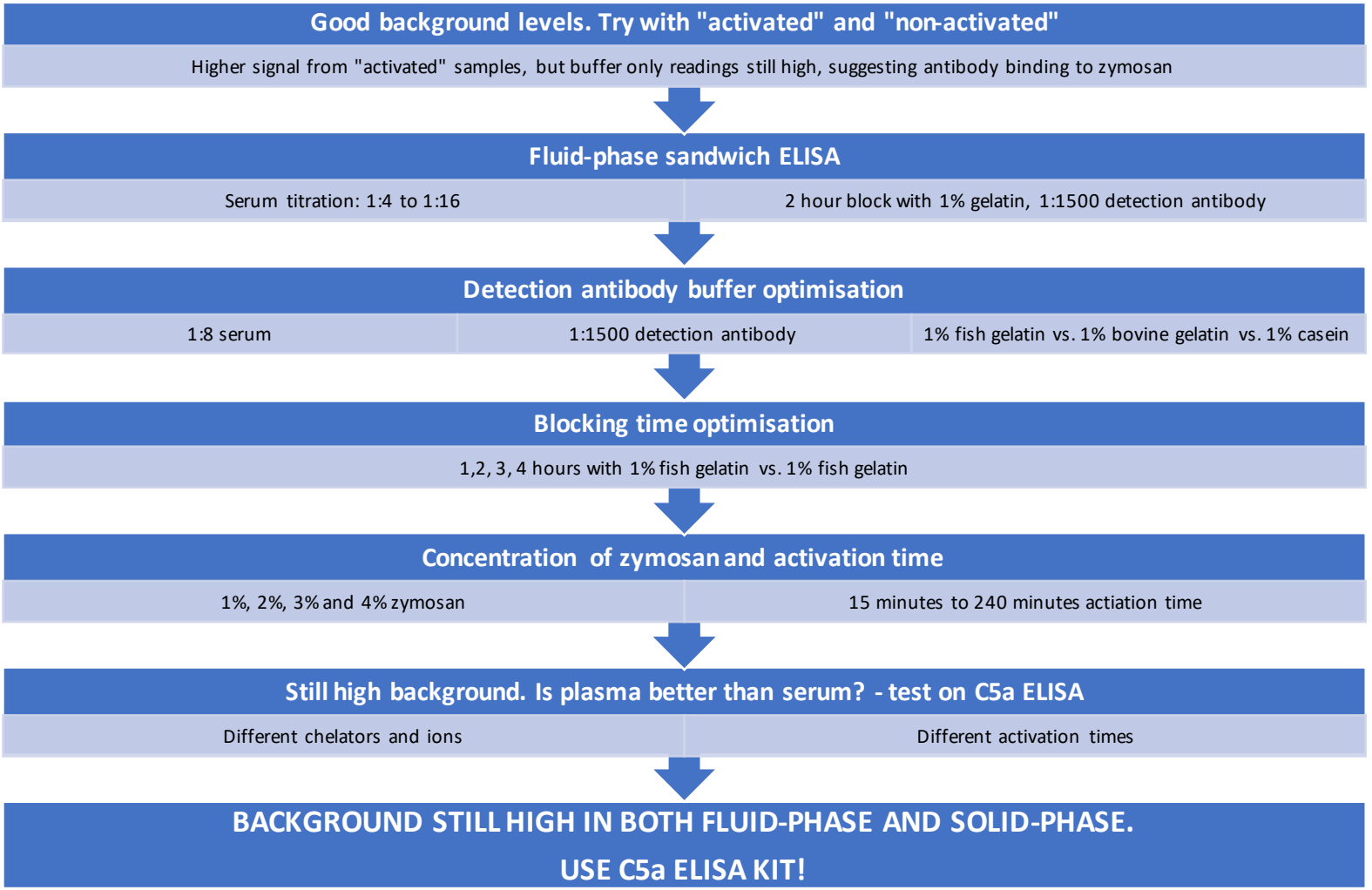


Figure 3.9 Flow diagram briefly outlining the optimisation steps taken to develop an *in-house* MAC ELISA to measure complement activation in serum/plasma.

3.3.2 C5a ELISA

The C5a ELISA kit is a useful tool readily available from Abcam. It is used for the quantitative measurement of human C5a from serum or plasma, as well from cell culture supernatants. The C5a ELISA was employed as C5a is useful to measure as a marker of complement activation, downstream of the C3 convertase therefore this assay can be utilised to determine the effect of compounds targeting the C3 convertase on complement activation. An anti-human C5a antibody is immobilised to the microtitre plate surface and the sample is probed with a biotinylated anti-human C5a antibody. Recombinant C5a standards were provided with the kit, which are manually titrated. The signal is produced using a 3,3",5,5"-Tetramethylbenzidine (TMB) substrate solution, which is stopped with a 'Stop Solution' provided in the kit, and the intensity of colour is read at 450 nm using a plate reader. This ELISA kit was used to determine the effect of the compounds on C5a generation in a serum or plasma-based system, thus allowing the measurement of complement activation, and determination of the effect of the compounds on complement activity.

3.3.2.1 C5a ELISA Optimisation

An optimisation step was performed to determine the optimum concentration of serum to be employed, and the effect that DMSO may have on the assay (Figure 3.10). Complement was activated by incubation of serum with zymosan A for 1 hour, stopping the reaction using a final concentration of 20 mM EDTA. Serum was titrated from 1 in 10 to 640 (2-fold serial titration) in the diluent provided, and samples were analysed according to manufacturer's instructions.

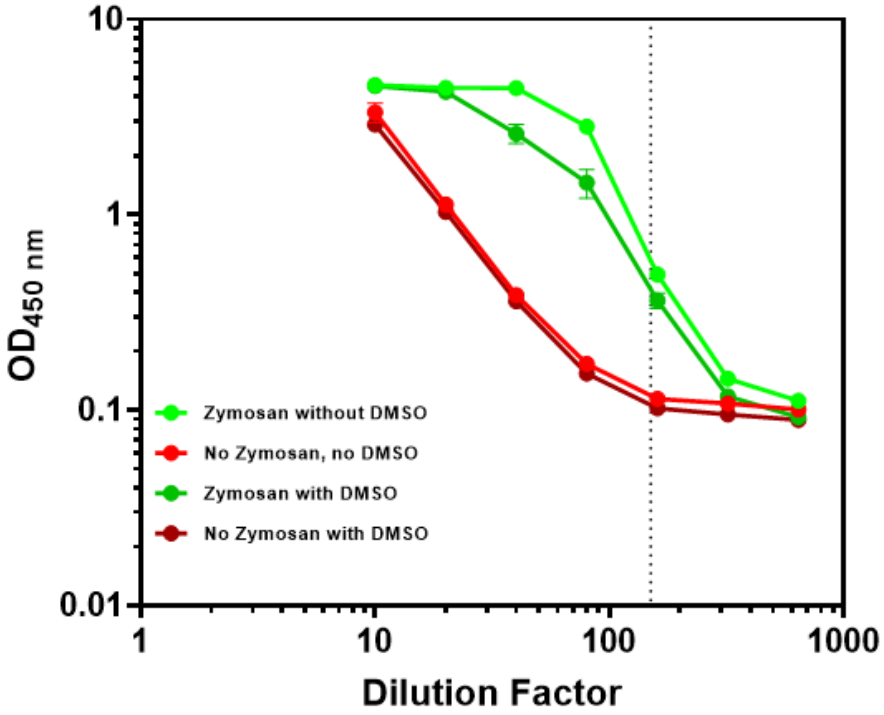


Figure 3.10 Serial dilution of activated and non-activated serum, with and without DMSO using the C5a ELISA.

Serum (6 donors) was incubated with 1% (w/v) zymosan A, in the presence and absence of [1% (v/v) DMSO] for 60 minutes. The reaction was stopped with 20 mM EDTA and the serum was titrated from 1 in 10 to 640 (2-fold serial titration) and the samples assayed using the C5a ELISA kit. The dotted line represents where $x = 150$. The assay was run in triplicate on a single plate.

Compounds were solubilised in 100% (v/v) DMSO and utilised in experiments using a final concentration of 1% (v/v) DMSO as a vehicle. A titration of serum was performed to determine the optimum concentration of serum to use for the C5a assay, and to determine the effect that DMSO might have on the results (Figure 3.10). The curves representing the activated serum (green) show a sigmoidal curve, suggesting the titration range was ideal to determine the optimum concentration. A concentration within the linear range was chosen (1 in 150) to ensure the assay was not saturated but remained sensitive enough to detect changes in C5a levels.

DMSO had little effect in the serum that was not activated by zymosan A. There was a lower absorbance for the serum activated by zymosan A in the presence of DMSO, however there was still a difference between activated and not activated. The difference may be due to the solubility of zymosan A being affected by DMSO, however it is more likely that due to the insolubility of zymosan A, there may be errors in pipetting the solution as the zymosan A settles, which could be indicated by the error bars.

The compounds were next included in the C5a ELISA assay in serum to determine the effect of the compounds on overall complement activation. In order to determine the effect of compounds on the binding of the C3 convertase complex, the complement pathway in serum was activated with zymosan in the presence and absence of compounds, and the samples were subjected to the C5a ELISA to determine the downstream consequences on C5a production.

3.3.2.2 The effect of Small Molecules on Complement Activation in Serum

The dilution of human serum chosen from the optimisation experiment (Figure 3.10) was 1 in 150. This concentration of human serum ensured the signal from the ELISA was within the linear range for the plate reader at 450 nm (Figure 3.10) and gave sufficient variability in signal strength for an increase or decrease in C5a production, furthermore there was also minimal background at this dilution.

The twelve compounds chosen to screen in the next phase were then run on the C5a ELISA assay (Figure 3.11). Compounds were used at a final concentration of 100 µM and at a final concentration of 1% (v/v) DMSO (vehicle) and were incubated for 10 minutes at 37°C. Serum samples were incubated with a final concentration of 1% (w/v) zymosan A or the equivalent volume of saline. Samples were then diluted in the diluent provided by the ELISA kit, and manufacturer's instructions (Appendix 3) were subsequently followed to completion.

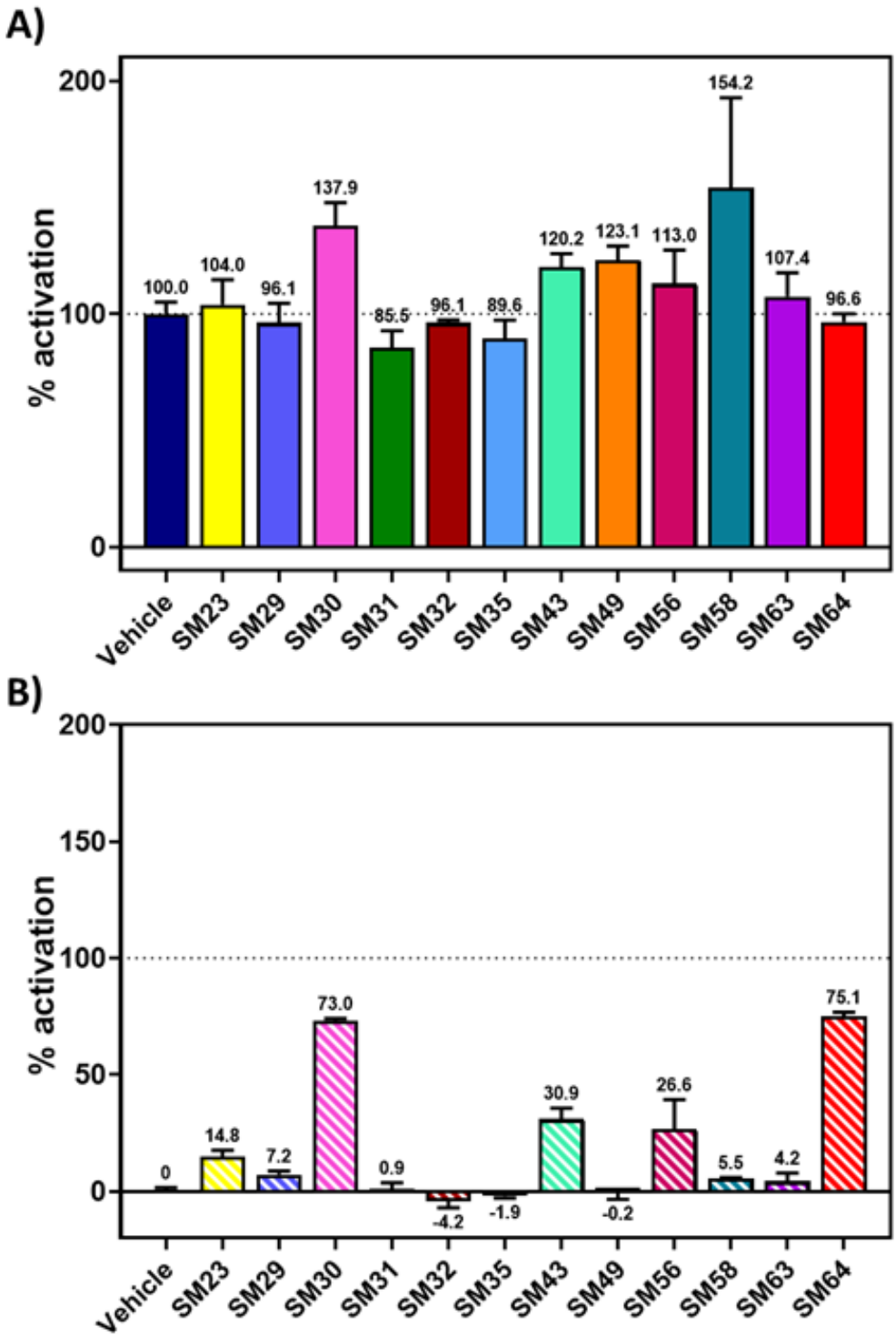


Figure 3.11 The effect of twelve small molecules on complement activation in serum, measured using a C5a ELISA kit.

Serum (6 donors) was incubated with (A) or without (B) zymosan A, in the presence of small molecules (SM). Vehicle controls were [1% (v/v) DMSO]. Values represent % activation normalised to the respective vehicle control.

The results from the C5a ELISA small molecule screen show that the effect of the compounds was variable and did not entirely follow the trends observed in the SPR data. The intra-assay coefficient of variation (CV) is <10% (Appendix 3) therefore any results within the range of the CV were considered to have no effect on complement activation measured by C5a levels. In 1% (w/v) zymosan A-treated serum SM58, SM63, SM49, SM30, SM23, SM56 and SM43 all enhanced C5a levels when compared to serum with vehicle alone, with SM58, SM49, SM30 and SM43 enhancing activation monitored by C5a levels by more than 20%. In contrast, SM32, SM31, SM35, SM29 and SM64 all decreased C5a levels with SM31 and SM35 decreasing complement activation by more than 10%. Interestingly, in the non-activated serum, some compounds appear to enhance C5a levels with SM30, SM56, SM43 and SM64 appearing to induce activation, determined by an increase in C5a levels by over 25%.

SPR is a purified system and the lack of inhibition in the C5a serum ELISA may be due to non-specific binding of the small molecules to plasma proteins such as albumin; plasma protein binding (PPB) modulates the amount of free drug available for pharmacologically efficacy. Variation in the level of PPB between compounds could potentially explain the differences observed between the purified SPR and serum systems. Another potential factor that could explain differences between the serum experiments and SPR data is the effects of naturally occurring regulators of the AP that are present in the serum, and potential effects on these regulators by the compounds. In the instances where complement appeared to be activated by the increased C5a levels (above the vehicle control) (Figure 3.11), this could be due to potential activation caused by the compounds, or it could be the enhancement of complement activity. During serum preparation, coagulation is activated by the presence of silica in the tube. This leads to contact activation of FXII, and it is already known that FXIIa can activate complement via the CP (Renne et al., 2012).

Given that serum is comprised of many activated coagulation and potential complement factors, experiments were performed to determine if plasma would be a better choice for examining the effects of compounds on complement activation.

3.3.2.3 C5a ELISA Optimisation

Serum is the blood component often used for complement assays. However, this may not be the most physiologically relevant way to test complement activation as FXII is activated during the serum separation process which may lead to downstream activation of complement via the CP. There may also be greater formation of inhibitor complexes during the serum preparation process from whole blood, therefore affecting complement activation in serum. Subsequently, normal pooled plasma (NPP) was investigated to determine its suitability for the use in small molecule screening assays.

Plasma was tested against serum. All samples were tested with and without 1% (w/v) zymosan A and incubated for 1 hour at 37°C (Figure 3.12A). NPP was treated with the synthetic peptide Gly-Pro-Arg-Pro (GPRP) to inhibit fibrin polymerisation. GPRP works by mimicking the activity of the E region; after release of FpA from the N-terminal sequence of Aα chain, a new N-terminal sequence is revealed, Gly-Pro-Arg. Therefore, GPRP works by inhibiting fibrin polymerisation by interfering with knob-hole interactions between the and E and D region (Lorand et al., 1998).

The dilution factor was reduced to 1 in 16 to ensure a signal as detected from plasma for Figure 3.12A, and to 1 in 32 for Figure 3.12B as it was expected that recalcification may result in a higher OD as complement may be further activated by activated clotting factors.

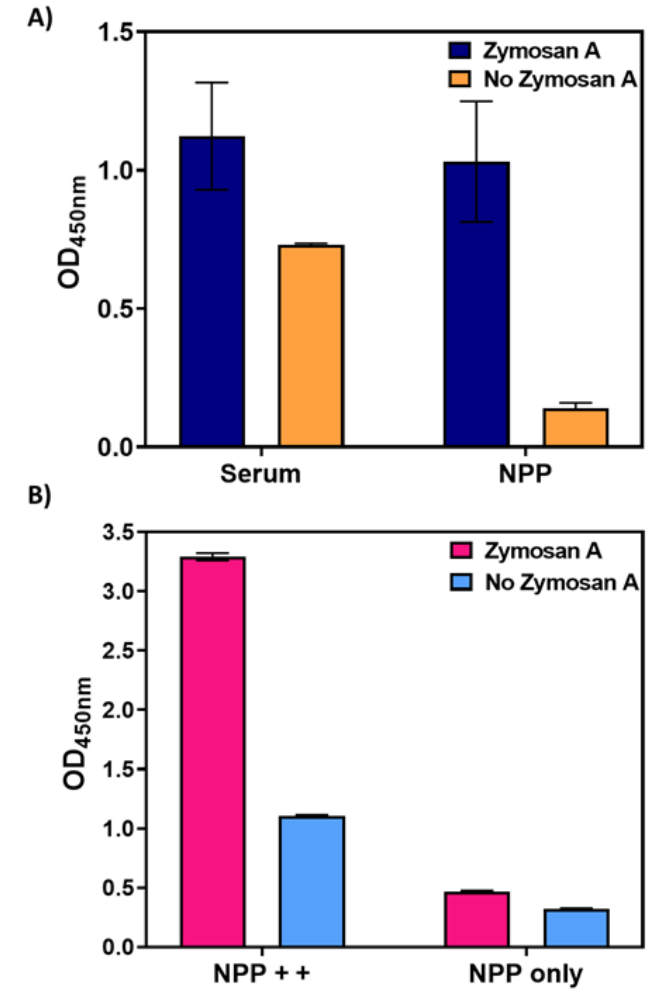


Figure 3.12 C5a ELISA using activated and non-activated serum and NPP.

A) Serum and citrated NPP were both diluted to 1 in 150 and 1 in 16, respectively. Samples were incubated with 1% (w/v) zymosan A or with saline (control) for 60 minutes. B) Citrated NPP was diluted to 1 in 32. Samples were incubated with 1% (w/v) zymosan A or with saline for 60 minutes. NPP was "recalcified" (NPP++) or was left citrated (NPP only) to allow activation of components that depend on ions.

Figure 3.12A shows that using serum may not be the best blood component for complement activity screening. There is a higher background signal with serum that is not activated by zymosan A when compared to NPP. This is likely due to the presence of the chelator, sodium citrate, in the NPP preventing the activation of clotting factors when plasma is static. The activation of clotting factors such as thrombin may result in complement activation, thus a higher level of C5a.

The effect of recalcification on C5a levels in NPP was then investigated to determine if the effect seen in serum (Figure 3.12A) was dependent on clotting factor activity and the resulting complement activation (Figure 3.12B).

Complement is activated in the recalcified NPP without incubation with zymosan A. This is likely due to activation of clotting factors such as thrombin. It would be useful to explore this mechanism as a time course and compare results with serum to evaluate whether this is underlying complement activation or if it is a result of thrombin generation over time. The results show that recalcified NPP treated with GPRP gives the biggest signal to noise ratio over the background. This was preferable to serum despite the limitation that there was background generation of C5a in NPP, which was most likely triggered from activation of coagulation after recalcification.

The plan was to test the small molecules in a NPP system; however, the supply of the compounds was extremely limited, and it was more important to use them in different assays. The next step was to break down the complement activation into the separate activation pathways.

3.3.3 WIESLAB® Functional ELISA

The WIESLAB® functional ELISA is a full complement screening test that can be used for diagnostics as well as for research purposes. Each test is specific to an activation pathway. The ELISA is functional in plasma or serum as it tests complement activation, by initiating complement through mechanisms specific to each pathway, and with pathway specific buffers. Agonists are coated on the bottom of the wells, and the samples are incubated in the specific buffers. The functional ELISA detects MAC that has formed on the activating surface, a similar concept to the in-house MAC ELISAs that were trialled at the start of this section.

The CP screen is activated via wells coated with Ig, allowing binding and activation of the C1 complex, thus forming the CP C3 convertase. The LP assay is activated via mannose-coated wells which allow binding and activation of MBL/MASP complexes, which in turn lead to formation of the LP C3 convertase. AP is activated by LPS-coated wells, allowing formation of the AP C3 convertase on the LPS surface. The contents of the buffers are not given; however, it is likely that the buffers contain EDTA-Ca^{2+} for CP and LP, and EDTA-Mg^{2+} for the AP.

The assay was performed according to manufacturer's instructions (Appendix 2).

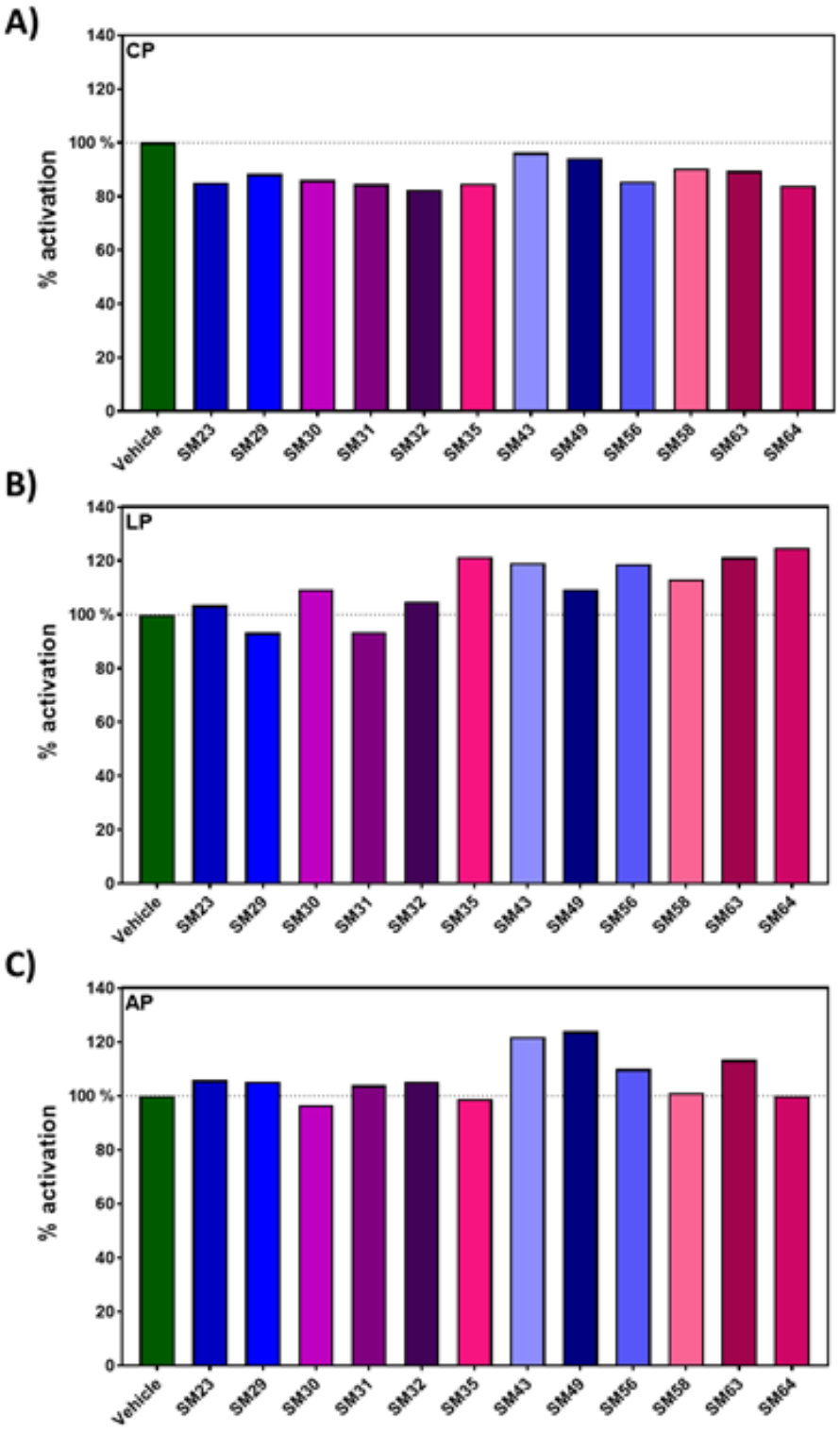


Figure 3.13 The effect of small molecules on complement activation via all three pathways.

Serum (6 donors) was incubated as per manufacturer's instructions (Appendix 4). Compounds were added at final concentrations of 100 µM compound and 1% (v/v) DMSO. A) Classical pathway (CP). B) Mannose-binding lectin pathway (LP). C) Alternative pathway (AP). Data were normalised with the vehicle as 100% activation.

The functional complement ELISA also showed variable results, with the highest level of inhibition achieved via the classical pathway (Figure 3.13A). The majority of the compounds appeared to inhibit CP activation by more than 10%, with only SM58, SM49 and SM43 less than 10% inhibition. LP activation was affected also (Figure 3.13B), however in this pathway, there was some increased activation, particularly with SM63, SM35 and SM64 which increased activation by more than 20%. None of the compounds inhibited LP activation by more than 10%. Surprisingly, AP was not inhibited by more than 5% by any compounds (Figure 3.13C), in fact most compounds enhanced AP activation. SM49 and SM43 enhanced activation by more than 20%.

This variability in results may be a sensitivity issue with the screening assay, however SPR is very different to a serum-based assay. There may be off-target effects of the compounds, which may modulate their effect, or the compounds may have differences in the amount of PPB, which may reduce the relative concentration of free, unbound compound. SPR on the other hand utilises purified proteins to investigate specific binding interactions between proteins or compounds. The compounds may also have a different effect on a protein that is immobilised when compared to fluid phase proteins in serum. It is also possible that crosstalk between coagulation and complement is taking place during serum preparation, and this may have an effect on the rate of complement activation as well as levels of activation. Inhibitory complexes may be form during the process which may prevent maximal complement activation; thus, the compounds may not appear effective.

3.4 C9 Western Blot

To further investigate the effects of the compounds of interest, a western blot was optimised to observe changes in C9 levels in serum. In theory, if a plate was coated with an agonist for complement activation and serum was incubated, the C9 would be pulled down to the surface as MACs were formed due to complement activation. This would therefore result in a decrease in C9 levels in the serum. The first step to this optimisation was to determine the optimal agonist. LPS and zymosan A are both used as agonists for complement activation (Figure 3.14).

As complement is activated by invading pathogens, the terminal pathway leads to MAC formation at the surface of the pathogen. MAC then inserts into the cell wall to form pores and induce lysis of the pathogens. In this assay, the surrogate pathogenic surface is the well coated with zymosan A or LPS. In theory, MAC would bind to the surface and would be pulled down out of serum, leading to a reduction in levels of serum MAC components, including C9, which can then be detected through immunoblotting. There may still be C9 in serum as MAC can also form as a soluble version which is available to be inhibited by vitronectin (Preissner et al., 1989) and clusterin (Tschopp et al., 1993), preventing MAC binding to the pathogenic surfaces.

Serum was incubated on a 96-well plate coated with 1 µg/mL zymosan A, 1 µg/mL LPS or vehicle (154 mM NaCl) for 1 hour at 37°C. The reaction was stopped using 20 mM EDTA and the serum was carefully removed from the wells to ensure the surface was not disturbed and was diluted to 1:10 before Western blot analysis.

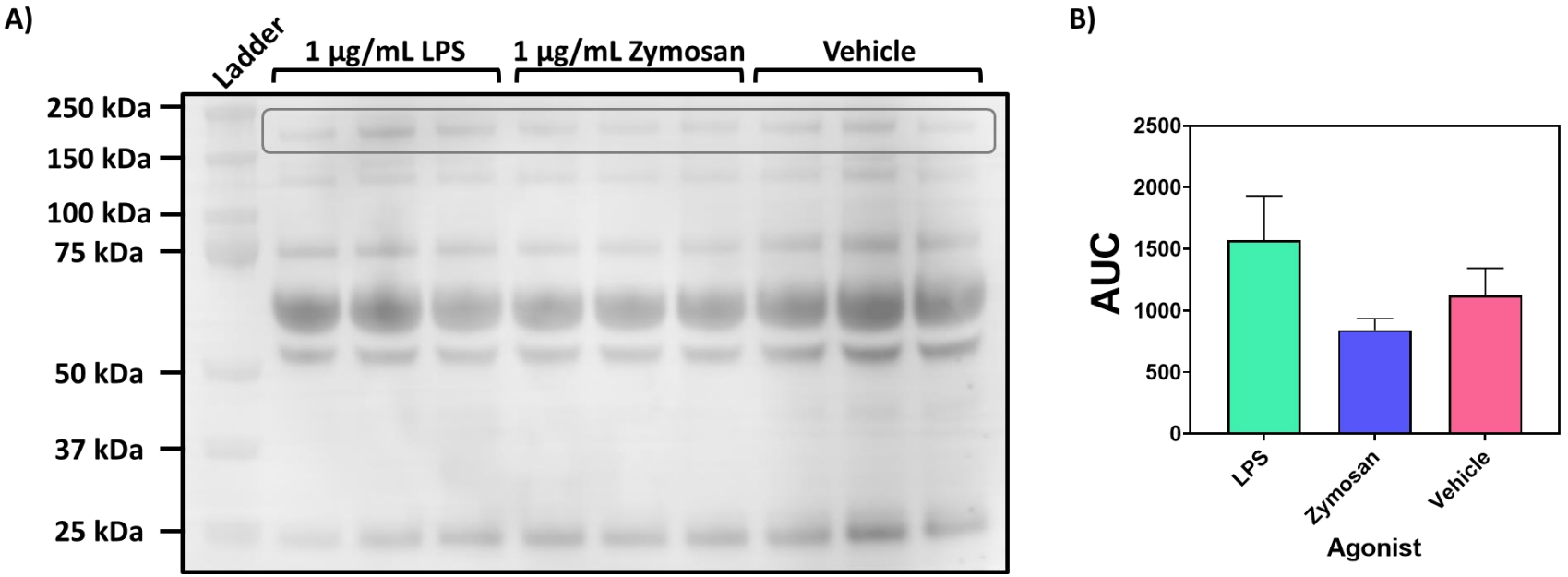


Figure 3.14 Zymosan A is a better agonist to induce depletion of C9 from serum.

Loss of C9 from serum (6 donors) was measured using Western blotting by determining the area under the curve (AUC) via band analysis in ImageJ. Serum was incubated with 1 µg/mL LPS, 1 µg/mL zymosan A or vehicle for 60 minutes at 37°C. A) C9 western blot. The grey box represents bands used for analysis. Each condition was run in triplicate as indicated. B) AUC represented as mean ± SEM.

The C9 antibody is a polyclonal antibody, therefore it is expected to see several bands. C9 has molecular weight of 71 kDa. However, during complement activation, C9 can polymerise giving rise to bands at higher molecular weights. The decision was made to analyse the poly-C9 bands, at around 210 kDa, as these appear to be more sensitive to the reaction.

Figure 3.14 shows that zymosan A can lead to partial depletion of C9 from serum, therefore this was the agonist carried forward for further experimentation. Zymosan A was titrated to determine the optimal concentration, and a C5a ELISA was run alongside the western blot to ensure the effects that were observed tallied with the C5a generation seen during complement activation (Figure 3.15).

NPP was used in the following assay, treated with GPRP to inhibit fibrin clot formation. Using NPP is more suitable than using serum, as previously described.

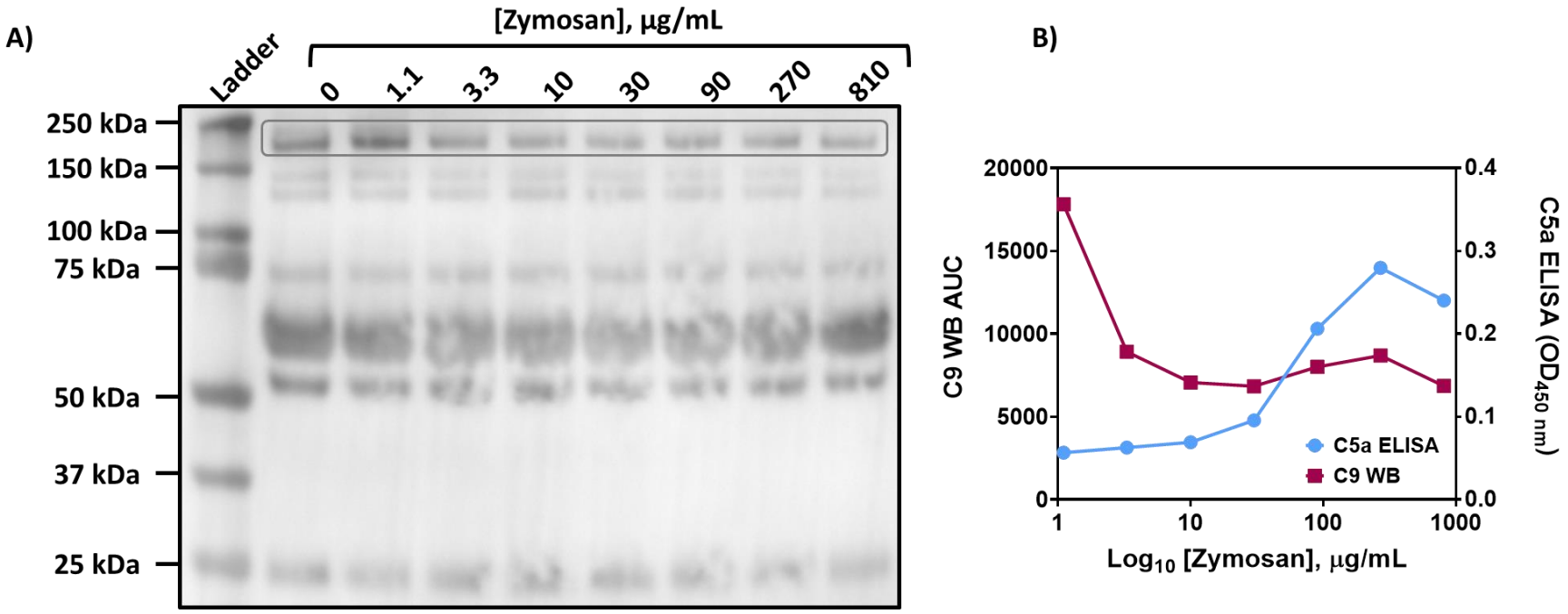


Figure 3.15 C9 depletion from NPP induced by zymosan A demonstrates a trend towards with increased NPP C5a, suggesting complement activation.

NPP was incubated with zymosan A titrated and coated on a 96 well plate. Samples were then taken and were diluted appropriately for analysis by western blot and ELISA. A) C9 western blot. Bands used for analysis are highlighted within the grey box. B) Dose dependent depletion of C9 is detected by western blot analysis, plotted as area under the curve (AUC) on the left Y axis. Dose dependent C5a generation was measured using ELISA, plotted as the optical density reading at 450 nm on the right Y axis (n=2).

The western blot analysis determines that complement activation by zymosan A does lead to partial depletion of C9 from NPP. This was supported by the dose-dependent generation of C5a measured using ELISA. Optimisation of this western blot to ultimately characterise compounds was put on hold, due to limited access to compounds.

It would be important to investigate different antibodies to ensure the best signal is achieved from the Western blot. A monoclonal antibody may be more useful as it would reduce background signal from multiple bands, making the assay more sensitive. It is clear however that the C5a ELISA demonstrated more sensitivity and would be a more useful tool in detecting effects of compound on overall complement activation, downstream of the AP C3 convertase.

3.5 Discussion

Complement is an innate immune response responsible for the initial recognition and opsonisation of pathogens and foreign molecules. Complement proteins have been conserved throughout evolution and is important for microbial recognition, phagocytosis and inflammation across many different species. Throughout evolution, the complement system has become more diverse, with the cascade separating into three activation pathways resulting in a terminal pathway necessary for pathogen lysis. This diversity not only allows direct pathogen recognition and clearance but is also required for immune cell regulation.

The classical and lectin pathways are best known for their “danger sensing” properties. C1q recognises antigen-antibody complexes on the surface of pathogens, which subsequently leads to activation of the CP (Thielens et al., 2017). C1q can also recognise misfolded proteins, such as β-amyloid fibrils seen in Alzheimer’s disease, and apoptotic cells via gC1qR and gC1qRp receptors, initiating apoptotic clearance via phagocytosis (Gaboriaud et al., 2011). MBL recognises carbohydrate complexes on pathogenic surfaces inducing the LP. MBL and C1 are similar complexes, which both recognise alternative structures (Bohlson et al., 2007).

Although there is some evidence to suggest that the AP may be activated in a similar way to CP and LP via pathogen recognition by FP (Spitzer et al., 2007), this regulatory protein is more commonly associated with the stabilisation of the C3/C5 convertase (Fearon and Austen, 1975a). Properdin enhances the activity of the convertase ten-fold and deficiencies in FP lead to susceptibility to meningococcal infections (Linton and Morgan, 1999). The AP has a different mechanism of activation to the CP and LP. C3 undergoes a “tick-over” (Lachmann and Nicol, 1973) or spontaneous hydrolysis leading to the exposure of an internal thioester bond, creating C3(H_2O). C3(H_2O) is rapidly degraded in the absence of a surface, however when a pathogen is present C3(H_2O) can bind to the surface via amide or ester bonds

with amino groups and carbohydrates on the pathogen surface (Ricklin et al., 2016b); FB and FP are then rapidly recruited inducing the assembly of the initial C3 convertase. Properdin has also been shown to bind modulate antimicrobial activity of natual killer (NK) cells (Narni-Mancinelli et al., 2017).

All three pathways lead to the terminal pathway where C5a and the lytic MAC are produced. C5a is a trigger for neutrophil activation, culminating in the adhesion of neutrophils to the vascular endothelial wall. C5a acts as a chemoattractant, recruiting immune cells to sites of injury and infection and MAC will induce lysis of pathogens via pore formation in the cell wall (Dunkelberger and Song, 2010).

Complement deficiencies often lead to an increased susceptibility to infections, particularly respiratory infections. CP and LP deficiencies share similar characteristics, as the C3 convertase components are the same. Autoimmunity is often a phenotype of patients with deficiencies, with those deficient in C1q being predisposed to systemic lupus erythematosus (SLE); patients with C2 and C4 deficiency are also at risk of SLE but this is much lower than C1q (Truedsson, 2015). MBL deficiency is relatively common, affecting up to 1 in 4 people. It often goes unnoticed, but patients with MBL deficiency may have recurrent infections and have a higher susceptibility to meningococcal meningitis and infections following bone marrow transplantation. MBL has been demonstrated to also have anti-inflammatory properties thus deficiency may influence the production of proinflammatory cytokines in monocytes and neutrophils (Dean et al., 2011).

Dysregulation of complement is a critical player in many conditions including AMD, aHUS and thromboinflammatory disorders such as DIC. This makes complement a promising target for novel therapeutics. AMD causes blindness and involves genetic variations of a variety of complement components including C3, FB, and C2, but also the key regulators FH and FI. AMD is classed as a chronic inflammatory disease and

is mostly caused by dysregulation of the AP, leading to a proinflammatory environment resulting in progressive damage to the retinal pigment epithelial cells. The border between these cells and the Bruch's membrane can rupture, which results in a loss of vision. Rupture of this border allows the development of yellow lipid deposits, drusen, and this can be observed in early AMD. Drusen have been reported to contain many complement components and share this characteristic with other diseases including atherosclerosis and amyloidosis (Mullins et al., 2000). C3 plays an essential role in the development of AMD, with C3 knockout murine models demonstrating a resistance to choroidal neovascularisation (CNV) thus it is a promising therapeutic target for the development of AMD (Bora et al., 2005). C5 is also an attractive target that is downstream of C3 thus a safer target to limit inhibition of important mechanisms such as opsonisation of pathogens however research is conflicting with some claiming that C5a inhibition is effective at reducing vascular leakage and CNV in mice (Brockmann et al., 2015), but others stating that it is insufficient to inhibit AMD progression (Toomey et al., 2018). FD is a key component that circulates as an active serine protease necessary for AP activation via rate-limiting cleavage of FB. Inhibition of FD would inhibit the AP only, without affecting the CP and LP, unlike inhibiting C3 or C5, and is therefore a target of high interest (Wu and Sun, 2019). Properdin is the only positive regulator for complement and works via an amplification loop in the AP. Evidence suggests that inhibition of FP in AMD may reduce CNV. This is important as 50% of CNV membranes in AMD patients contain FP and this correlates with the patient having worse symptoms (Wolf-Schnurrbusch et al., 2009). Variations of FB have been demonstrated to be associated with the development of AMD. FB is the serine protease that amplifies the AP, thus may be a sensible therapeutic target for AMD (Gold et al., 2006).

PNH is a complement-mediated haemolytic disorder that arises as a result of an acquired deficiency of complement regulators due to the lack of the lipid anchor

glycosylphosphatidylinositol (GPI) (Miyata et al., 1993). GPI is an anchor that attaches to the C-terminus of a protein during post-translational modification. It allows a variety of proteins to bind at the cell surface including complement regulatory proteins CD55 and CD59 (Schubert and Röth, 2015). CD55 accelerates the degradation of the C3 convertase complex (Murray et al., 2000), thus the loss of CD55 from the membrane of erythrocytes culminates in uncontrolled complement activation at the cell surface. Opsonisation by complement fragments is increased which leads to MAC formation. The lack of CD59 results in dysregulated MAC formation as C9 can aggregate in the absence of the inhibitor forming the lytic pore, resulting in intravascular haemolysis and anaemia (Karbian et al., 2018). The main complement-directed treatment is eculizumab, targeting C5, inhibiting the formation of C5a and C5b, which ultimately prevents MAC formation, reducing the haemolysis and anaemia. Research has recently demonstrated that PNH may be treated using mAbs targeting FP which may also have a higher efficacy than eculizumab (Gullipalli et al., 2018). This may be due to the difference in plasma concentrations, with FP being relatively low, thus less inhibitor would be needed to generate a similar effect. Properdin also only plays a role in AP activity, although a critical component, inhibition of FP would not affect other complement activation mechanisms.

DIC is a serious thromboinflammatory complication that arises as a result of dysregulation in coagulation and inflammation pathways caused by several issues including sepsis, trauma and cancer. Complement dysregulation has been observed in DIC as a result of sepsis and C3 convertase inhibition efficiently inhibits sepsis induced complement activation in a baboon sepsis model, reducing organ damage by the deposition of C3b, MAC and MBL (Silasi-Mansat et al., 2010). Silasi-Mansat et al. also demonstrated that inhibition of the C3 convertase reduced thrombocytopaenia and DIC in the baboons. Complement can directly affect the thrombotic phenotype of DIC, through platelet activation (Polley and Nachman, 1983)

and increased TF expression (Esmon, 2004). In addition, C5a can indirectly inhibit fibrinolysis via the upregulation of PAI1 release from immune cells (Wojta et al., 2002). Properdin knockout murine models are resistant to DIC in the localised Shwartzman reaction (Ali, 2005) suggesting that FP is a key player in the culmination of microthrombi, however the mechanisms behind this remain unknown.

Complement inhibition is clearly supported by a variety of research in a plethora of disease states, however there are risks. Complement deficiencies lead to a higher prevalence of bacterial infections, particularly meningococcal infection leading to respiratory tract illnesses including pneumonia. Therefore, it is important to compare risks versus benefits. In many diseases, treatment via complement inhibition will be time-dependent, and will need prophylactic antibiotic treatment to prevent bacterial infection (Silasi-Mansat et al., 2010).

Many complement therapeutics, including eculizumab, are biologic treatments such as monoclonal antibodies. There are many advantages to a biological agent as a therapeutic. They are very specific and have minimal off-target effects. They do however require invasive administration techniques, such as subcutaneous or intravenous injection. Small molecules are chemical compounds that can be manipulated to have high specificity and they can be administered orally, making them more desirable to the patient. They are also small enough to be able to cross cell membranes if necessary, however they may have unwanted off target side effects.

Drug discovery and development provides insight into the affinity and specificity of small and large molecules towards their targets. It allows investigation into toxicity and off-target effects and is therefore a vital stage in the drug development process to ensure novel therapeutics have the necessary adsorption, distribution, metabolism and excretion (ADME) properties. Binding assays such as SPR are an important first

stage of the screening process to ensure compounds with poor binding properties and undesirable effects can be eliminated (Bio-Rad, 2020).

SPR is often used to determine interactions between small molecules and their targets. It provides high-throughput kinetic data in real-time to generate affinity values and aids in determining the specificity of binding partners. Using SPR, it is possible to screen large libraries of small molecules in a relatively short time, allowing quantitative ranking of chemical compounds, and elimination of those that are less desirable. The platform used during this project is the Pioneer, which differs from other SPR platforms in that kinetic values can be obtained with one single inject instead of manually titrating the analytes which reduces error. The Pioneer uses Taylor dispersion to automatically titrate the analyte over the immobilised ligand, causing a concentration gradient within the flow cell. This technique requires a lower volume of analyte and does not require steady state to be reached to obtain the kinetic information. The Qdat software, specific for Pioneer platforms, recognises the OneStep® inject and processes the data to calculate the analyte concentration over time.

First, two proof of concept assays were performed to determine the binding properties of the C3 convertase components of the AP. It was confirmed that FP binds with high affinity to C3b, with a KD of $2.24x10^{-20}$ M. Properdin was also shown to enhance the binding of FB to C3b. The binding interaction of FB alone with C3b had a KD of $3.9x10^{-6}$ however when the C3b was coated with FP, the KD of FB to the complex increased to $2.5x10^{-7}$, demonstrating the positive regulation necessary for AP activity.

Small molecules were subsequently screened using these components of the AP C3 convertase. C3b was immobilised while FB and FP were injected together in the presence and absence of the compounds, to determine the effect of the small molecules on the binding of the complex. Three plates of compounds were provided

by the School of Chemistry, from the compound library at the University of Leeds. Controls of FB & FP were run throughout each assay to detect chip degradation, which is common after regeneration steps. Results were normalised to these controls and were plotted as % binding. Forty-one of ninety-five compounds were chosen to be taken to the next stage of the SPR screening; nine inhibited complex binding by 5% or more, three were negative controls that did not affect complex binding and twenty-nine enhanced the binding of complexes by 5% or more. At this stage, any interaction was of interest as it showed the potential for the compounds to modulate the interaction between the complement components.

An affirmation screen was then performed to determine the effects of the forty-one chosen small molecules. Plates of compounds from the library were again screened in a similar manner to the previous plates, after which twelve compounds were taken forward to the third stage.

To determine if the orientation of the complex affected the interaction between the proteins and the small molecules, FP was immobilised, and C3b&FB were the analytes in the presence and absence of the twelve compounds. Interestingly, eleven compounds inhibited complex formation by more than 60%, whereas one enhanced complex formation by almost 200%.

To further investigate the interactions that were taking place, a OneStep® assay was performed to determine the binding kinetics of the small molecules to C3b and FP. C3b was immobilised to FC1 and FP to FC3. The compounds were then injected using the OneStep® method and kinetic data were calculated. All compounds bound to both C3b and FP. SM29 bound with highest affinity to C3b and FP with a KD of 1.8x10-7 and 4.6x10-11 respectively. Interestingly, SM64 which increased the complex interaction previously, bound tightly to both C3b and FP with a KD of 4.6x10-7 and 4.8x10-6 respectively, but the RU was also greatly increased when binding to

FP. This may partly explain the increased response observed in the previous assay where SM64 appeared to increase the response of the complex components by almost 200%.

The components of the C3 convertase were then separated, to determine the effects of the small molecules on each binding partner. C3b was immobilised and FP, FB and FP&FB were injected as analytes in the presence and absence of the compounds. Eleven of the small molecules demonstrated an inhibitory effect between FP and C3b. SM30 had an interesting effect on the binding of FP and C3b. only the dissociation was affected, which suggests that the complex between C3b and FP could still form, however it was less stable and degraded much faster. SM64 had the opposite effect on the FP and C3b interaction that was previously observed when FP was immobilised, and in this instance, it acts as the best inhibitor. It is important to take note of this, as there may be different mechanisms at play depending on which protein is immobilised or surface bound. Many of the compounds had little effect on the binding of C3b and FB, however SM23 and SM35 appeared to slightly enhance the response of the interaction. This may be due to the binding of the compound to C3b enhancing the response. All twelve of the compounds have an inhibitory effect on total complex formation, when FP&FB are injected together. SM64 demonstrated the most significant inhibitory effect on complex formation. This change in the interaction between the C3 convertase complex and SM64 may have been due to the centrifugation of FP before the assay to eliminate non-physiological aggregates. The aggregation of FP may have increased the affinity of FP towards SM64.

The next phase of the screening was to optimise ELISAs to observe the downstream effects of the compounds in a more physiological setting. SPR is a purified system and does not take into account the entire interactions in the physiological setting.

Optimisation of an ELISA to measure MAC formation was initiated, to determine the effect of the compounds on complement activation in serum. The theory being that if a plate was coated with a complement agonist, MAC would either form at the surface and could be measured using an antibody to detect C9, a major component of the MAC; the serum could also be decanted and used in a sandwich ELISA, where the MAC could be captured by a C5 antibody, and detected with the same C9 antibody. Many issues arose during the optimisation process. High background, with low signal, suggesting off target interactions of the detection antibody. Many blocking methods were tried and tested to no avail, as well as wash methods and activation times. It was eventually decided that an alternative approach should be pursued, and a commercial C5a ELISA kit was purchased. C5a is a useful component to measure complement activation, as it is also classed as a terminal product of complement activation. Commerical ELISA kits are often used in clinical and research settings as they are already validated.

Zymosan A, a polysaccharide from yeast cell walls, was used as an agonist for complement activation. It is commonly used as a complement activator and can activate all three pathways. Zymosan A is composed of mannan allowing LP activation and it also acts as a surface for AP activity (Harboe et al., 2012). Although less efficient, most humans have antibodies against yeast which ultimately causes CP activation via antibody-antigen complexes.

As the small molecules are solvated in DMSO, an important step was to analyse the effect of DMSO on the serum activation of complement. It was determined that DMSO did not affect the C5a levels in serum that was not incubated with zymosan A, however there was a small difference when complement was activated. The error bars were larger for the serum incubated with DMSO and zymosan A suggesting intra-assay variation. This assay also allowed the determination of the optimal dilution of serum to use in future experiments as the serum was titrated. A dilution factor

within the linear range was chosen (1 in 150) to ensure saturation was not reached and sensitivity was not lost; background absorbance was also lowest at this concentration and below.

The compounds were then screened using the C5a ELISA kit, at a final concentration of 100 µM. The serum was incubated with either 1% zymosan A or the equivalent volume of saline, to allow sufficient activation of complement. The reaction was stopped with EDTA to ensure further complement activity did not occur on the ELISA plate. The effect of the small molecules was not consistent with the SPR data, suggesting off-target effects. Over half of the compounds enhanced complement activation, with four of them enhancing the activation by more than 20%. Four of the compounds also appeared to increase the activation by more than 25% in the absence of zymosan A, suggesting potential complement activation properties of the compounds. The lack of inhibition seen in the ELISAs is likely a result of PPB, reducing the amount of free drug concentration. The serum may also have residual complement activity due to the serum preparation process which may lead to variation of results.

NPP was next investigated to reduce the potential background complement activity that may result in the inconsistent C5a levels. GPRP was used to ensure fibrin clot formation was inhibited, which allows the plasma to remain in a fluid state which is easier to manipulate and test using an ELISA. The optimisation step determined that complement activated more efficiently in NPP than in serum. This may be due to the consumption of complement components during the serum separation process or the formation of inhibition complexes but may also be explained by the presence of fibrin(ogen) aiding the complement activation mechanism. Fibrin(ogen) has been shown to augment the LP in murine plasma via interactions with ficolin and MBL (Endo et al., 2010). Endo et al. demonstrated that fibrin(ogen) enhanced the binding of ficolin and MBL to *Staphylococcus aureus* which led to enhanced C3 and C4

cleavage. As LP can be activated by zymosan A, this is a reasonable explanation as to why there is an enhanced signal for C5a in plasma, when compared to serum which is depleted of fibrin(ogen) in the serum preparation process.

Subsequently, a commercial ELISA kit was sourced that had been optimised to test the activity of all three pathways of complement separately. The WIESLAB® Functional ELISA screens each pathway using serum. For this assay, NHS was prepared. The kit provides negative and positive controls, however in-house controls were also used. The kit works by immobilising pathway specific agonists to the bottom of the wells, in a similar manner to the theory behind the MAC ELISA previously described. Serum was then diluted in pathway specific buffers to ensure activation of the other pathways is inhibited using chelators and ions. MAC deposition on the surface was then measured using a MAC specific antibody that detects a neoepitope on C9, which is exposed upon C9 polymerisation.

The compounds were incubated with the serum in accordance to manufacturer's instructions (Appendix 4). The small molecules appeared to inhibit the CP more than the LP or the AP. The LP showed some increased activation with ten of the compounds which was unexpected, and the AP mostly exhibited increased activity in the presence of compounds. Interestingly, SM64 had no effect in the AP, but increased the LP by almost 25% and decreased CP activity by over 15%. It is important that purified SPR assays are followed up by serum/plasma-based assays to detect the influence of compounds on other mechanisms.

Structural and functional modifications of small molecules can be performed to manipulate the binding, increasing specificity and affinity for their targets which may only be determined once these serum and plasma assays are completed (unless structurally related potential off-target proteins are known and can be tested in purified systems).

The optimisation of Western blots to measure complement activation was started to be able to determine further how the compounds were working. Serum and plasma can be used in Western blots due to the specificity of antibodies. Western blots are a useful tool in research as they allow several mechanisms to be explored. Detection of specific proteins can be performed, to ensure samples contain the target of interest and protein abundance can be determined. Cleavage and aggregation of proteins can be tested, and this can help to elucidate activation and inhibition profiles. A Western blot was optimised to determine the polymerisation of C9, and loss of C9 in serum and plasma, to evaluate small molecule inhibition of MAC formation. Serum was incubated with zymosan A, LPS or the vehicle and was prepared for Western blot analysis. This was tested in triplicate. LPS is a well-known activator of complement, especially the AP. The results revealed that zymosan A was a better activator of total complement when compared with LPS and the vehicle. The density of the bands that represent polymerised C9 was performed to determine complement activation. The more activity of complement, the lower the density of the bands as MAC deposited on the agonist surface. NPP was then used in the following assay, as complement activation was more efficient as previously described. This assay was run alongside a C5a ELISA to support what was being observed; C9 clearance from NPP followed a trend with an increase in C5a.

However, the data generated suggests that the use of plasma rather than serum was better for detection of monitoring the effects of compounds on complement activation and unfortunately due to a limited access to the compounds and the laboratory closing due to COVID-19, these experiments were unable to be continued within the time frame for this thesis.

3.6 Considerations for Future Work

This chapter has highlighted how a small molecule screening programme is optimised, and the importance of purified and serum/plasma-based assays in the elucidation of the mechanisms of action. This work is still in the initial stages of a compound screening project, and though access to the compounds may be limited, there is potential here that warrants further investigation and will be pursued in our laboratory.

Analogues of the small molecules that have been used in this project are available by the School of Chemistry to be evaluated. As these assays are now optimised, this can be done relatively quickly and efficiently to determine which compounds should be taken through a screening cascade for a drug discovery programme.

It is also important to investigate how these compounds may affect coagulation and therefore thrombosis and would be a component of a future drug discovery programme to ensure the compounds do not interfere in coagulation using i*n vitro* and *in vivo* models available at the University of Leeds. There are many basic clotting assays that can be utilised, including turbidity & lysis, and assays to measure clotting times. These assays can be initiated via both pathways of coagulation and can therefore be used to determine specific mechanisms of action, if any exist.

An important part of this investigation is to determine how the compounds may affect cell to cell interactions, i.e. platelets interacting with neutrophils. Research states that these interactions are important in the development of DIC. This can be evaluated using *in vivo* models of sepsis and DIC but can also be investigated using washed platelets and leukocytes.

A different model of DIC may be useful to examine with what cause of DIC this therapy may be most useful. The localised Shwartzman reaction is an important tool

to research DIC however it uses purified initiators, LPS and TNF-α. It would be interesting to optimise a sepsis or trauma model that leads to DIC.

In hindsight, endpoint assays such as the ELISAs and Western blots used in this chapter, may not be the most useful tool to investigate a complement therapeutic. However, there does appear to be utility in the use of plasma activation of complement followed by the use of a C5a ELISA for best sensitivity of compound efficacy. The AP in the initial complement activation pathway, surveying the environment for pathogen and spontaneously activating. This is followed by activation of the CP and LP, thus investigating different time points may be more beneficial to evaluate the effect of the compounds on complement activity. This could still be performed using the ELISAs and Western blots, with modifications to the existing protocols.

These studies would support the need for the generation of a novel therapeutic to target DIC and would elucidate some of the mechanisms behind the crosstalk of complement and coagulation pathways.

Chapter 4 A New Connection between the Complement and Coagulation Cascade

4.1 Introduction

It has long been known that crosstalk between the complement and coagulation pathways exists, yet there remains a lack of understanding of the ways in which these pathways interact. Dysregulation of each pathway can give rise to life-threatening consequences and can have an impact on the other pathway. Important interactions take place between the contact activation pathway of coagulation and the CP and LP of complement, but research to determine the role of the AP of complement in coagulation is limited. The serine protease cascades of the complement and coagulation systems play a central role in thromboinflammation and these systems are descended from a common ancestor (Krem and Di Cera, 2002). The two systems should not be considered as separate entities, even though the interactions between the two are yet to be completely defined (de Bont et al., 2019). Proteases from each cascade have similar structural and functional characteristics. Some interactions have been defined previously such FXIIa activation of the CP of the complement system (Ghebrehiwet et al., 1981). The common pathway of complement can also be initiated through the cleavage of C3 by PKa (Irmscher et al., 2018). It has also been demonstrated that the LP of complement may interact with the KKS through MASP-1 cleavage of HK leading to BK release (Dobo et al., 2011). The main route of coagulation activation by a complement component is the cleavage of prothrombin to thrombin by MASP-2 (Krarup et al., 2007). It is well known that the primary inhibitor of the classical pathway, C1-INH can also inhibit the intrinsic pathway of coagulation (Davis et al., 2010) through inhibition of FXIIa, FXIa and PKa (Davis et al., 2008), demonstrating clearly how both systems are intricately related and have functional similarities. This chapter aims to better define the relationship between components of the AP of complement and coagulation factors from the contact pathway specifically.

4.2 FXI and FXIa Bind to both C3b and FP with High Affinity

Initially SPR was utilised to investigate whether any coagulation factors could be potential binding partners for C3b and/or FP.

After chip installation and preconditioning with 0.1% SDS, 10 mM HCl, 50 mM NaOH, C3b and FP were immobilised at the sensor surface on FC1 and FC3 respectively, with the treated blank FC2 as the reference channel. Once the ligand was immobilised the sensor-chip was primed three times and the matrix was preconditioned with 1, 2 and 3 M NaCl, and with 1, 2 and 3 mM NaOH to prepare the protein for regeneration. The assay was run using RBTM++ as the running buffer. Analytes were dialysed into running buffer. C3b and FP were immobilised to FC1 and FC3 to 85.90 RU and 194.02 RU respectively. FC2 was used as the reference channel with no protein immobilised.

Analytes (Table 4.1) were injected using a OneStep® 100% of loop inject at a flow rate of 30 µL/min, with a dissociation period of 300 seconds (Figure 4.1). The chip was regenerated using a fast injection of 5 µL regeneration cocktail of 1 M NaCl, 3 mM NaOH 60 µL/min with a dissociation time of 30 seconds.

Table 4.1 Analytes used in the SPR assays determining the binding of coagulation factors to immobilised C3b and FP.

Analyte	Final Concentration (nM)
FXI	50
FXII	100
IF1 purified fibrinogen (IF1-FBG)	50
Prekallikrein (PK)	100
Prothrombin (FII)	500
Plasminogen (PLG)	500
FIX	100
FX	250
FVII	250
FP	25
FXIa	50

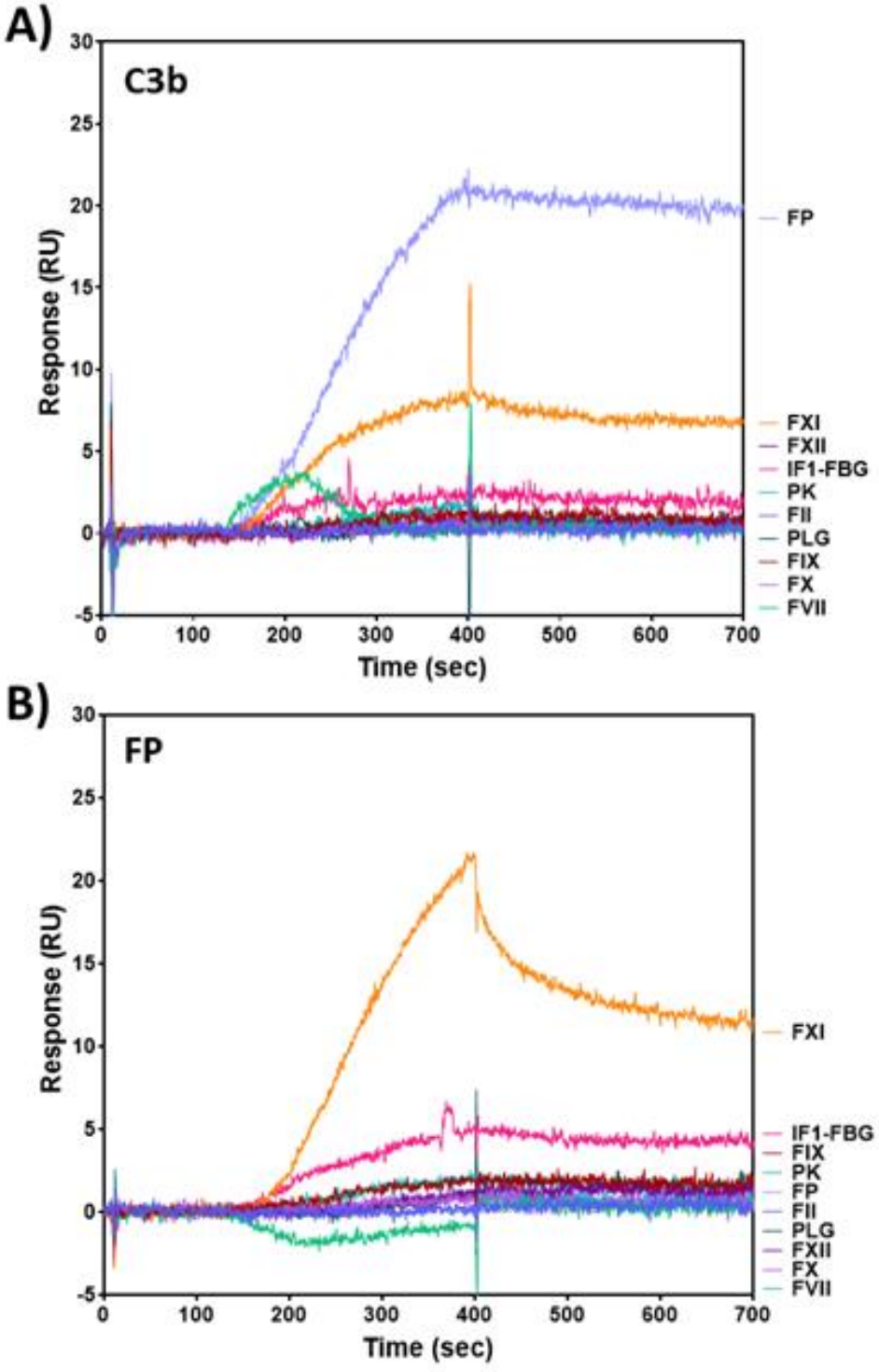


Figure 4.1 SPR reveals FXI binds to both C3b and FP.

A) C3b and B) FP were immobilised on the sensor surface. Factors of the coagulation cascade were injected over the surface at various concentrations. A concentration scout was performed before this assay. 25 nM FP was injected to serve as a positive control. Data were analysed using Qdat software, and GraphPad Prism v8 was employed for graph editing. Curves represent the mean of triplicates run in one assay. *IF1-FBG, fibrinogen purified with an IF1 column. PLG, plasminogen.*

SPR revealed that FXI binds to both C3b and FP. It also revealed that fibrinogen might bind to both FP and C3b, with a maximum response of about 3 RU and 5 RU respectively, however this is yet to be explored further. A higher concentration of fibrinogen may be necessary to be able to determine the relevance of this binding. Properdin was also injected as a control to check the viability of the chip, and this bound with high affinity to C3b, but not to immobilised FP. Factor XI appeared to bind with high affinity to both C3b and FP, with a maximum response of around 17 RU and 22 RU respectively. Further to this, FXIa was also injected in a separate assay to determine if the active form of FXI would also bind to C3b and FP (Figure 4.2).

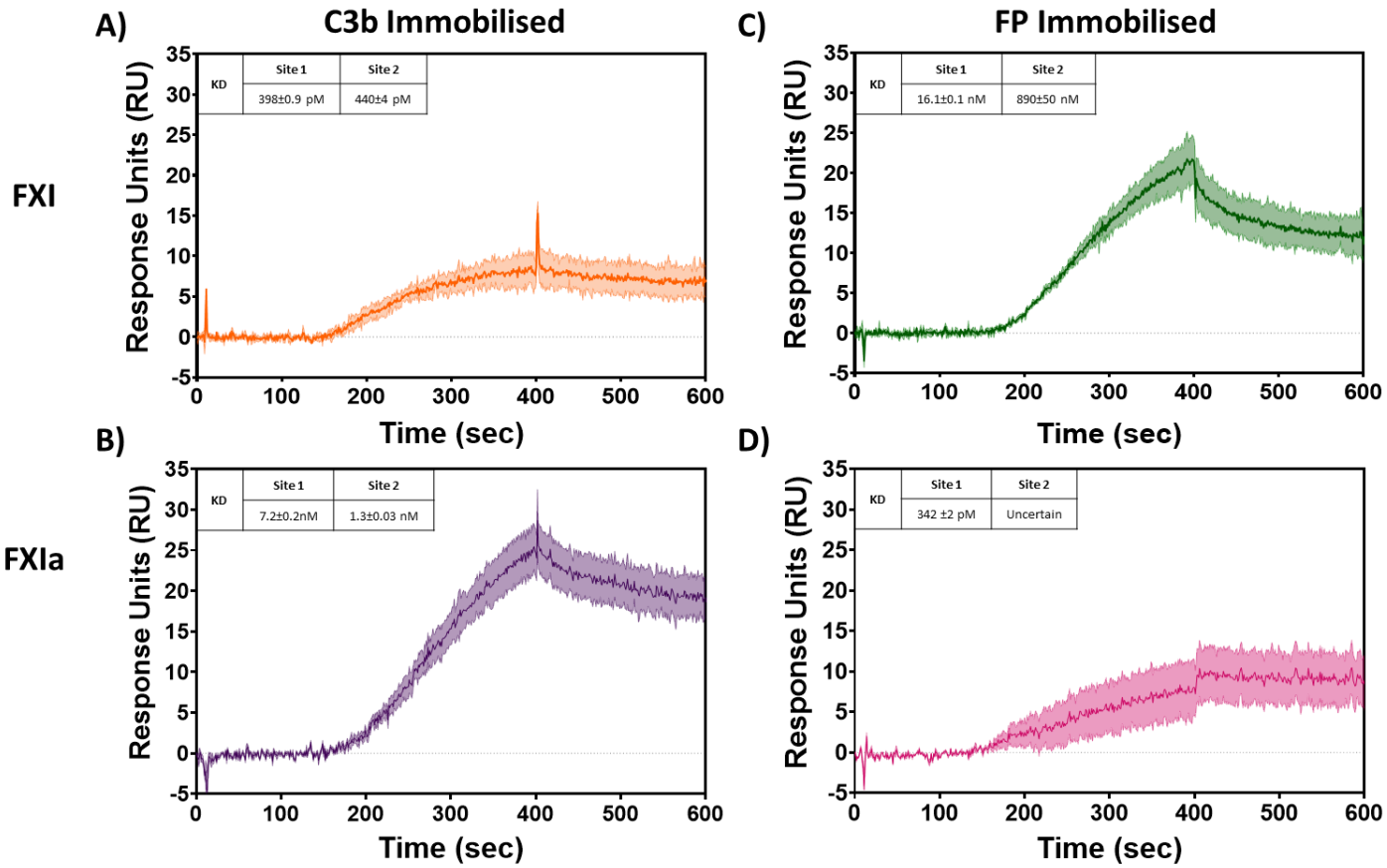


Figure 4.2 SPR determines that FXI and its activated form, FXIa, bind to C3b and FP.

This figure is composed of two separate assays. “A)” and “B)” are results from the previous assay. “C)” and “D)” are results from a new assay, using the same chip. A) FXI binds with high affinity to C3b. B) FXI binds with high affinity to FP. D) FXIa binds with high affinity to C3b. D) FXIa binds with high affinity to FP. Data are expressed as mean of triplicates run in a single assay ± SEM. SEM are represented by the lightly coloured areas around the curves. Binding affinity is represented by KD ± SEM, calculated using Qdat.

SPR revealed that FXI and FXIa both bind to C3b and FP when immobilised to a sensor surface (Figure 4.2). Analysis of the data in Qdat was performed to a 2-site model, however, Site 1 is of most importance. Factor XI bound with high affinity to C3b and FP with a KD of 398±0.9 pM and 7.2±0.2 nM respectively. Factor XIa also bound to C3b and FP with high affinity, with a KD of 16.1±0.1 nM and 342±2 pM respectively. The high affinity of this interaction suggests the possibility of it being a physiological mechanism as does the higher affinity of FP for FXIa when compared to FXI, therefore this was further explored using FP. Properdin was taken forward as the main molecule of interest, as it is the only known positive regulator of complement and it may be a novel regulator of intrinsic pathway activity.

To understand why the binding interactions of FXI and FXIa to FP may be important, chromogenic assays were employed. Dextran sulfate (DXS) is a glycosaminoglycan (GAG), often used in the literature as a surface for contact activation of the intrinsic pathway, and as a mediator for C1-INH inhibition of FXI (Wuillemin et al., 1997). Factor XII can gain activity at the surface of DXS, and it has also been demonstrated that DXS acts as a template for FXI autoactivation (Naito and Fujikawa, 1991a). The template mechanism (Figure 4.3) works as an activation, as well as an inhibition mechanism, and this is demonstrated by a bell shaped or a Gaussian curve (Figure 4.3). The surface acts as a template for the enzyme and substrate to colocalise, culminating in substrate cleavage. However eventually, the surface concentration can increase to a point where the reaction is hindered and effectively blocks the enzyme/substrate interaction.

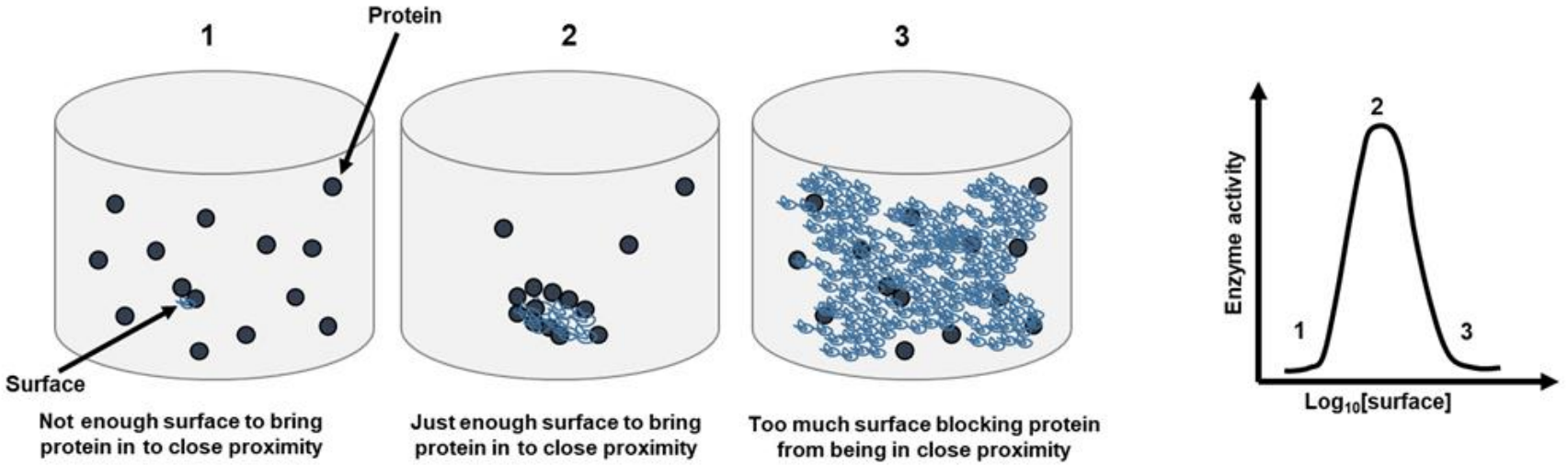


Figure 4.3 The template effect.

Surfaces can exert a template effect – a template for enzyme activation to take place. There is a fine balance where an optimal concentration of surface is needed in order to achieve maximal enzymatic activity

4.3 DXS acts as a Template for FXI Autoactivation

In the first instance, it was important to perform a proof of concept assay, to ensure the experimental conditions were optimal. The optimum concentration of DXS was determined by employing a chromogenic autoactivation assay of FXI (Figure 4.4). Titrations of 40 kDa DXS (DXS_{40kDa}) and 500 kDa DXS (DXS_{500kDa}) were incubated with FXI zymogen in HBS pH 7.4, with 500 µM S-2288 and readings were taken over a course of up to 5 hours. Assays were run in the presence of 1% (w/v) PEG to ensure surface interactions with the well were minimal.

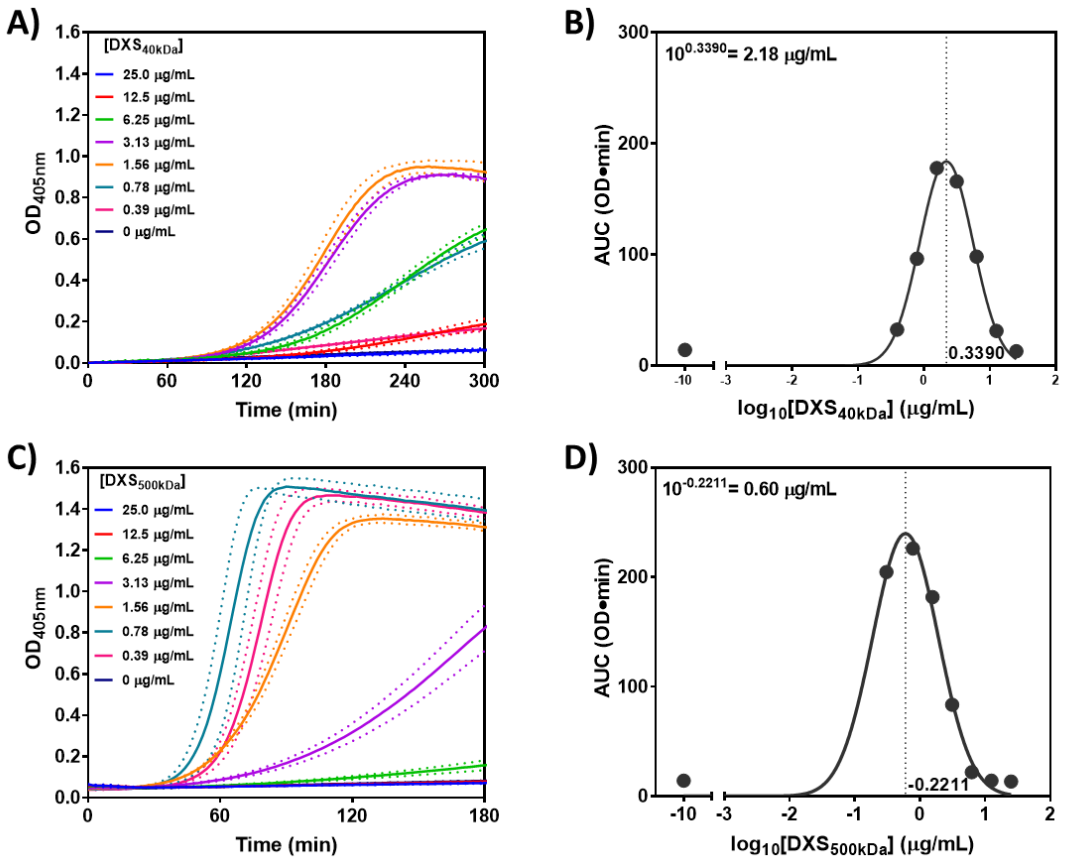


Figure 4.4 DXS causes autoactivation of FXI via a template mechanism.

30 nM FXI was incubated with DXS_{40kDa} (A and B) or DXS_{500kDa} (C and D) and autoactivation of FXI was measured by cleavage of 500 µM chromogenic substrate S-2288 over a course 5 and 3 hours respectively. A) Baseline-corrected raw data of FXI autoactivation with a serial dilution of DXS_{40kDa}. B) Bell-shaped curve demonstrating the template effect of DXS_{40kDa} and an optimal concentration of 2.18 µg/mL for autoactivation. C) Baseline corrected raw data of FXI autoactivation with a serial dilution of DXS_{500kDa}. D) Gaussian curve demonstrating the template effect of DXS_{500kDa} and an optimal concentration of 0.6 µg/mL for autoactivation. Raw data were plotted as optical density (OD) read at 405 nm vs time (minutes). The area under the curve (AUC) was calculated using GraphPad Prism v8. The curves were plotted against log_{10}[DXS] (µg/mL) and fitted using a Gaussian model. The mean of this model was generated to determine the peak of the curve, allowing the calculation of the optimal concentration of DXS for FXI autoactivation. Assay was run in triplicate, on one plate and data are shown as mean ± SEM.

As seen in Figure 4.4, DXS acts as a surface for FXI to autoactivate, and this works in a template mechanism. The optimum concentrations of DXS_{40kDa} and DXS_{500kDa} are 2.18 µg/mL (Figure 4.4B) and 0.6 µg/mL (Figure 4.4D) respectively. This was determined by interpolation of a standard curve of maximum rate of reaction vs. concentration of DXS (Figure 4.4B & D). As the larger molecular weight DXS is more efficient, this was the polyanion that was taken forward through future assays.

4.4 FP Modulates Cleavage of S-2288 by FXIa Autoactivated by DXS_{500kDa}

The interactions between FXI and polyanions was further explored in the presence of FP, as FP can also bind to polyanions such as GAGs and sulfatides as verified in the literature (Kemper et al., 2008, Kouser et al., 2013). The first step of this investigation was to determine if FP influences the autoactivation mechanism of FXI by the presence of DXS_{500kDa} (Figure 4.5).

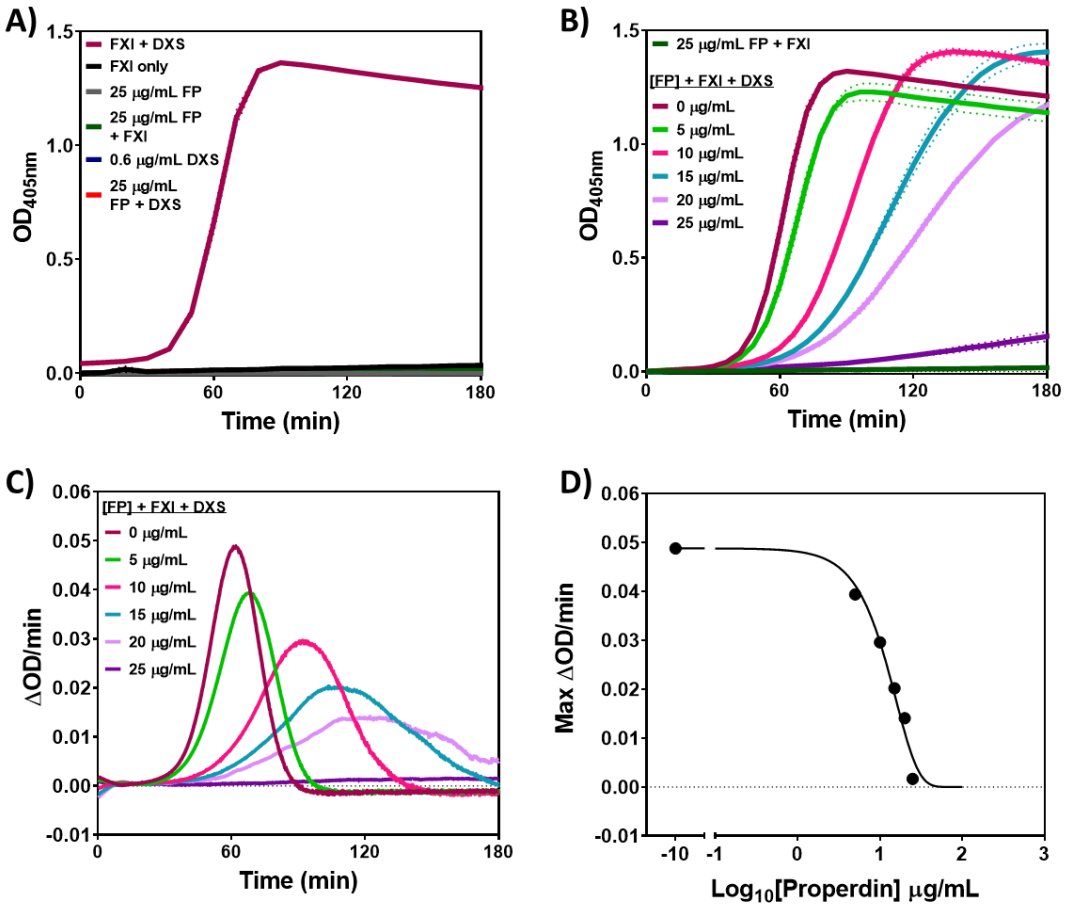


Figure 4.5 FP modulates activity of FXIa autoactivated using DXS_{500kDa} in a dose dependent manner.

30 nM of FXI zymogen was incubated with 5 concentrations of FP (5-25 µg/mL) in the presence of 0.6 µg/mL of DXS_{500kDa} (DXS). Autoactivation of FXI was measured by cleavage of 500 µM chromogenic substrate S-2288 over a course of 3 hours. A) Control samples. B) Dilution of FP incubated with FXI and DXS_{500kDa}. Negative and positive controls are also shown. C) The first derivative of the data in "B)" to determine the rate of S-2288 cleavage over time. D) Dose response curve of FP inhibition of FXI autoactivation, calculated by determining the maximum rate of change of OD per minute from graph "C". Raw data were plotted as optical density (OD) read at 405 nm vs time, the first derivative and maximum rate of substrate cleavage was calculated using GraphPad Prism v8. Assay was run in triplicate, on one plate and data are shown as mean ± SEM. *Error bars may not be visible due to small SEM values.*

The presence of DXS_{500kDa} rapidly induced amidolytic activity of FXI(a) towards S-2288 (Figure 4.5A & B). The addition of physiological concentrations of FP to the autoactivation reaction drastically reduced cleavage of S-2288 in a dose dependent manner (Figure 4.5C & D), with the top concentration of FP (25 µg/mL) almost completely eradicating the cleavage of S-2288 indicating that FP may inhibit FXI autoactivation on a surface. The first derivative was calculated to determine the rates of reaction (Figure 4.5C). The maximum rates of reaction were then plotted (Figure 4.5D) and determined that FP reduced the rate of S-2288 cleavage by autoactivated FXI in a dose dependent manner. These data suggest two hypotheses: 1) a potential regulatory mechanism of the complement regulatory protein, FP, which appears to have an inhibitory effect on the autoactivation mechanism of FXI or 2) a change in substrate specificity of FXIa away from the chromogenic substrate in the presence of both FP and DXS_{500kDa}.

Properdin is known to have anion binding properties and the highly positive charge of FP at pH 7.4 would suggest that FP is binding to DXS_{500kDa}, effectively blocking the interaction of FXI and DXS_{500kDa}, and thus inhibiting autoactivation. Although FXI can undergo autoactivation in the presence of DXS_{500kDa}, the literature states that DXS also inhibits FXIa in an allosteric manner (Sinha et al., 2004).

To investigate whether this “inhibitory” activity of FP was due to the differences in charge of DXS_{500kDa} and FP, a similar assay was performed using protamine sulfate (Figure 4.6), a cationic protein that is used clinically as a reversal or neutralising agent for heparin (Jaques, 1973).

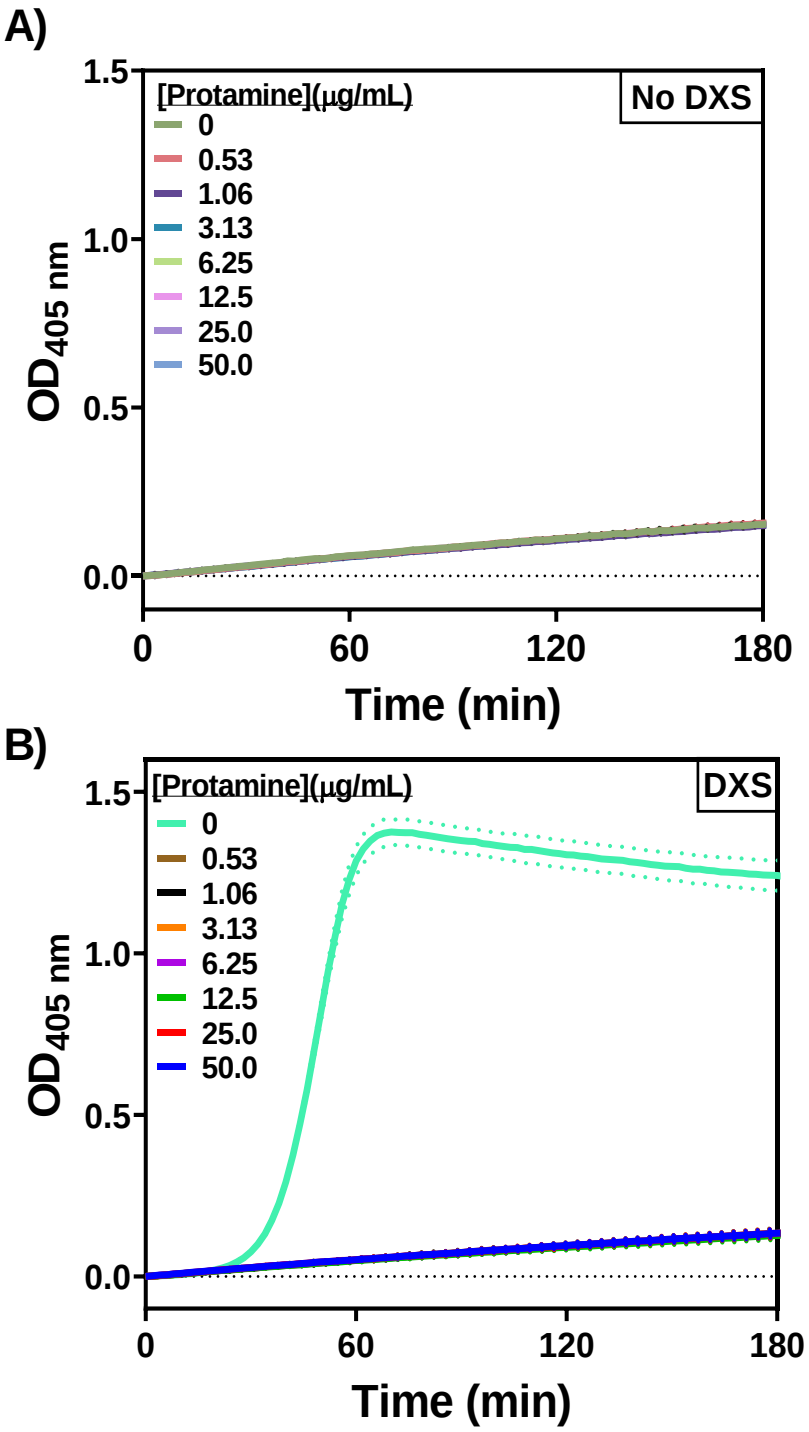


Figure 4.6 Protamine sulfate exhibits an inhibitory effect on FXI autoactivation by DXS_{500kDa}.

30 nM of FXI zymogen was incubated with a titration of protamine sulfate in the absence (A) and presence (B) of 0.6 µg/mL of DXS_{500kDa} (DXS). Autoactivation of FXI was measured by cleavage of 500 µM chromogenic substrate S-2288 over a course of 3 hours. Assay was run in triplicate, on one plate and data are shown as mean ± SEM. *Error bars may not be visible due to small SEM values.*

The chromogenic assays revealed that FP (Figure 4.5) may be acting in a similar manner to protamine sulfate (Figure 4.6), suggesting that FP may be binding to the anionic surface and blocking the interaction of FXI with DXS_{500kDa}, thus inhibiting the autoactivation mechanism and reducing the cleavage of S-2288. Though this is a logical explanation, it was important to investigate the interaction further as FP inhibited in a different, dose dependent manner, where the rates of reaction were affected, but the maximum absorbance was not. This would suggest that the effect that FP had on the autoactivation mechanism was more likely to be an effect on the amidolytic activity of FXIa in the presence of DXS_{500kDa}. This was further explored using Michaelis-Menten kinetics of FXIa, in the presence of FP, DXS_{500kDa} and FP & DXS_{500kDa}.

To further explore this mechanism, a more physiological environment was optimised, using sulfatides from bovine brain. Sulfatides are found in the myelin sheath and are abundant in the brain but can also be found in the kidneys and the spleen. They play an important role in haemostasis and thrombosis, having an anticoagulant effect yet enhancing thrombosis, suggesting a role involving the intrinsic pathway of coagulation (Takahashi and Suzuki, 2012). Properdin has been shown to initiate complement at the surface of late apoptotic cells for clearance, and FP has sulfatide binding properties that may be involved in this complement activation mechanism, though DNA is the predominant binding partner (Xu et al., 2008). To explore this effect of FP on autoactivation of contact pathway zymogens, FXII and PK were also incubated with sulfatides, using FXI as a control (Figure 4.7).

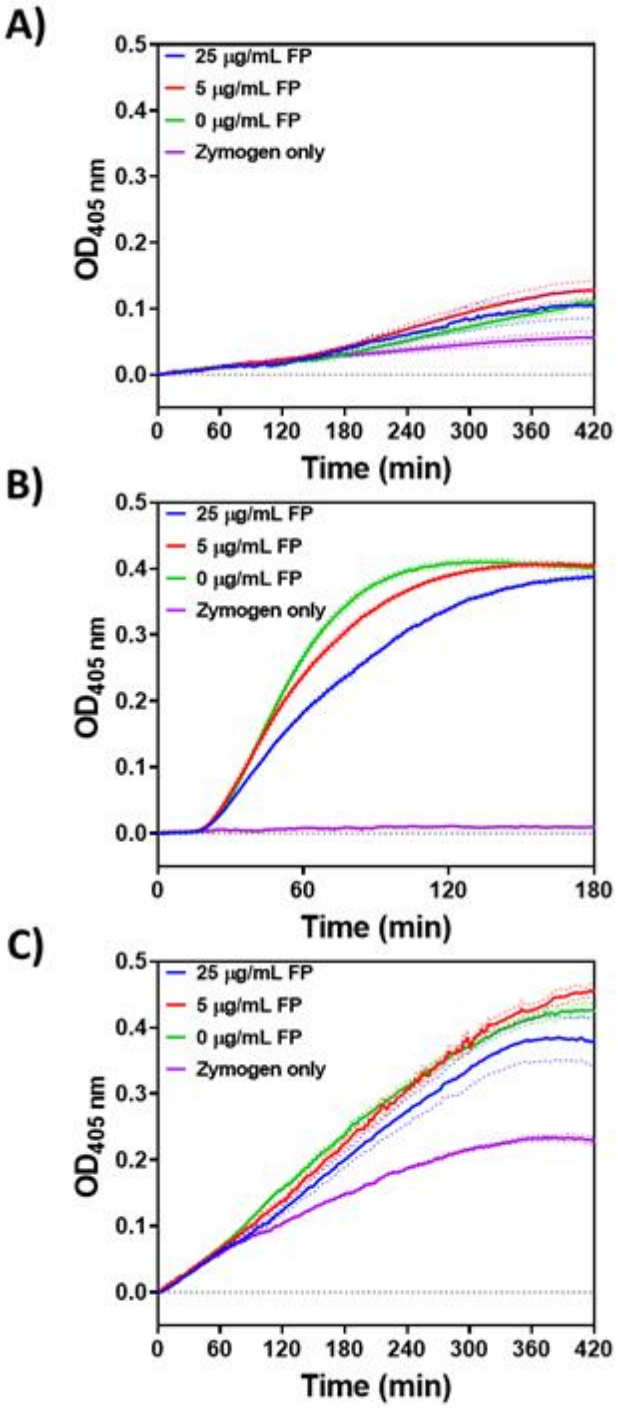


Figure 4.7 FP modestly influences the autoactivation process of PK and FXI, but not FXII.

Reactions containing 100 nM FXII, 100 nM PK, 30 nM FXI and 25 µg/mL sulfatides were incubated in the presence and absence of 0, 5 and 25 µg/mL FP. "Zymogen only" represents no FP present. A) FXII autoactivation measured over 7 hours, indicated by the cleavage of 200 µM S-2302. B) PK autoactivation measured over 3 hours, indicated by the cleavage of 200 µM S-2302. C) FXI autoactivation measured over 7 hours, indicated by the cleavage of 500 µM S-2288. Assay was run in triplicate, on one plate and data are shown as mean ± SEM.

FP is known to have sulfatide binding properties as previously mentioned and appears to modulate autoactivation of PK and FXI in the presence of sulfatides. It is interesting that FP does not appear to interfere in the same way with PK as it does with FXI. This may be due to differences in the mechanisms of autoactivation. Factor XI is a dimer whereas PK is a monomer, and structural studies may reveal the answer to the differences in the interactions.

Two assays were performed to determine the effect of the physiological cofactor of FXI, HK, on FXI autoactivation using DXS_{500kDa} and sulfatides as agonists (Figure 4.8 & Figure 4.9).

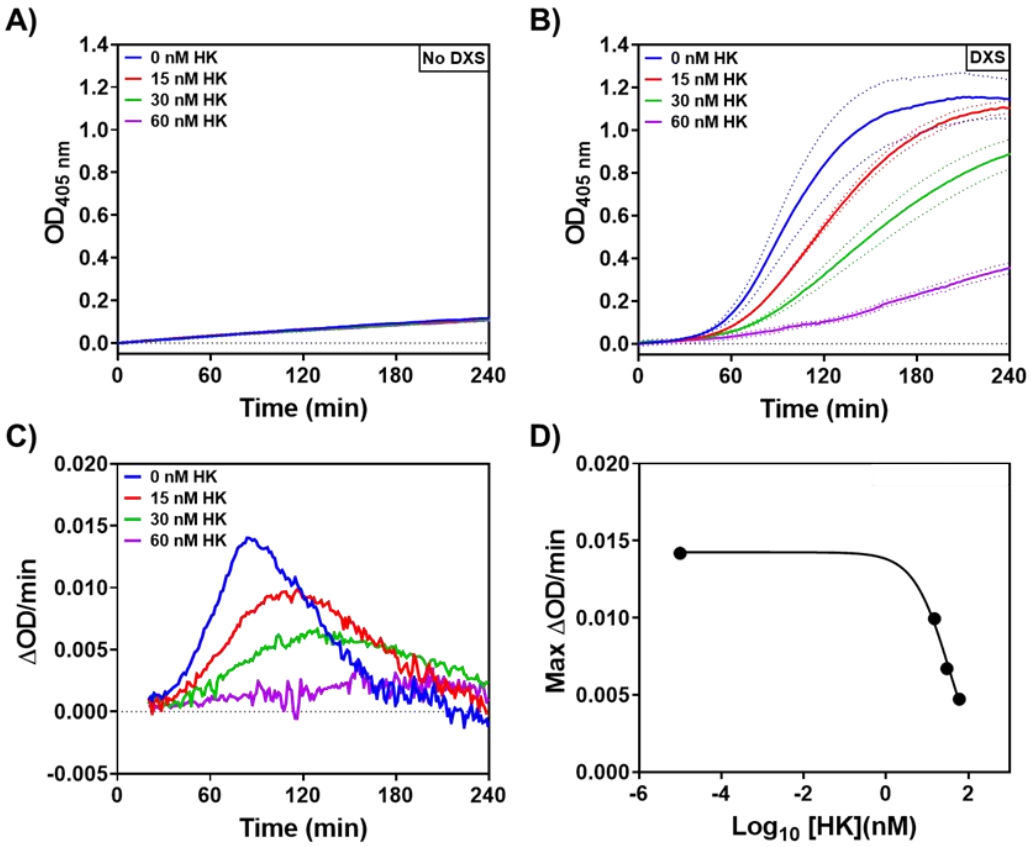


Figure 4.8 Activity of FXI autoactivated by DXS_{500kDa} is modulated by HK in a dose dependent manner.

30 nM of FXI zymogen was incubated with a titration of HK in the absence (A) and presence (B) of 0.6 µg/mL of DXS_{500kDa} (DXS). C) The first derivative of the data in "B)" to determine the rate of S-2288 cleavage over time. D) Dose response curve of HK calculated by determining the maximum rate of change of OD per minute from graph "C". Autoactivation of FXI was measured by cleavage of 500 µM chromogenic substrate S-2288 over a course of 3 hours. Assay was run in triplicate, on one plate and data are shown as mean ± SEM. *Error bars may not be visible due to small SEM values.*

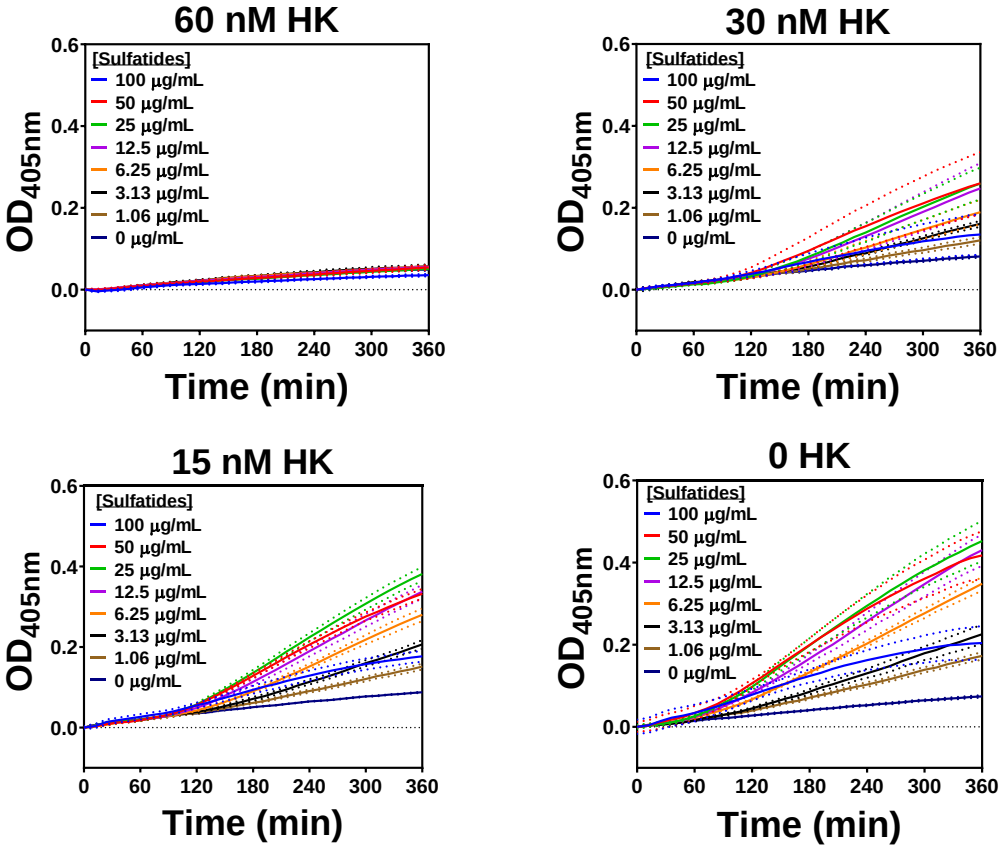


Figure 4.9 FXI can autoactivate in the presence of sulfatides however HK reduces the cleavage of the chromogenic substrate in a dose dependent manner.

30 nM of FXI zymogen was incubated with a titration of sulfatides and HK. Autoactivation of FXI was measured by cleavage of 500 µM chromogenic substrate S-2288 over a course of 6 hours. Assay was run in triplicate, on one plate and data are shown as mean ± SEM.

The presence of HK reduces the activity of autoactivated FXI towards the chromogenic substrate in the presence of both DXS_{500kDa} (Figure 4.8) and sulfatides (Figure 4.9). The inhibition of FXI autoactivation was previously demonstrated by Ivanov et al. (2017), where the presence of HK prevented FXI autoactivation by nucleic acids. It was, however, shown that FXII and thrombin could overcome the inhibitory mechanism of HK and lead to the generation of FXIa (Ivanov et al., 2017b). As HK can act as a substrate for FXIa (Scott et al., 1985) it is possible that the decrease in cleavage of the chromogenic substrate may be due to a change in substrate specificity, and the target for FXIa amidolytic activity may be HK rather than S-2288.

To elucidate whether the reduced rate of S-2288 cleavage was a result of FXI(a) cleaving HK in the presence of DXS_{500kDa}, SDS-PAGE was performed (Figure 4.10) to analyse the production of any cleavage products.

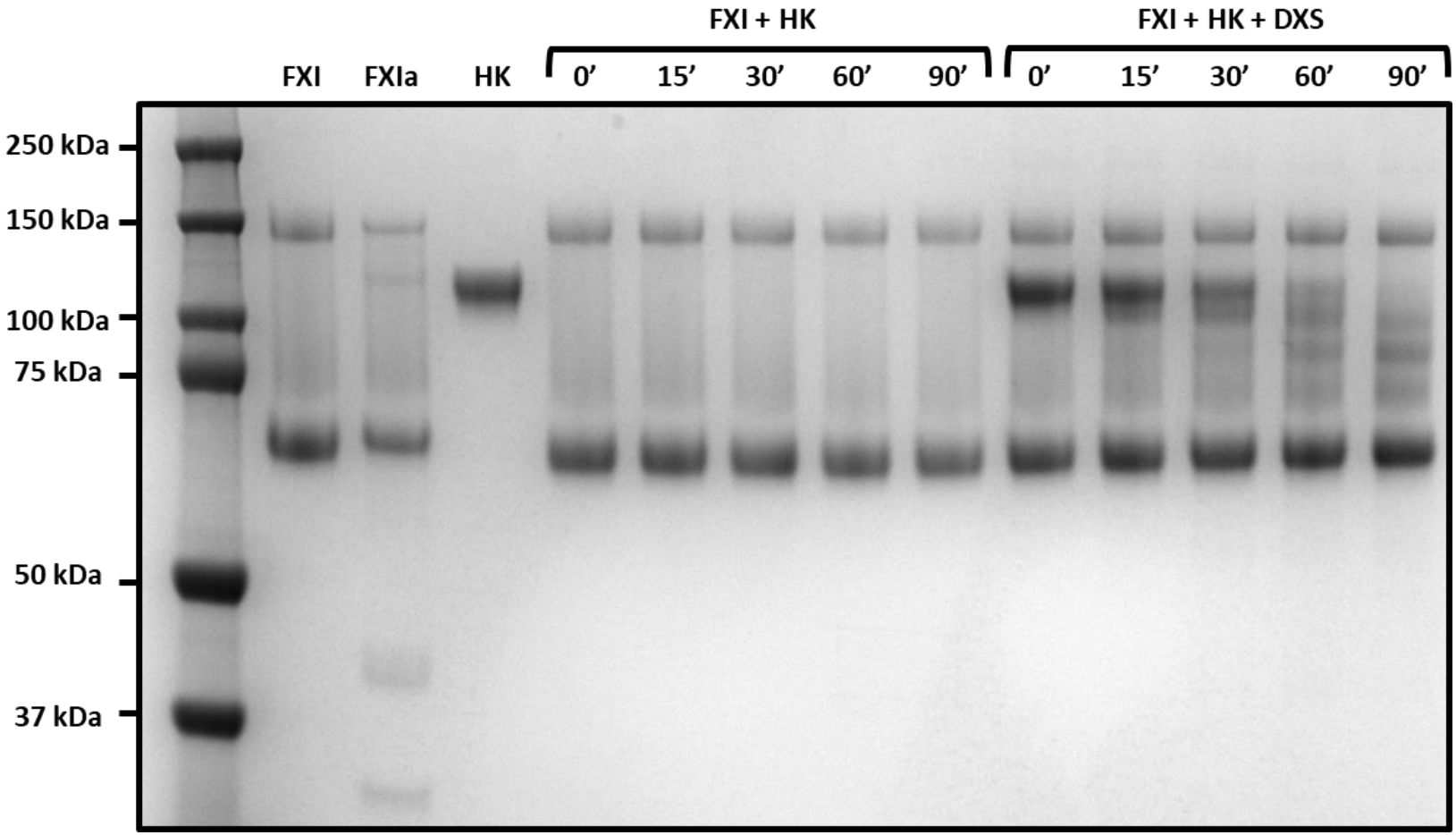


Figure 4.10 Non-reducing SDS-PAGE reveals potential cleavage of HK in the presence of FXI and DXS_{500kDa}.

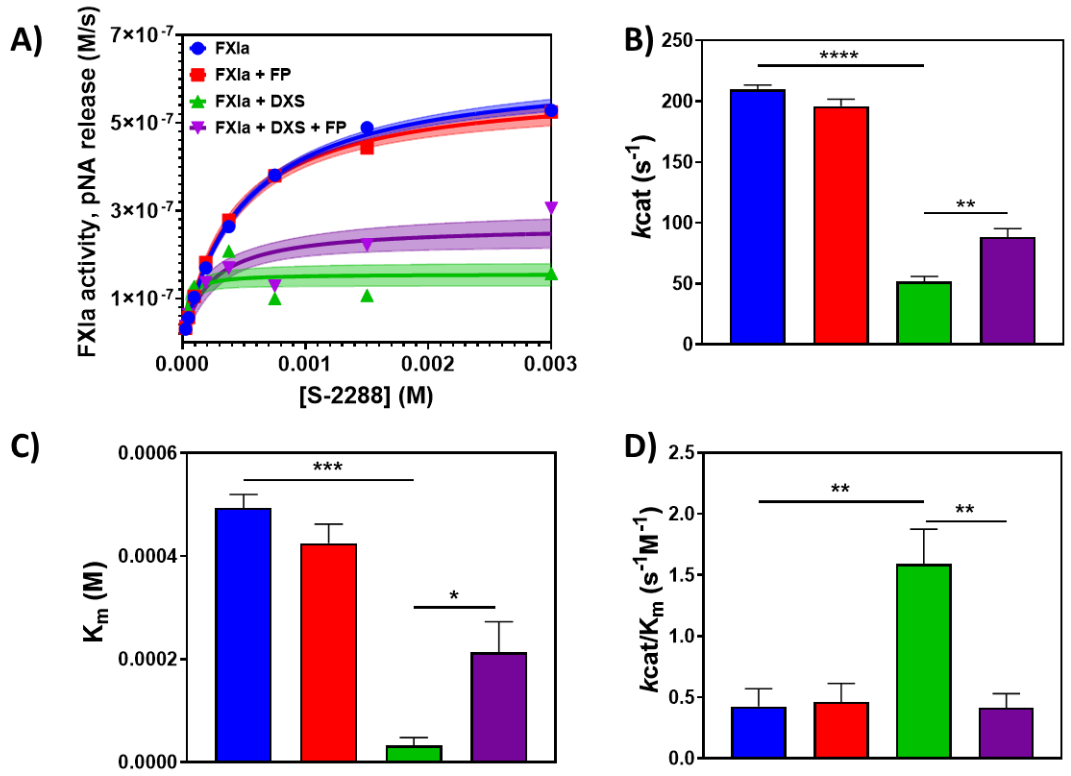
Reactions of 100 µg/mL FXI, 100 µg/mL HK and 12.5 µg/mL DXS_{500kDa} were incubated for 90 minutes at 37°C in HBS, Samples were vortexed at each time point. Samples were taken at time points between 0 and 90 minutes and were diluted two-fold and separated by non-reducing SDS-PAGE.

Figure 4.10 revealed that FXI that was autoactivated by DXS_{500kDa} could cleave HK, demonstrated by the disappearance of the HK band. This supports the hypothesis that the reduction of S-2288 cleavage in Figure 4.8 and Figure 4.9 could be a result of a change in substrate specificity away from the chromogenic substrate and towards HK. To elucidate how DXS_{500kDa} was affecting FXIa activity, Michaelis-Menten kinetic analysis was performed.

4.5 Kinetic Analysis of FXIa

Autoactivation of FXI by DXS_{500kDa} was modulated in a dose dependent manner when in the presence of physiological concentrations of FP. Figure 4.5 clearly shows DXS_{500kDa} initiating FXI activity towards chromogenic substrate S-2288. The maximum absorbance seen in Figure 4.4 was not lowered which led to the hypothesis that FP was interfering with the amidolytic activity of FXIa when in the presence of DXS_{500kDa} Figures 4.8, 4.9 and 4.10 show a change in substrate specificity away from the chromogenic substrate and towards HK suggesting that DXS_{500kDa} is altering activity of FXIa. . This was further explored to determine the interactions between FP, FXIa and DXS_{500kDa}.

Subsequently the effect of DXS_{500kDa} and FP on FXIa amidolytic activity was explored. Reactions of FXIa, DXS_{500kDa} and FP were incubated with a titration of S-2288 to allow Michaelis-Menten kinetic analysis in GraphPad Prism v8 (Figure 4.11).



	FXIa	FXIa + FP	FXIa + DXS_{500kDa}	FXIa + FP + DXS_{500kDa}
k_{cat} ± SEM (s^{-1})	209.5 ± 3.8	196.1 ± 5.6	51.8 ± 4.3	88.4 ± 6.9
K_m ± SEM (M)	$4.9x10^{-4} \pm 2.6x10^{-5}$	$4.3x10^{-4} \pm 3.7x10^{-5}$	$3.3x10^{-5} \pm 1.5x10^{-5}$	$2.1x10^{-4} \pm 5.9x10^{-4}$
V_{max} ($M.s^{-1}$)	$6.3x10^{-7}$	$5.9x10^{-7}$	$1.6x10^{-7}$	$2.7x10^{-7}$
K_{cat}/K_m ($s^{-1}M^{-1}$)	$4.24x10^{5}$	$4.61x10^{5}$	$1.59x10^{6}$	$4.14x10^{5}$

Figure 4.11 FP reduces the inhibitory effect of DXS_{500kDa} on FXIa catalytic activity.

Michaelis-Menten kinetic analysis was performed to determine how FP affected the previously reported inhibitory action of DXS_{500kDa} on FXIa. Reactions of 0.6 µg/mL DXS_{500kDa} and 3 nM FXIa were incubated with or without 25 µg/mL of FP, with 500 µM S-2288. Factor XIa catalytic activity was measured by cleavage of 500 µM chromogenic substrate S-2288 over 3 hours. Optical density was read at 405 nm, at 12 second intervals. Kinetic analysis was performed using the k_{cat} model on GraphPad Prism v8. A) Catalytic activity defined by amount of pNA released per second (M/s). B) k_{cat} (s^{-1}). C) K_m (M). D) k_{cat}/K_m ($s^{-1}M^{-1}$). Statistical differences were analysed by one-way ANOVA, differences between columns were detected by Tukey's multiple comparisons test. **** $p \leq 0.0001$, ** $p \leq 0.01$, * $p \leq 0.05$.

Michaelis-Menten kinetic analysis (Figure 4.11A) showed a decrease in K_m and V_{max} when FXIa was incubated with DXS_{500kDa}, suggesting uncompetitive inhibition by DXS_{500kDa}. The k_{cat} (Figure 4.11B) of FXIa in the presence of DXS_{500kDa} alone was reduced from 209.5 to 51.76 s^{-1} suggesting that the turnover rate of S-2288 by FXIa was reduced in the presence of DXS_{500kDa} supported by a decrease in V_{max} from $6.3x10^{-7}$ to $1.6x10^{7}$ $M.s^{-1}$ at concentrations above the K_m. The K_m (Figure 4.11C) was decreased from $4.9x10^{-4}$ to $3.3x10^{-5}$ M signifying a higher affinity for S-2288. The decrease in k_{cat} is compensated for by the decrease in K_m leading to an overall increased k_{cat}/K_m (Figure 4.11D). The addition of FP to the reaction did not significantly modulate FXIa amidolytic activity alone, however it partially reversed the effect that DXS_{500kDa} had on the k_{cat}. k_{cat} was increased from 51.76 to 88.4 s^{-1} ($p=0.0051$) and K_m was increased from $3.3x10^{-5}$ to $2.1x10^{-4}$ M ($p=0.393$). The increase in K_m and k_{cat} in the presence of FP suggest that FP may be interfering with the interaction between FXIa and the polyanionic surface presented by DXS_{500kDa}.

4.6 FP Acts as an Alternative Substrate for FXIa in the presence of DXS

FXIa actvity (k_{cat}/K_m) was not affected by FP (Figure 4.11). It has previously been demonstrated that DXS inhibits FXIa through an allosteric mechanism (Sinha et al., 2004). Properdin appears to partially reverse the inhibitory effect observed in the previous assay. This may be explained by FP binding and coating DXS_{500kDa} and blocking the binding of FXIa to DXS_{500kDa}. This interaction was further explored by incubating FXI and FXIa with DXS_{500kDa} and FP and examining the results using SDS-PAGE.

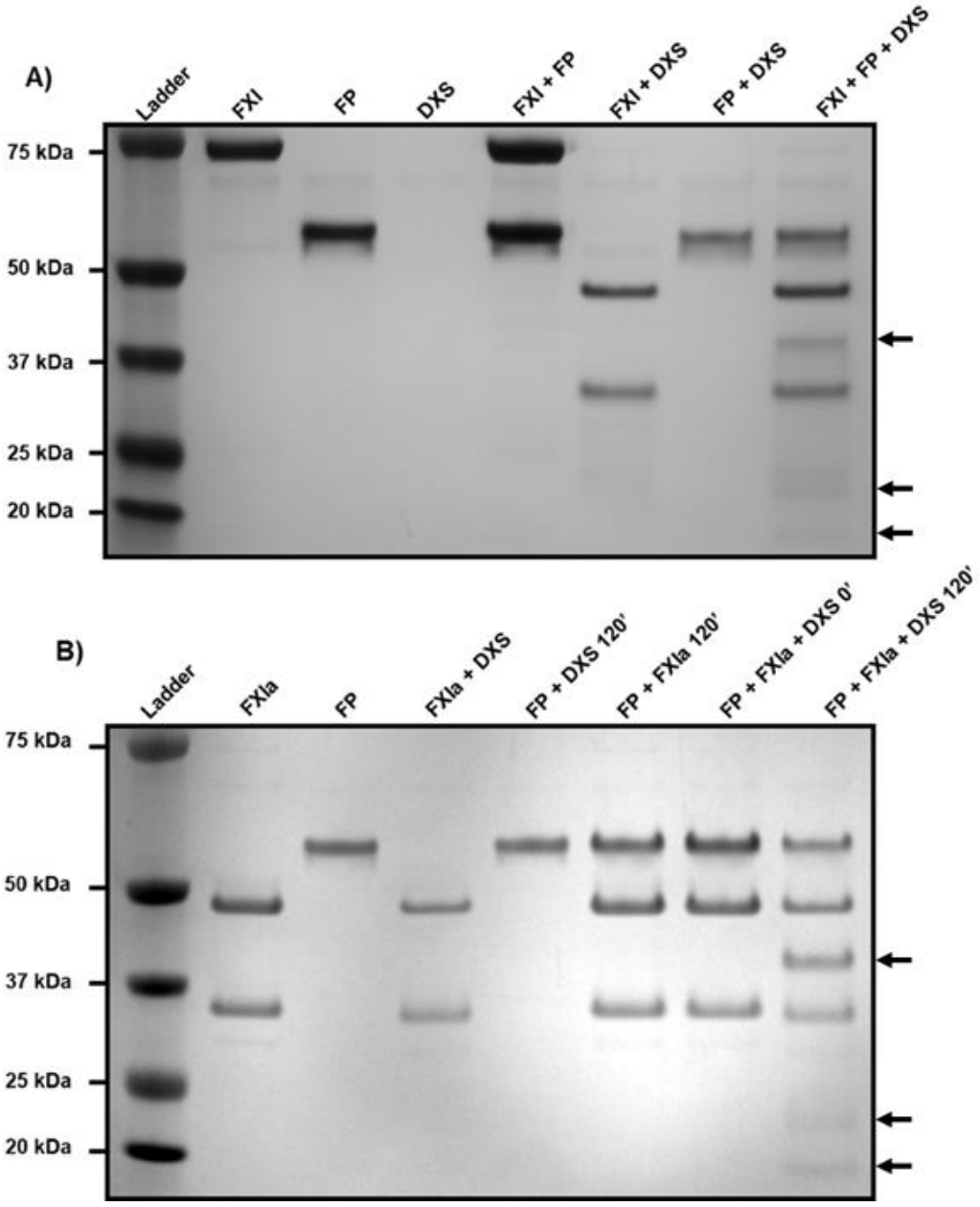


Figure 4.12 SDS-PAGE reveals three unknown bands when FP and FXI(a) are incubated with DXS_{500kDa}.

Reactions of 100 µg/mL FXI (A) or FXIa (B), 200 µg/mL FP and 12.5 µg/mL DXS_{500kDa} were incubated for 120 minutes in HBS. Samples were taken at 0 and 120 minutes. Samples were then diluted two-fold and separated by reducing SDS-PAGE.

The reactions analysed using SDS-PAGE (Figure 4.12) disproves the first theory; FP inhibits the autoactivation of FXI by DXS_{500kDa}. This is clear on the electrophoresis images as FXI becomes activated in the presence of DXS_{500kDa}, shown by the reduction of FXI (the 80 kDa band) to form two new bands at around 50 kDa and 30 kDa, which represent FXIa. Interestingly, three unknown bands were revealed in the presence of FP and DXS_{500kDa}. These new bands are revealed during autoactivation (Figure 4.12A) and during a FXIa activity assay (Figure 4.12B), thus a substrate specificity change is suspected, and it is hypothesised that FXIa can cleave FP, in the presence of DXS_{500kDa}. This hypothesis was explored by sending the gel to the University of Leeds Mass Spectrometry Facility (Figure 4.13).

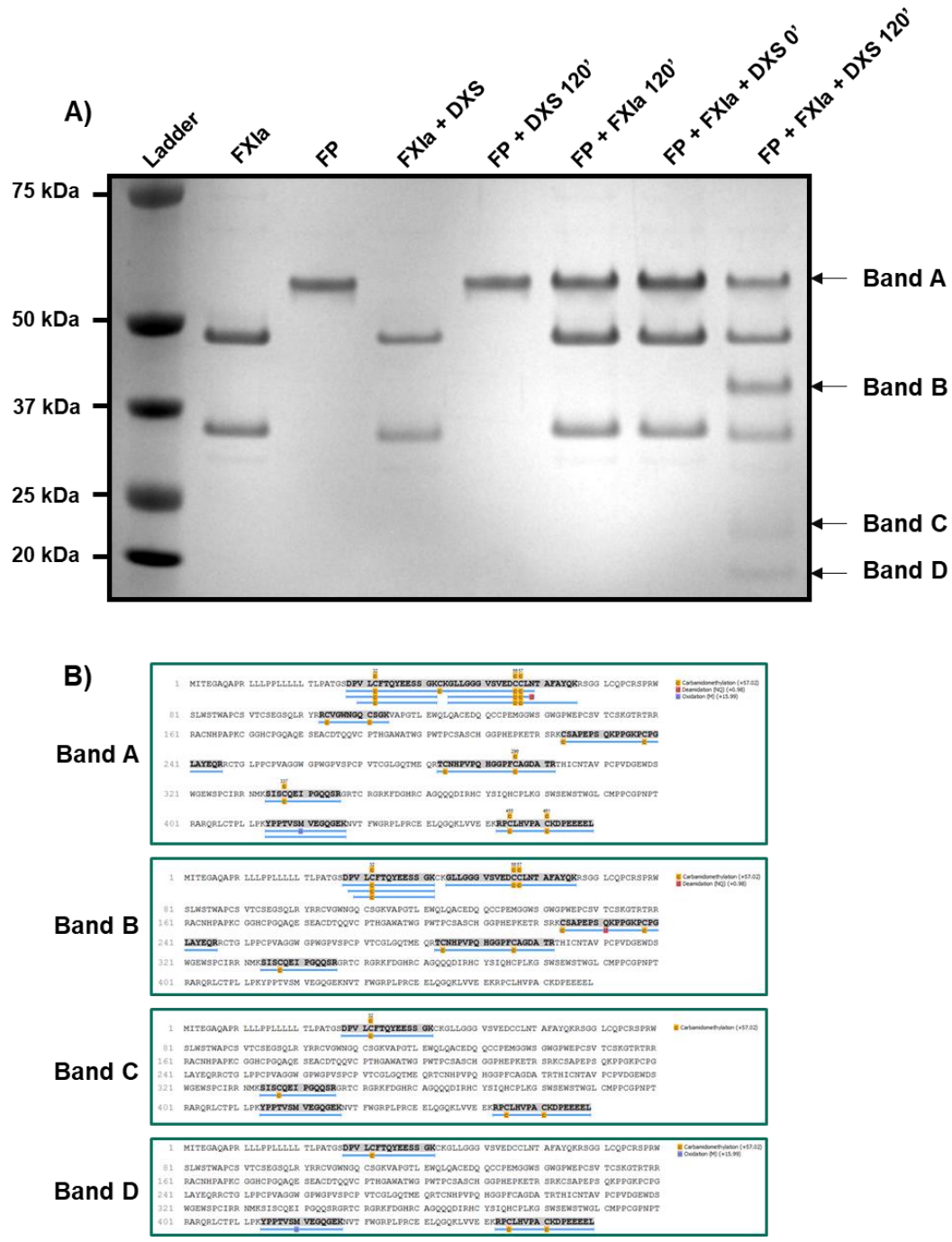


Figure 4.13 Mass spectrometry analysis reveals unknown bands are FP cleavage products.

A) Reactions of 100 µg/mL FXIa, 200 µg/mL FP and 12.5 µg/mL DXS_{500kDa} were incubated for 120 minutes in HBS. B) Mass spectrometry (facility at the University of Leeds) aligns sequences from unknown bands (bands B, C and D) with the full-length sequence of FP (band A), revealing that FP is being degraded in the presence of DXS_{500kDa} and FXIa.

The cleavage bands that are shown suggest that there is a substrate specificity change of FXIa. MS revealed that the bands are indeed FP products. Properdin and DXS_{500kDa} alone do not show the bands of FP, though the density of the FP band at around 53 kDa does appear lower than FP alone. This may be due to FP binding to DXS_{500kDa} and not entering the gel due to the size of the complex being too large. The FP products are only visible when both FXIa and DXS_{500kDa} are present suggesting that FP is being cleaved by FXIa. MS revealed that the sequences of the bands were unique to FP.

There are multiple hypotheses to be drawn from these data: 1) FXIa is modulating FP through cleavage in the presence of an anionic surface, 2) FP binds to DXS_{500kDa} and is therefore more amenable to cleavage by FXIa, 3) anionic surfaces induce a substrate specificity change in FXIa.

It was next determined if the substrate specificity change of FXIa prevented the cleavage of its main physiological substrate, FIX.

4.7 FXIa Cleaves FIX in the presence of FP

The main physiological substrate of FXIa is FIX which goes on to form the intrinsic tenase complex and induces the common pathway of coagulation through FX activation. It is important to distinguish the possible effects of FP on the coagulation cascade by investigating FIX activation by FXI(a). A chromogenic assay (Figure 4.14) and SDS-PAGE analysis (Figure 4.15) were employed to determine the downstream effects of FP and of FXIa substrate specificity changes.

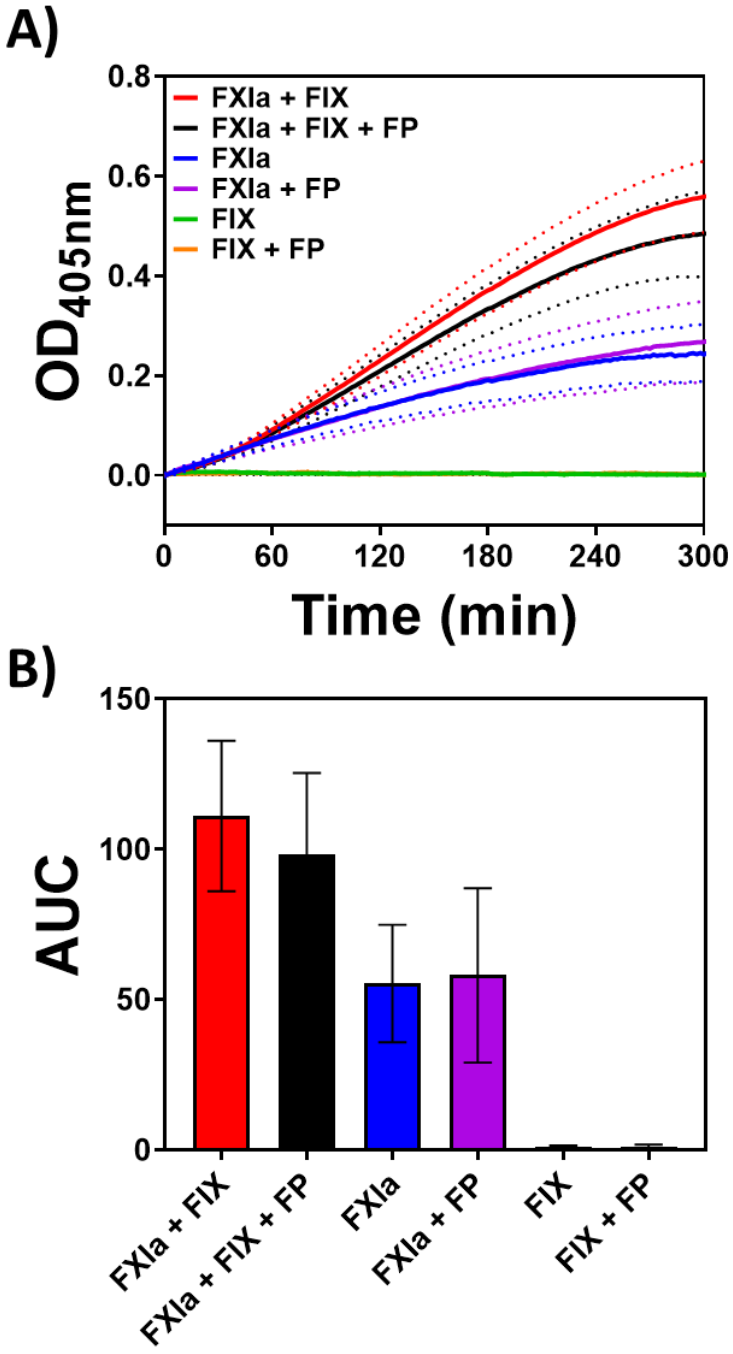


Figure 4.14 FP does not affect FXIa cleavage of FIX.

Reactions of 125 pM FXIa, 3nM FIX, 25 µg/mL FP 1.5 mM $CaCl_2$ and 40 µM $ZnCl_2$ were incubated for 5 hours. Factor IX activation was measured by cleavage of 1 mM chromogenic substrate S-2765. A) Baseline corrected data showing optical density read at 405 nm vs. time. B) Area under the curve (AUC) calculated from "A".

FXIa cleavage of FIX was not affected by FP alone (Figure 4.14). A surface was then introduced to determine if the substrate specificity changes seen previously with DXS_{500kDa} may affect FIX activation, and thus demonstrate downstream effects of coagulation.

The following experiments to investigate FIX activation used SDS-PAGE to determine FIX activation, as the chromogenic assay is not sensitive, demonstrated by the reactions containing FIX not reaching a max absorbance that was clearly higher than the background absorbance from FXIa alone. It was demonstrated that FXI autoactivated by $DXS_{500kDa,}$ could cleave FIX, thus suggesting that this FXIa had similar functionality to FXIa produced by FXI cleavage by FXIIa (Figure 4.15).

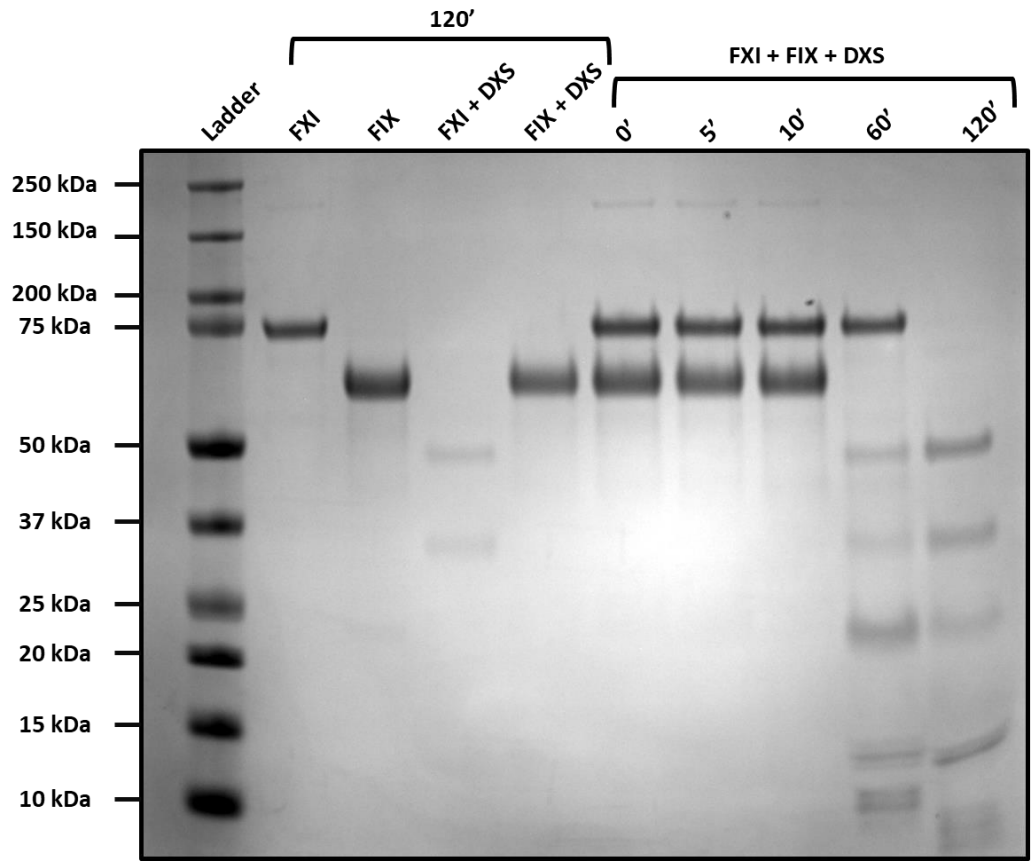


Figure 4.15 SDS-PAGE reveals that FXI autoactivated by DXS_{500kDa} can cleave FIX.

Reactions of 100 µg/mL FXI, 12.5 µg/mL DXS_{500kDa} and 100 µg/mL FIX were incubated for up to two hours and samples were taken over a time course. Samples were reduced and analysed using SDS-PAGE.

4.8 Investigation of FXIa Substrate Specificity

It was demonstrated that the change in substrate specificity did not stop autoactivated FXIa from cleaving FIX. This suggests that the autoactivation process does not alter FXIa when compared to FXIa produced via FXI cleavage by FXIIa. Properdin was then introduced to these reactions to determine if there were downstream consequences of the substrate specificity change seen previously. SDS-PAGE was again employed to analyse reactions of FXIa, FIX, DXS_{500kDa} and FP (Figure 4.16).

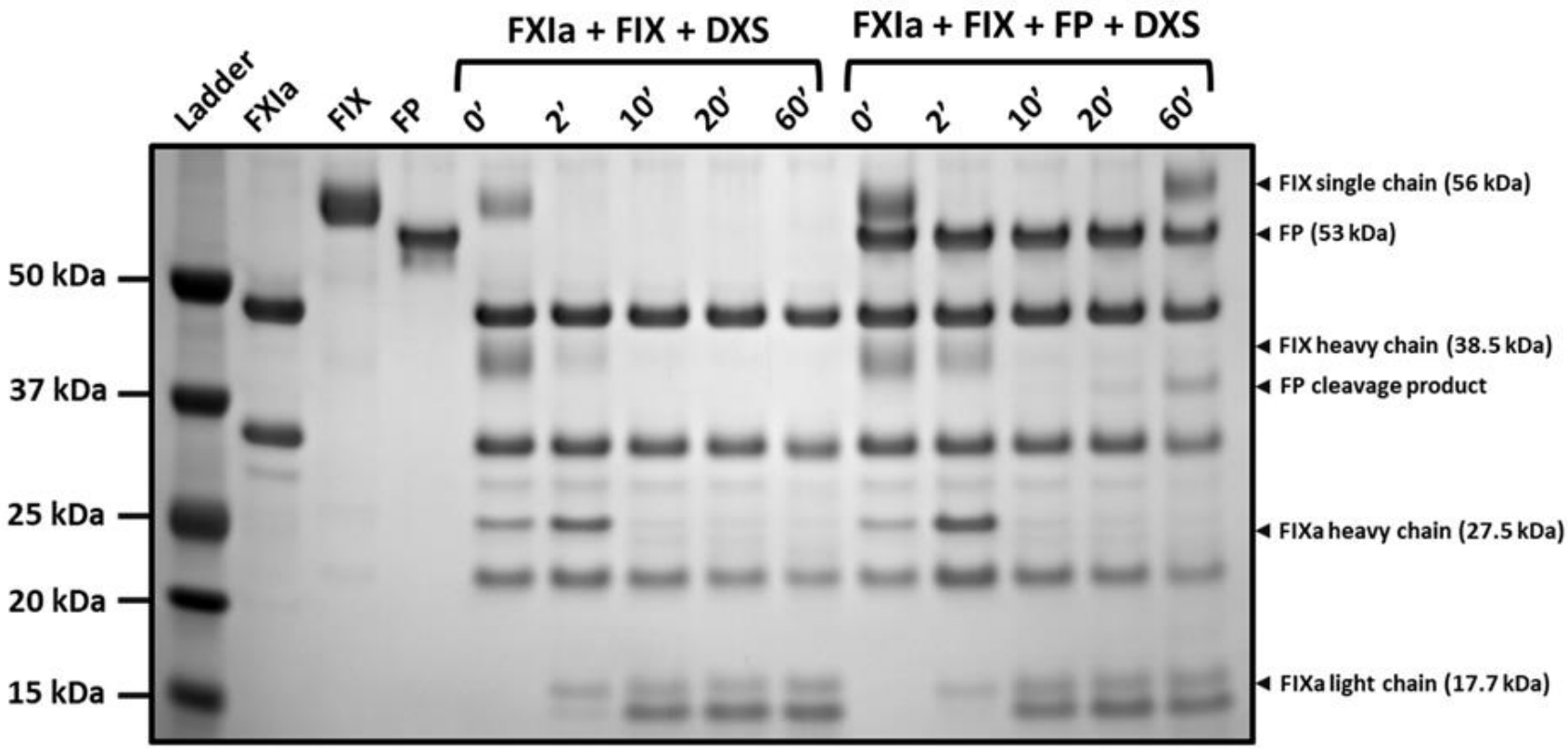


Figure 4.16 FXIa cleaves FIX in the presence of DXS_{500kDa} and FP.

A) Reactions of 100 µg/mL FXIa, 12.5 µg/mL DXS_{500kDa}, 100 µg/mL FIX and 200 µg/mL FP were incubated for up to one hour and samples were taken over a time course. Samples were reduced and analysed using SDS-PAGE.

FXIa can still cleave FIX in the presence of DXS_{500kDa} and FP. Figure 4.16 shows a time course of these reactions. By observing the disappearance of the FIX single chain, and the appearance of cleavage products it can be determined that FIX is being cleaved by FXIa in the presence of DXS_{500kDa}. The appearance of FP cleavage products in the reaction of FXIa, DXS_{500kDa} and FIX is seen demonstrating the substrate specificity change of FXIa, however this does not inhibit FIX activation by FXIa. There is a band that appears at the top of the lane in the 60-minute time point in the reaction containing FXIa, DXS_{500kDa}, FP and FIX. The band has not been further analysed; however, the band appears to be higher than the FIX band alone and may represent the appearance of a new complex possibly containing derived cleavage products.

These results may suggest that FP is cleaved by FXIa, but this cleavage does not inhibit intrinsic pathway activation of coagulation through surface mediated substrate specificity changes of FXI(a).

FP may become more amenable to cleavage by FXIa when bound to a surface. It is likely that FP undergoes conformational changes, altering the structure of the protein, revealing cryptic cleavage sites. As DXS_{500kDa} is not a physiological surface, more polyanions were investigated.

4.9 Do Other Surfaces lead to the Cleavage of FP?

To investigate if FXIa was cleaving FP in the presence of other polyanionic surfaces, reactions were incubated with a variety of synthetic and physiological surfaces and were analysed using SDS-PAGE (Figure 4.17).

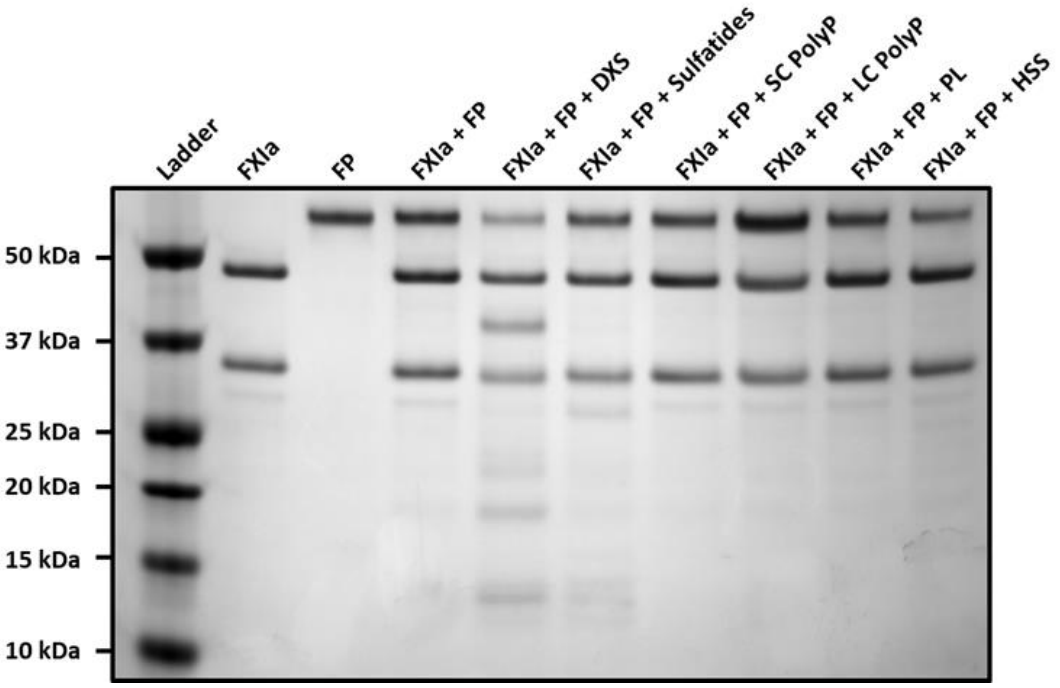


Figure 4.17 FP cleavage products are revealed only in the presence of DXS_{500kDa}.

Reactions of 100 µg/mL FXIa and 200 µg/mL FP were incubated with various surfaces. 12.5 µg/mL DXS_{500kDa}, 125 µg/mL bovine sulfatides, 50 µM short chain polyphosphate (SC PolyP), 50 µM long chain polyphosphate (LC PolyP), 300 µM phospholipids (PL), 5 µg/mL heparin sodium salt (HSS). Reactions were incubated for 2 hours and were reduced to run analyse using SDS-PAGE. DXS_{500kDa} was used as a control.

Cleavage bands of FP were observed when FXIa and FP were incubated with DXS_{500kDa}. The presence of sulfatides also revealed potential bands for further investigation.

With this information in mind, it may be plausible to suggest a potential regulatory mechanism of complement via FXIa.

Another alternative substrate for FXIa is FX (Matafonov et al., 2013b). The effect of FP on the cleavage of FX by FXIa was explored using PL as the anionic surface (Figure 4.18).

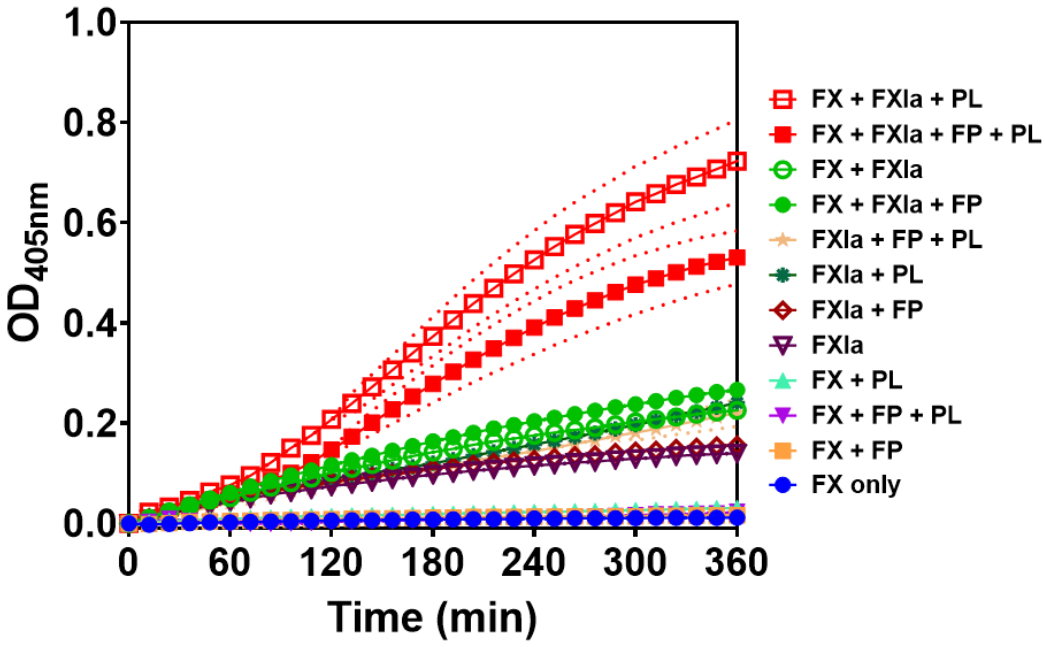


Figure 4.18 FP modulates FX cleavage by FXIa in the presence of PL.

Reactions of 30 pM FXIa, 10 µM PL, 30 nM FX and 25 µg/mL FP were incubated. Factor X activation was measured by the cleavage of 350 µM S-2765. Assay was run in triplicate on one plate and data are shown as mean ± SEM. *Error bars may not be visible due to small SEM values.*

Interestingly, FP appeared to modulate the activation of FX by FXIa, but only in the presence of PL. This supports the hypothesis that FP is interacting with FXI in a surface dependent manner. It also suggests that there may be a physiological role for FP within the coagulation cascade with downstream effects.

4.10 FP Modulates FXII(a) Activation of FXI in the presence of an Intrinsic Agonist

It is important to outline the physiological relevance of the interaction between FP and the intrinsic pathway. As previously discussed, the autoactivation of FXI by a negatively charged surface may not be a physiologic occurrence. Therefore, our next aim was to explore the effect of FP on the activation of FXI by FXIIa, in the presence of HK. Factor XII zymogen was used at a low concentration of 2 nM, to ensure there

was no background signal from FXII(a) alone cleaving the substrate, S-2288. PTT automate was the agonist used, which is also used in clinical tests for determining aPTT. This contains the negatively charged surfaces, silica and PL, to initiate surface activation of FXII, thus leading to the downstream activation of FXI. HK was included as a cofactor for FXI at a 1:1 stoichiometry to FXI (Figure 4.19).

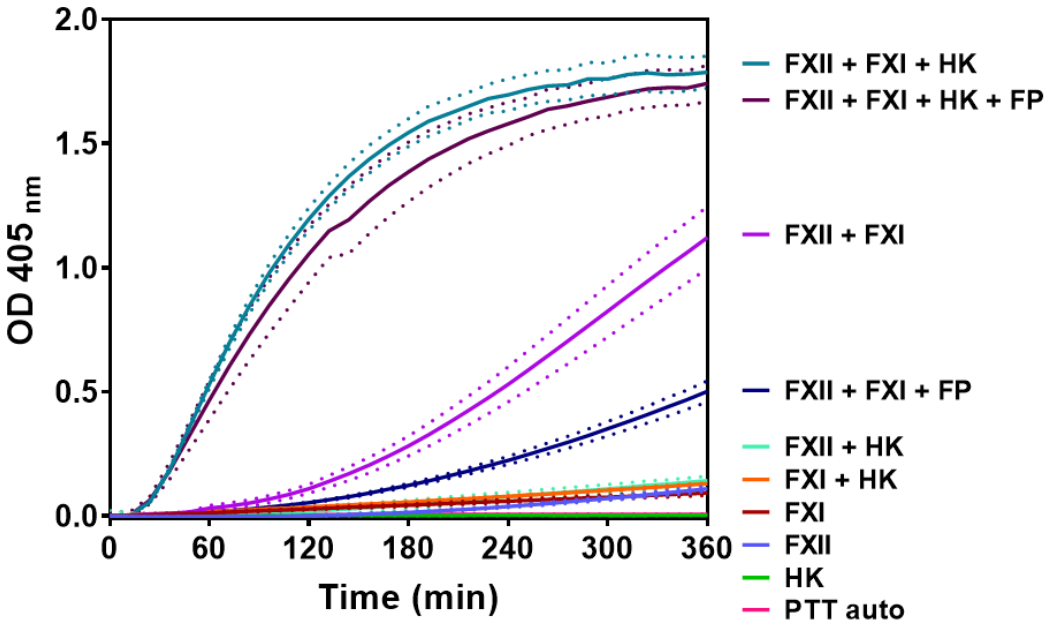


Figure 4.19 FP modulates the intrinsic pathway by inhibiting FXI activity or its activation by FXIIa (in the absence of HK) in a chromogenic assay.

Reactions of 2 nM FXII, 30 nM FXI, 30 nM HK and 1% PTT automate (PTT auto) were incubated over a course of 6 hours at 37°C in the presence and absence of 25 µg/mL FP. Raw data were plotted as optical density (OD) read at 405 nm vs time (minutes); graphs were generated using GraphPad Prism v8. Assay was run in triplicate, on one plate and data are shown as mean ± SEM.

In the presence of the intrinsic pathway agonist, PTT automate, FXII becomes activated and cleaves FXI. The addition of HK greatly accelerates this reaction. HK binds to surfaces, pulling down FXI, allowing the interaction between FXII and FXI, leading to intrinsic activation. The presence of FP reduced the activation of FXI by autoactivated FXII when HK was not present. It could potentially indicate that FP is acting at the same site on FXI as HK but with a lower affinity, however, it is more likely that FP is blocking the interaction of FXI with the negative surface, and this blocking action is overcome in the presence of HK (Figure 4.19).

HK is an important cofactor for the surface dependent activation of PK, and a similar theory is postulated for FXI (Wiggins et al., 1977). HK mediates surface binding of FXI allowing FXII to bind in proximity, leading to activation of FXI. HK has also been shown to prevent cleavage of a chromogenic substrate by FXI autoactivated by polyanions (Ivanov et al., 2017b).

To further understand the mechanism behind the chromogenic assay, incubations were performed and were analysed using SDS-PAGE (Figure 4.20).

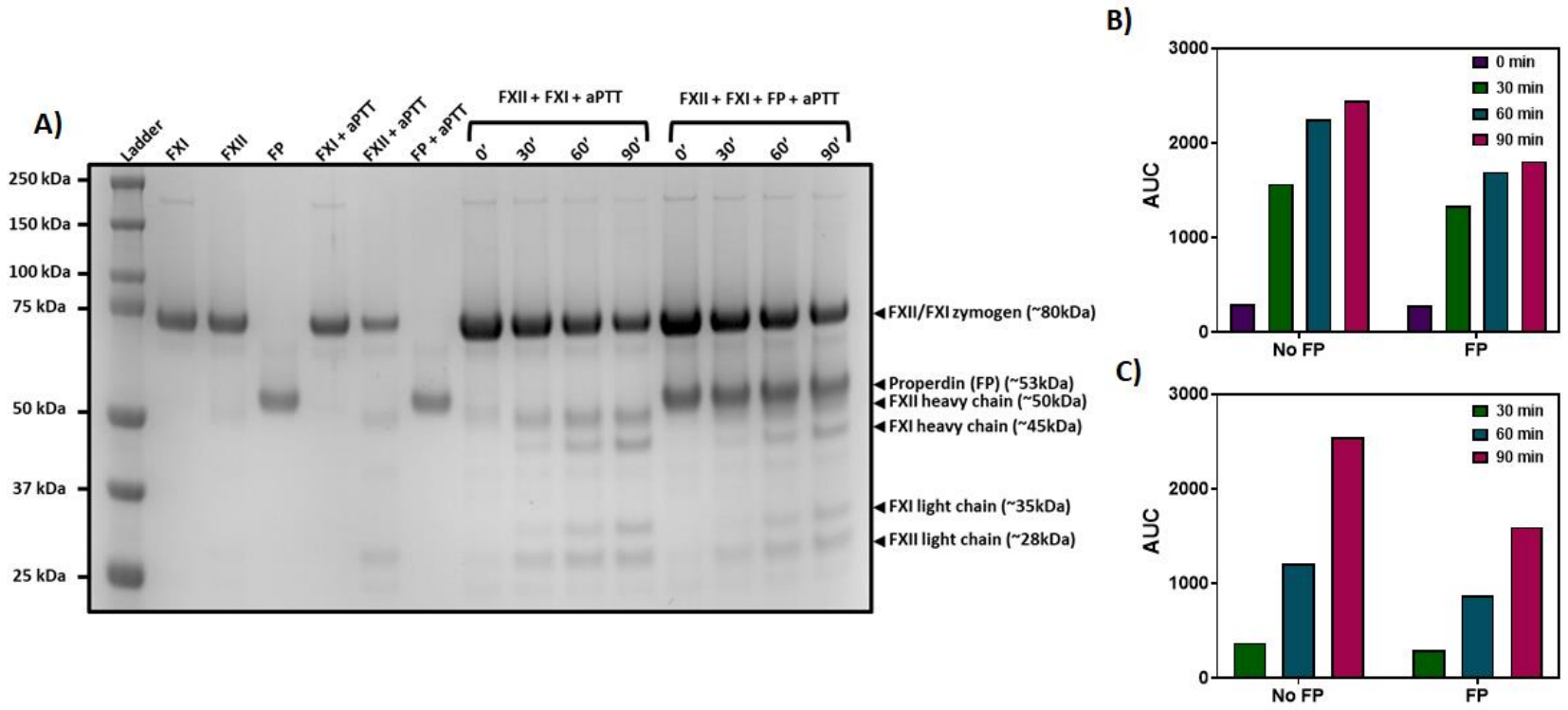


Figure 4.20 FP modulates FXII activation, thereby affecting FXI activation.

Reactions of 2 µg FXII, 2 µg FXI, 4 µg FP and 5% aPTT were incubated for 90 minutes with samples taken at 30-minute intervals. A) SDS-PAGE of reactions. B) FXIIa light chain. C) FXIa heavy chain. Samples were run using reducing SDS-PAGE and band analysis was performed using ImageJ and GraphPad Prism v8. AUC was plotted vs. incubation time. Reading could not be taken for 0 min on FXI heavy chain band as it was not yet present.

SDS-PAGE revealed that there may be an underlying mechanism of FP that affects FXII activation, thus affecting the downstream activation of FXI (Figure 4.20). This assay was performed in the absence of HK, as the bands may have overlapped on the gel after staining. The band intensity was measured by calculating the area under the curve using ImageJ which was then plotted against incubation time. The intensity of FXIIa light chain and FXIa heavy chain were used to determine the effect of FXI and FXII activation, respectively. The reduced band intensity of both FXII and FXI in the presence of FP when compared to its absence suggests a modulation of FXII activation. There is no apparent cleavage of FP in the SDS-PAGE, suggesting that in this case there is no change in substrate specificity. This supports the hypothesis that FP is modulating the interactions between coagulation factors and polyanionic surfaces and is not inducing substrate specificity changes.

The effect of sulfatides was then investigated as a more physiological surface, with HK present, in reactions of FXII, FXI and FP.

4.11 FP Modulates FXI Activation by FXII Activated by Sulfatides

To further characterise the physiological relevance of these data, the next surface tested was sulfatides which are expressed on a variety of cell surfaces and are involved in several processes including cell- adhesion and nerve conduction. Sulfatides can interact with many molecules including P-selectin (Merten et al., 2005) and vWF (Data et al., 1991). It has been speculated that sulfatides may have both coagulant and anticoagulant properties, suggesting an important role in haemostasis and/or thrombosis. Properdin has specific binding sites for sulfatides on the TSR_5 (Higgins et al., 1995). The intrinsic pathway is also initiated by sulfatides via activation of FXII (Tans and Griffin, 1982), thus it was important to investigate this interaction in the presence of FXI, FXII and HK. For this investigation, 50 µg/mL sulfatides from bovine brain were incubated with FXII and FXI, in the presence and absence of HK. Properdin was titrated into this reaction using a physiological concentration range (1-25 µg/mL), and cleavage of the chromogenic substrate was recorded over 3 hours (Figure 4.21).

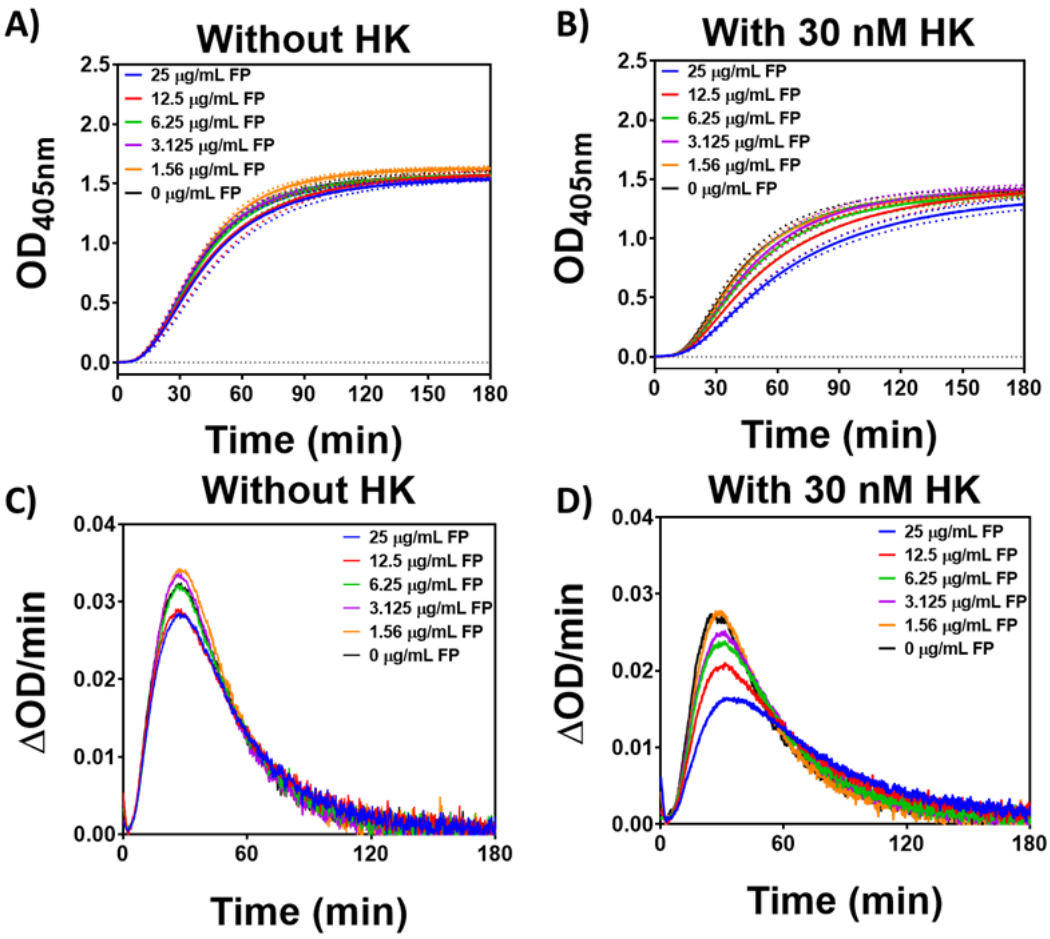


Figure 4.21 FP modulates FXI activation by FXII activated by sulfatides in the presence of HK.

Reactions of 30 nM FXI and 2 nM FXII were incubated with a titration of FP, in the presence and absence of 30 nM HK, with 1% (w/v) PEG and 10 µM $ZnCl_2$. A) No HK present. B) 30 nM HK present. C) First derivative of "A". D) First derivative of "B". Raw data were plotted as optical density (OD) read at 405 nm vs. time (min); graphs were formed using GraphPad Prism v8. Assay was run in triplicate, on one plate and data are shown as mean ± SEM.

In the presence of 50 µg/mL bovine sulfatides, the small amount of FXII readily gained activity and activated FXI. The rate of reaction was decreased in the presence of 30 nM HK. The presence of FP appears to modulate this reaction, showing a dose dependent decrease in the rate of reaction, demonstrated by the lower peak of the first derivative results supporting the hypothesis that the interaction is surface dependent.

4.12 FP modulates inhibition of FXIa by C1-INH and DXS_{500kDa}

To further investigate the mechanisms behind how FP affects FXI and FXIa, a chromogenic assay was developed to determine the effect of FP on FXIa inhibition. C1-INH is one of the main inhibitors of FXIa in plasma and this is greatly enhanced in the presence of GAGs; in this instance DXS_{500kDa} was used to remain consistent with previous experiments. The functions of C1-INH demonstrate the overlap between complement and coagulation.

Firstly, optimisation steps were performed to determine the optimal concentrations of C1-INH and DXS_{500kDa} (Figure 4.22). Reactions of FXIa, C1-INH and DXS_{500kDa} were pre-incubated for two hours and the assay was started with the addition of chromogenic substrate S-2288. C1-INH and DXS_{500kDa} were titrated with a two-fold serial dilution. In FXIa activity assays, 3 nM FXIa was used, which is a ten-fold dilution of the plasma concentration of FXI. A maximum of 180 nM C1-INH was used as this is around ten-fold less than the plasma concentration to keep physiological stoichiometric ratios within the assay.

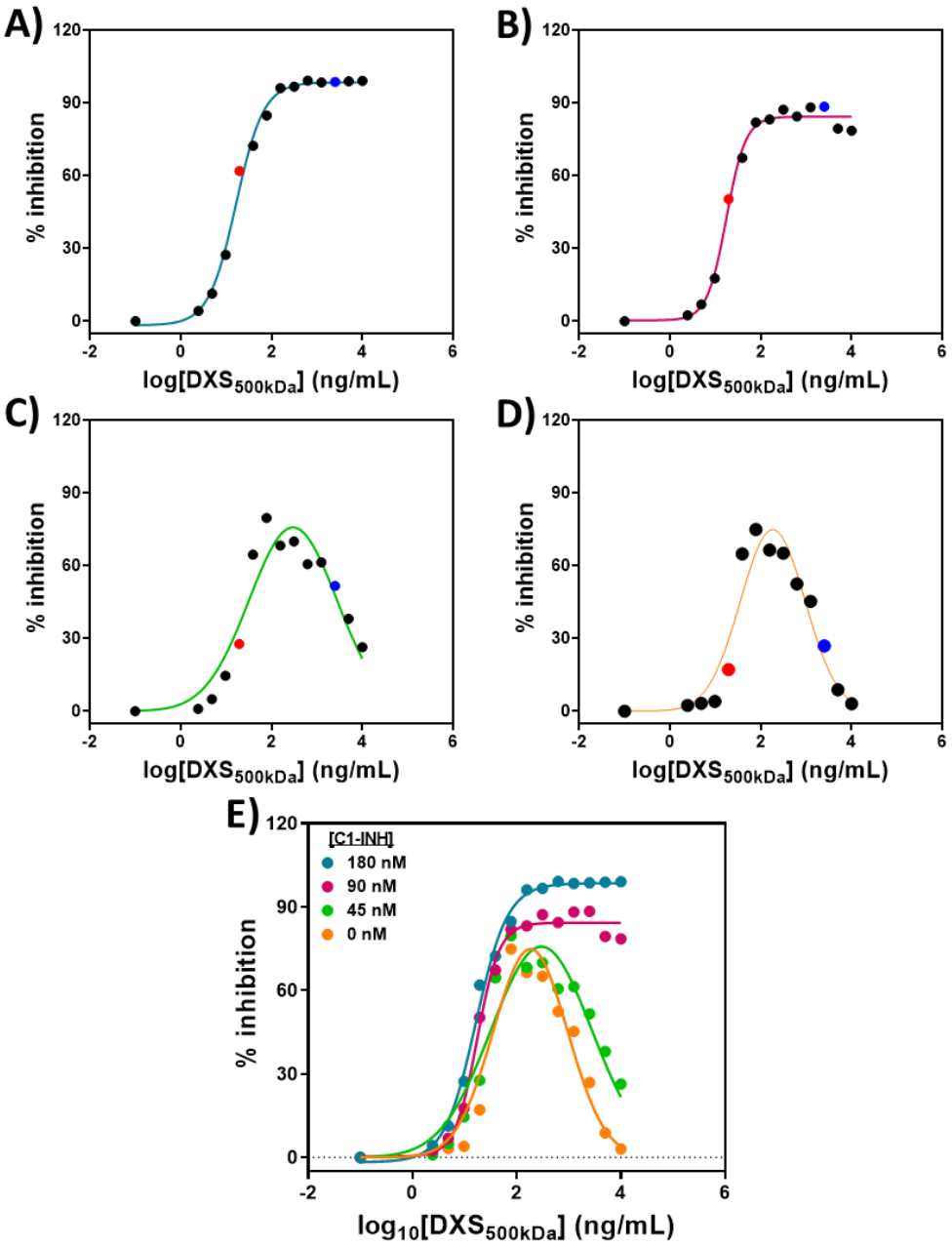


Figure 4.22 FXIa is dose dependently inhibited by C1-INH and via a template mechanism by DXS_{500kDa}.

Reactions of 3 nM FXIa with serial dilutions of C1-INH and DXS_{500kDa} were incubated for two hours prior to adding chromogenic substrate S-2288. Once substrate was added, readings were taken at 12 second intervals. Data were normalised to the lowest and highest absorbance values of the data set as 100% and 0% inhibition respectively. A) 180 nM C1-INH. B) 90 nM C1-INH. C) 45 nM C1-INH. D) 0 nM C1-INH. E) Merged data from A, B, C and D. Curves A and B were fit with a nonlinear model (variable slope [four parameters]) and curves C and D were fit with the Gaussian model, all using GraphPad Prism v8. Red (0.0195 µg/mL) and blue (2.5 µg/mL) points highlight two concentrations of DXS_{500kDa} of interest for future experiments. Assay was performed in triplicate on two plates. Each normalised to controls on specific plate. 0 µg/mL DXS_{500kDa} is represented as –1 for curve fitting.

Figure 4.22 shows dose-dependent inhibition of FXIa by C1-INH, as well as a template mechanism of inhibition by DXS_{500kDa}. Inhibition is most effective when using the top concentration of C1-INH (180 nM) with a range of concentrations of DXS_{500kDa} (0.156-10 µg/mL). These data show the importance of the interaction between C1-INH and GAGs for efficient FXIa inhibition.

Concentrations of DXS_{500kDa} were taken from opposite sides of the template curve (Figure 4.22) to help us delineate whether FP would affect C1-INH interactions or DXS_{500kDa} interactions only; 0.0195 µg/mL and 2.5 µg/mL were used.

Reactions of FXIa, FP, C1-INH and DXS_{500kDa} were pre-incubated for two hours at 37°C prior to adding the chromogenic substrate. The chromogenic substrate was then added to start the reaction (Figure 4.23).

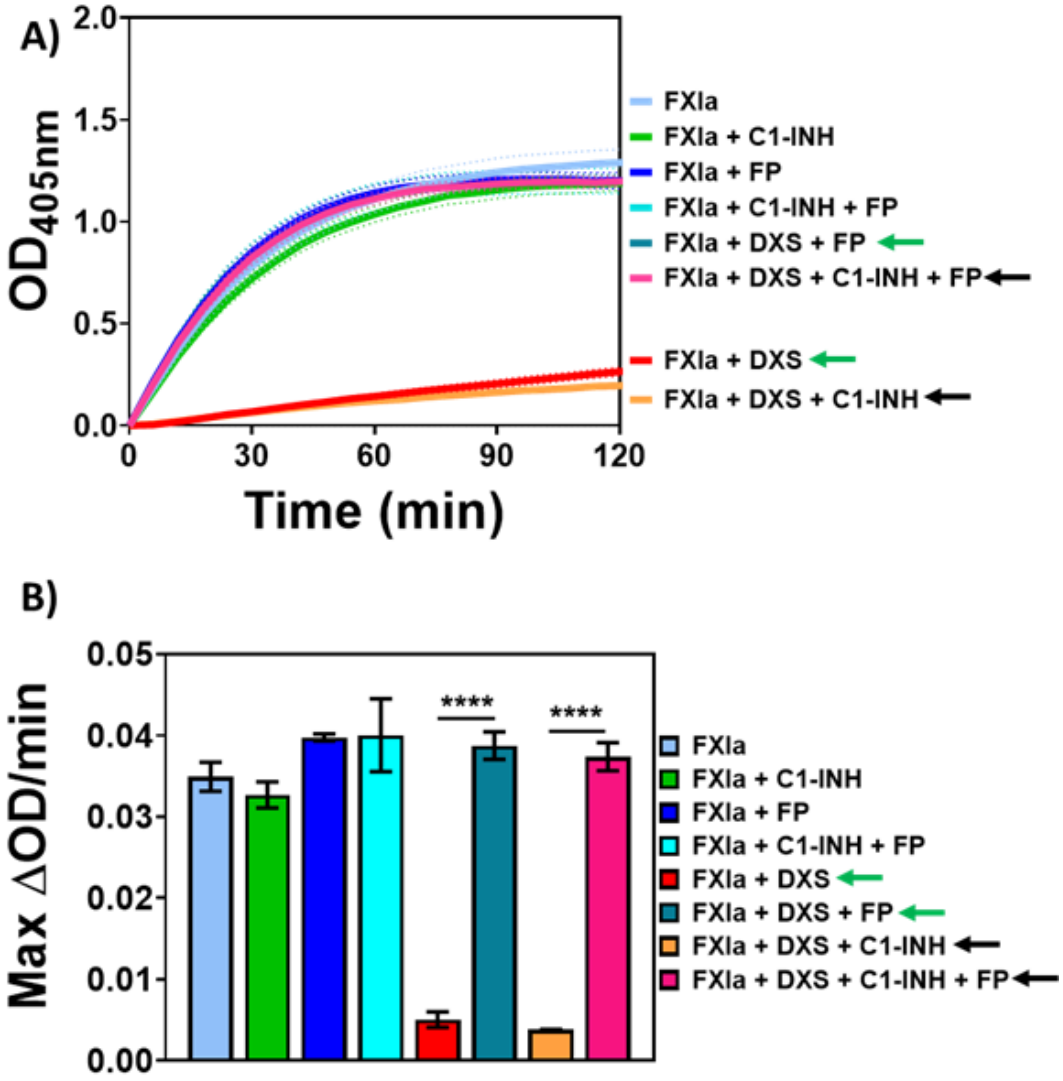


Figure 4.23 FP reverses inhibition of FXIa by C1-INH and DXS_{500kDa}.

Reactions of 3 nM FXIa, 45 nM C1-INH, 19.5 ng/mL DXS_{500kDa} and 25 µg/mL FP were preincubated at 37°C for 2 hours. S-2288 was then added to start the reaction, and optical density (OD) readings were measured at 405 nm for 2 hours. A) Baseline subtracted data. B) Maximum rate of reaction calculated from the first derivative of "A". Data represented as mean of triplicates from one assay ± SEM. **** p≤0.0001. *Arrows used to highlight data of interest.*

Inhibition of FXIa by C1-INH is poor in these assays, and the presence of a surface is necessary for complete inhibition. This experiment involved a very low concentration of DXS_{500kDa}, and FXIa was completely inhibited, regardless of the presence of C1-INH. Properdin does not affect the interaction between C1-INH and FXIa alone, however in reactions of FXIa, C1-INH and 19.5 ng/mL $DXS_{500kDa,}$ 25 µg/mL FP completely reverses the inhibition of FXIa ($p \leq 0.0001$). Due to the major inhibitory effect of DXS_{500kDa} on FXIa regardless of C1-INH, it was important to evaluate whether the observation is FP interacting directly with the surface. The experiment was repeated, using a higher concentration of DXS_{500kDa} on the opposite side of the template model of inhibition, alongside a titration of C1-INH (Figure 4.24).

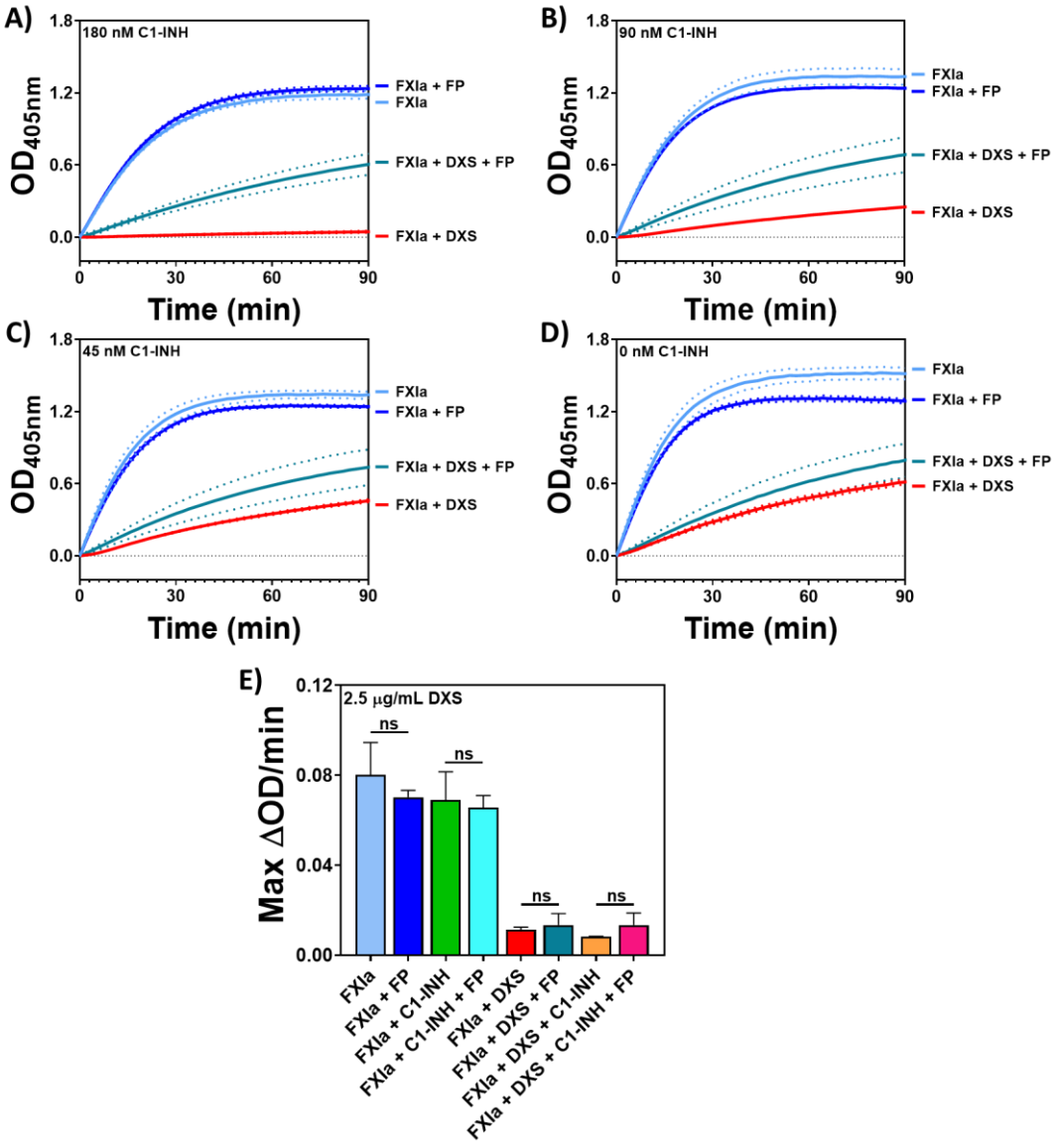


Figure 4.24 FP does not reverse the inhibition of FXIa by C1-INH when DXS_{500kDa} is at a higher concentration.

Reactions of 3 nM FXIa, titration of C1-INH, 2.5 µg/mL DXS_{500kDa} and 25 µg/mL FP were preincubated at 37°C for 2 hours. S-2288 was then added to start the reaction, and optical density (OD) readings were measured at 405 nm for 1.5 hours. A) Baseline subtracted data at 180 nM C1-INH. B) Baseline subtracted data at 90 nM C1-INH. C) Baseline subtracted data at 45 nM C1-INH. D) Baseline subtracted data at 0 nM C1-INH. E) Maximum rate of reaction calculated from the first derivative of "C". Data represented as mean of triplicates from one assay ± SEM. *ns=not significant.*

C1-INH was titrated in reactions in the presence of 2.5 µg/mL DXS_{500kDa}, 3 nM FXIa and 25 µg/mL FP. C1-INH inhibited FXIa in a dose dependent manner, and this was greatly enhanced in the presence of DXS_{500kDa}. Properdin alone did not significantly modulate the inhibition of FXIa by C1-INH, however in the presence of DXS_{500kDa}, FXIa inhibition was reduced and this was dependent on the concentration of C1-INH. This suggests that the reduced FXIa inhibition caused by the presence of FP is surface dependent and relies on an interaction between FP and DXS_{500kDa}.

To test this in a more physiological setting, this experiment was repeated in the presence of C1-INH enhancer, heparin sodium salt (HSS). Heparin binds to a multitude of complement proteins with a high affinity for C2 and FB (Yu et al., 2005). Heparin is also an important cofactor for serpins, increasing their inhibitory effect. Heparin increases the inhibition of C1s by C1-INH, but also aids the inhibition of FXI by AT-III and C1-INH (Wuillemin et al., 1997, Wuillemin et al., 1996).

FXIa, HSS, FP and C1-INH were incubated as previously described and readings were taken over 2 hours (Figure 4.25).

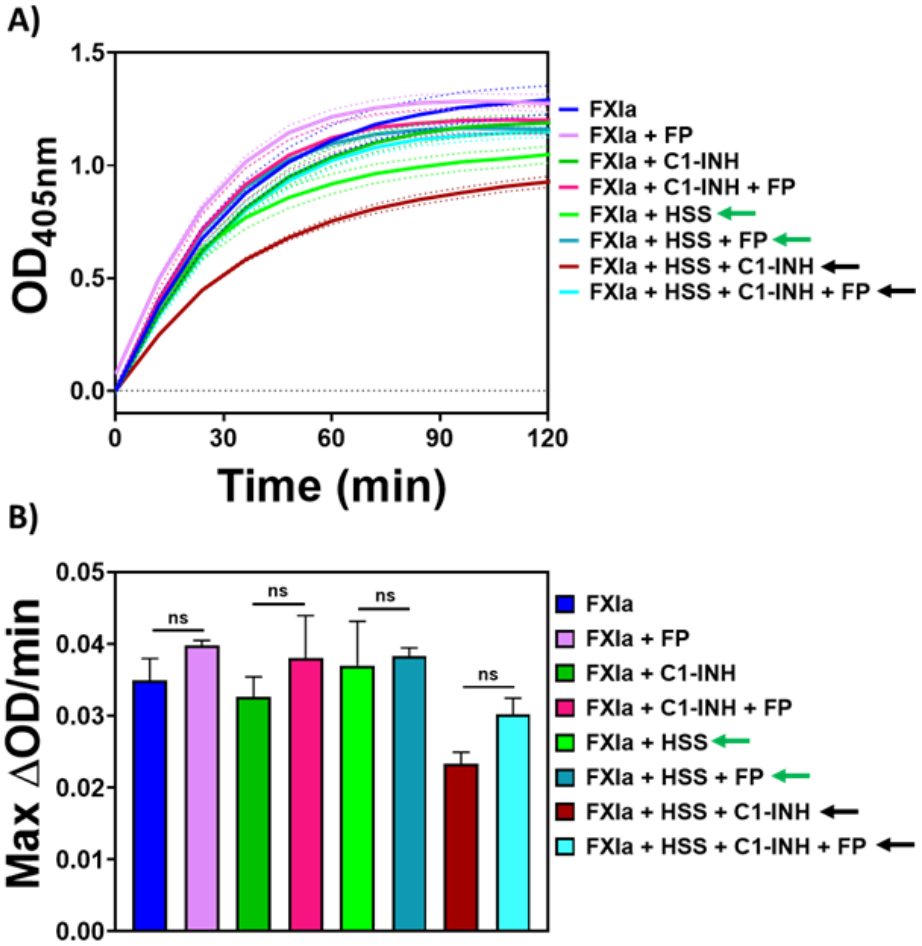


Figure 4.25 FP reverses inhibition of FXIa by C1-INH and HSS.

Reactions of 3 nM FXIa, 45 nM C1-INH, 100 pg/mL HSS and 25 µg/mL FP were preincubated at 37°C for 2 hours. S-2288 was then added to start the reaction, and optical density (OD) readings were measured at 405 nm for 2 hours. A) Baseline subtracted data. B) Maximum rate of reaction calculated from the first derivative of "A". Data represented as mean of triplicates from one assay ± SEM. *Arrows used to highlight data of interest.*

FXIa inhibition by C1-INH alone was not significant, however this was increased by the presence of HSS. Properdin had no inhibitory effect of C1-INH with FXIa alone. Properdin was able to partially reduce the inhibition of FXIa by C1-INH in the presence of HSS, though this was not significant, it does follow a similar trend as seen with DXS_{500kDa}. This supports the concept that FXIa inhibition can be modulated in a surface dependent manner by FP.

4.13 Discussion

Crosstalk between complement and coagulation has been researched for over 80 years (Wadsworth et al., 1937) yet the mechanisms behind the interactions are still not fully understood. Many thromboinflammatory events involve both cascades, suggesting that elucidating the key mechanisms may reveal potential therapeutic targets. Many therapeutics targets of coagulation and complement components already exist, however the effects of these on the opposing cascade are often overlooked.

Binding of coagulation proteins to complement factors was investigated using SPR. C3b and properdin were immobilised to the sensor surface and coagulation proteins were injected. Factor XI appeared to bind well to both C3b and FP, with fibrinogen also binding with a low response to both C3b and FP. Factor IX, PK, FII, plasminogen, FXII, FX and FVII demonstrated negligible binding, however this could be further explored using higher analyte concentrations. Factor XIa binding was then explored and it was elucidated that both FXI and FXIa could bind to C3b and FP, with high affinity in the nM and pM ranges. This high affinity binding observed suggests the potential of a physiological interaction.

The autoactivation of coagulation factors such as FXI and PK has long been investigated however the physiological relevance has been debated (Naito and Fujikawa, 1991a, Brunnée et al., 1993, Tans et al., 1987). The autoactivation of FXII has been well characterised and it has been suggested that FXII gains single chain activity once bound to a polyanionic surface through conformational changes, leading to the cleavage and activation PK and a downstream reciprocal activation mechanism (Griffin, 1978). It has also been observed that PK can autoactivate in the presence of sulfatide and DXS (Tans et al., 1987) however, this was in a purified system therefore the physiological relevance remains unclear.

FXI has two active forms where either one or two of the subunits are activated, 1/2FXIa and FXIa respectively. 1/2FXIa is formed on cleavage of one subunit by either FXIIa or thrombin and this conversion is quicker than the conversion of 1/2FXIa to FXIa suggesting a physiological importance for both species (Mohammed et al., 2018). As FXI monomers are activated much slower than the dimeric form, this suggests a *trans*-activation mechanism whereby the activating protease binds to one subunit whilst activating the other (Wu et al., 2008). Factor XI can be activated by both FXIIa and thrombin however this reaction is inefficient without polyanionic surfaces, such as DXS and polyP, which enhance the activation of FXI and promote FXI autoactivation (Geng et al., 2013a, Choi et al., 2011). It has been shown that although 1/2FXIa has some activity, monomeric FXI cannot be used to reconstitute FXI deficient mice, supporting the hypothesis that FXI is required to be in its dimeric form for *trans*-activation (Geng et al., 2013b). The autoactivation mechanism is still unclear but it is thought that this occurs due to residual FXIa in purified preparations of FXI.

To explore the consequences of this interaction, a chromogenic assay was optimised to investigate the effect of FP on FXI autoactivation induced by polyanions.

The first step to this investigation was optimised using the non-physiological GAG, DXS_{500kDa}. Highly sulfated GAGs are important in both complement and coagulation. They are linear polysaccharides comprised of repeating disaccharides containing an amino sugar and an hexuronic acid and is involved in infection, cell migration and cell growth (Quittot et al., 2017). GAGs aid in regulation of coagulation and are heavily involved in mechanisms of the intrinsic pathway. GAGs may be described as double-edged swords. They have a net charge which is highly negative, and this can culminate in FXII activity and autoactivation of FXI (Hojima et al., 1984). However, they also increase the inhibition of FXIa by C1-INH (Schoenfeld et al., 2016). Microbes bind to GAGs on self-surfaces, as do complement proteins, colocalising the

two and initiating complement activation at the microbial surface. Properdin binding to GAGs has also been demonstrated (Kouser et al., 2013).

DXS is a polymer of glucose containing 17-20% sulfur that has been used as an anticoagulant, but also as an agonist to induce an inflammatory response. It has been demonstrated to have the ability to inhibit the binding of HIV-1 to immune cells and has thus been investigated for use as an antiviral agent (Nakashima et al., 1989). It is commonly used as both an experimental and clinical laboratory reagent and research has been carried out on the potential use as a therapeutic for hyperlipidemia (Tsubamoto et al., 1994). DXS has been used to investigate contact activation of zymogens from the intrinsic pathway of coagulation for over 35 years (van der Graaf et al., 1982). DXS can induce activity of FXII, autoactivation of FXI and can act as an inhibitor of FXIa via an allosteric mechanism (Sinha et al., 2004). DXS can affect the activity of serpins, including C1-INH however this effect varies according to the target protease; this is dependent on the presence of basic side chains being neutralised, thus the repulsive forces being reduced by the negatively charged polysaccharide, bringing C1-INH and the protease together (Dijk et al., 2016).

The initial investigation was to characterise the autoactivation mechanism of FXIa by using two different polymer lengths of DXS: DXS_{40kDa} and DXS_{500kDa}. Factor XI zymogen was incubated with DXS and the autoactivation was measured by recording the cleavage of the chromogenic substrate by the change in absorbance at 405 nm over time. Both DXS_{40kDa} and DXS_{500kDa} resulted in a bell-shaped curve, demonstrating a template effect and allowing the optimum concentration of DXS to be calculated. DXS_{500kDa} was the more efficient agonist, resulting in faster autoactivation thus this was taken forward for future experiments.

It was then investigated how FP may interfere with the autoactivation mechanism of FXI. Using DXS_{500kDa}, a chromogenic assay was employed, involving a titration of

physiological concentrations of FP. The autoactivation of FXI was inhibited in a dose-dependent manner by the by FP, demonstrated by the reduced cleavage of the chromogenic substrate. The mechanism behind this was further explored as FP could be blocking the interaction between the surface and FXI, but the reduced cleavage of S-2288 may be due to a change in substrate specificity. Due to the known anion binding properties of FP it was most likely that FP was blocking the surface interactions of FXI.

An experiment was then performed to determine if this was due to the differences in charge between FP and DXS_{500kDa} by using protamine sulfate, most often used clinically as a reversal agent for the negatively charged anticoagulant heparin (Jaques, 1973). Protamine sulfate was titrated in the autoactivation assay, and cleavage of S-2288 was completely diminished, supporting the hypothesis that FP may be affecting FXI autoactivation via charge-mediated interactions.

This is not the first time that the autoactivation of FXI has been inhibited by plasma proteins. Scott & Colman (1992) revealed that in the presence of both fibrinogen and HK in plasma FXI would not be activated either via thrombin or through autoactivation (Scott and Colman, 1992). It has also been demonstrated that the presence of HK inhibits the autoactivation of FXI by nucleic acids (Ivanov et al., 2017b) and sulfatides.

It was shown using SDS-PAGE the FXI autoactivated by DXS_{500kDa} was able to cleave HK. This may be the cause of the reduction of cleavage of the chromogenic substrate. The change in substrate specificity of FXIa was later explored. This is not the first time that HK has been shown to be a substrate for FXIa (Gailani and Broze, 1991, Scott et al., 1985), bringing about important questions of whether FP was acting as a substrate for FXIa in the presence of an anionic surface.

To further elucidate the mechanism behind the interactions between FP and FXI(a) amidolytic activity, Michaelis-Menten kinetic analysis was performed. Kinetic analysis

is important to determine if FP was affecting FXIa amidolytic activity. S-2288 was titrated and FXIa activity was investigated in the presence of FP, DXS_{500kDa} and both FP & DXS_{500kDa}. DXS_{500kDa} alone displayed an uncompetitive inhibitory effect of FXIa, by decreasing the k_{cat} and the K_m resulting in less substrate cleaved over time but an increased affinity for the substrate. DXS_{500kDa} must therefore only bind to the enzyme substrate complex. Properdin alone did not affect FXIa amidolytic activity, however in the presence of DXS_{500kDa}, k_{cat} remained reduced and the reduction in K_m was reversed, suggesting FP is interfering with the surface interaction between FXI(a) and DXS_{500kDa}.

This raises the question of why DXS_{500kDa} can lead to the autoactivation of FXI but can also inhibit FXIa. It may be postulated that there are two binding sites for DXS_{500kDa} on FXI(a), but it could also be due to the dimeric structure of FXI(a). The binding of FXI to DXS_{500kDa} may have a high enough affinity that once autoactivated, the complex does not dissociate. This however needs to be further explored. To support these data, autoactivation of other contact zymogens was investigated, using sulfatides as the agonist. FP was also able to modulate PK autoactivation, but not FXII. As there are similarities in the characteristics of FXI and PK, it suggests an avenue worth investigating in the future for physiological relevance.

The substrate specificity of FXI autoactivated by DXS_{500kDa} was then explored using SDS-PAGE to determine if there may be a substrate specificity change of FXIa, leading to the decreased cleavage of S-2288. It was demonstrated that DXS_{500kDa} leads to the conversion of FXI to FXIa, and the addition of FP did not inhibit the autoactivation of FXI. The gel did reveal unknown bands, presumed to be cleavage bands of FP. The next experiment performed was to determine if there was a difference in autoactivated FXI and preactivated FXIa, by performing a similar experiment and incubating FXIa with DXS_{500kDa} and DXS_{500kDa} & FP. This experiment showed that FXI(a) was potentially cleaving FP in the presence of the anionic surface.

The gel was repeated and was analysed using the MS facility at the University of Leeds, where it was revealed that the cleavage products were FP. The cleavage products did not appear unless DXS_{500kDa} was present, suggesting that the cleavage of FP was dependent on the presence of DXS_{500kDa}. Due to this change in substrate specificity, it was important to then explore whether this prevented the cleavage of the physiological substrate of FXIa, FIX, to determine if this novel interaction may have a downstream effect in the coagulation cascade. The first experiment was a chromogenic assay utilising FXIa and incubating this with FIX, FP and FIX & FP. The cleavage of FIX was determined by an increase in optical density observed when the chromogenic substrate S-2765 was cleaved over and above that of FXIa alone. It was observed that there may be a minor effect in the cleavage of FIX, however this was not significance due to the large range of values. The interaction was further explored by SDS-PAGE and involved DXS_{500kDa} as it has already been demonstrated that the substrate specificity change is only present with the presence of the anionic surface. First the experiment was optimised to ensure the cleavage of FIX was observed. The experiment was then repeated in the presence of FP. Factor IX could still be cleaved in the presence of DXS_{500kDa} and the cleavage of FIX by FXIa was not affected by this. Properdin would therefore not act as an inhibitor of the intrinsic pathway through the surface mediated change in substrate specificity.

The cleavage of FP may be due to conformational changes of FP when bound to a surface. The high theoretical isoelectric point of FP makes it highly likely to bind to anionic surfaces as FP is highly positively charged at neutral pH. This attraction and binding may lead to the exposure of cleavage sites for FXIa on FP. The cleavage products of FP observed using SDS-PAGE could be explained by the exposure of cryptic disulphide bonds on FP once bound to a surface, however this is unlikely as they only appear in the presence of FXI(a), supporting the theory that FXI(a) can cleave FP. It would be interesting to explore the effect of these cleavage products to

elucidate the physiological need to cleave FP, however this is not possible at the current time.

More physiological surfaces were investigated, as DXS_{500kDa} is a synthetic polyanion, to determine if the cleavage of FP may occur under a more physiological environment. Sulfatides are predominantly found within components of the central nervous system including the brain but can also be found in other organs including the kidneys. The presence of sulfatides potentially revealed cleavage products of FP however this needs further investigation. PolyP can be found in many biological settings and can induce haemostasis, thrombosis and inflammation. PolyP come in a variety of lengths, with long-chain polyP found in pathogenic microorganisms being a potent agonist for the intrinsic pathway of coagulation. Short-chain polyP is secreted from activated platelets and aids in FV activation, clot structure and thrombin activation of FXI, while diminishing TFPI function (Morrissey et al., 2012). The SDS-PAGE showed that both long- and short-chain polyP did not lead to the cleavage of FP by FXIa. PL play an important role in both intrinsic pathway and complement activity. Phosphatidylserine (PS) is a major component of the PL membrane at the cell surface and is exposed upon platelet activation and induction of apoptosis. PL also act as cofactors throughout the coagulation cascade. The negative charge of PL may lead to FP binding and this interaction was explored with FXIa; no cleavage products of FP were observed. Heparin sodium salt (HSS) is a GAG that acts as a physiological inhibitor of the intrinsic pathway and is also utilised clinically as an anticoagulant. HSS aids in the inhibition of FXIa by C1-INH and it has been shown that FP can bind to HSS. GAGs, including heparin, regulate complement activity with heparin enhancing the inhibitory activity of C1-INH on C1 in the fluid phase (Caldwell et al., 1999). The presence of HSS did not reveal any cleavage product of FP.

It is interesting that reactions involving DXS_{500kDa} lead to FP cleavage by FXI(a), and potentially the presence sulfatide also leads to this novel phenomenon. Care must be taken when reviewing the literature that utilises DXS in experiments as it is not a physiological GAG and may provide misleading data. The presence of sulfatide in these incubations revealed the need for further investigation. It is interesting that there are potential cleavage products as the presence of DXS has been shown to interfere with the interaction between FP and sulfatide. It has been observed that DXS can inhibit FP binding to sulfatide (Holt et al., 1990), which suggests the possibility of a shared binding site on FP, which may be the key to revealing possible cryptic cleavage sites for FXIa.

To further investigate the interaction between FP and FXI, a chromogenic assay was optimised to induce contact activation of intrinsic pathway proteins using the clinical agonist PTT automate to activate FXII, which in turn activates FXI. Factor XI activity was measured by detecting the change of absorbance as pNA was released from the chromogenic substrate. This assay involved the physiological cofactor for FXI, HK. In the absence of HK, FP clearly reduces the activation of FXI by activated FXII however this is less obvious in the presence of HK. This may suggest that the binding site on FXI for FP is the same site used to bind to HK. It is known that the majority of FXI circulates in complex with HK thus these data may suggest that the interaction between FP and FXI is not physiological. There is speculation that platelets may express FXI and if this is released into the microenvironment, it may be an opportunity for binding of FP. There is evidence that FXI and PK compete to bind to HK and that their binding sites overlap (Thompson et al., 1979, Renné et al., 1999). This suggests a discontinuous binding of HK to FXI supporting the physiological relevance of FP binding to FXI. It would also be interesting to perform experiments using PK to reveal whether FP may exhibit similar effects on the autoactivation.

This was then further explored using SDS-PAGE. The mechanisms were determined by observing the density of the FXI and FXII bands. Cleavage bands of FP do not appear suggesting that PTT automate does not induce a substrate specificity change, however, there appears to be a lower band intensity in the samples containing FP compared to without FP, suggesting that less FXII activation and thus less FXI activation. This supports the hypothesis that FP is interfering with the interactions between the coagulation factors and the polyanion surfaces.

To create a more physiological environment, sulfatide was used in similar reactions. Sulfatide successfully induced contact activation of FXII leading to activation of FXI. HK had little effect on this alone however in the absence of HK, FP reduced FXI activity supporting the hypothesis that FP is affecting the interaction via the negatively charged surface.

As previously described, the inhibition of FXIa by C1-INH is modulated by anionic surfaces. It was therefore important to investigate whether FP would affect this interaction. It was clearly demonstrated that C1-INH alone is a poor inhibitor of FXIa and it is greatly enhanced in the presence of DXS_{500kDa}, where a template effect was observed once again. From these data it was possible to establish the optimum conditions to use in the next investigation. A chromogenic assay was employed to determine how FP effected the surface dependent inhibition of FXIa by C1-INH. Properdin completely reversed the inhibition of FXIa when both DXS_{500kDa} and C1-INH were present however it also completely reversed the inhibition by DXS_{500kDa} alone. Therefore, it was important to elucidate this interaction further and a higher concentration of DXS_{500kDa} was utilised. This assay revealed that FP could not inhibit C1-INH but was inhibiting the interaction between the DXS_{500kDa} and either C1-INH and/or FXIa. It appears likely that DXS_{500kDa} is effectively being coated by the highly positively charged FP. This was further investigated using the physiological cofactor, HSS. Although inhibition of FXIa is not as high with HSS when compared to

DXS_{500kDa}, there is still a reversal effect caused by FP again supporting the hypothesis that FP is exhibiting its effect at the anionic surface (Figure 4.26). This novel interaction is complex and open-ended. There is a lot of work that needs to be performed to understand the importance of what has been observed.

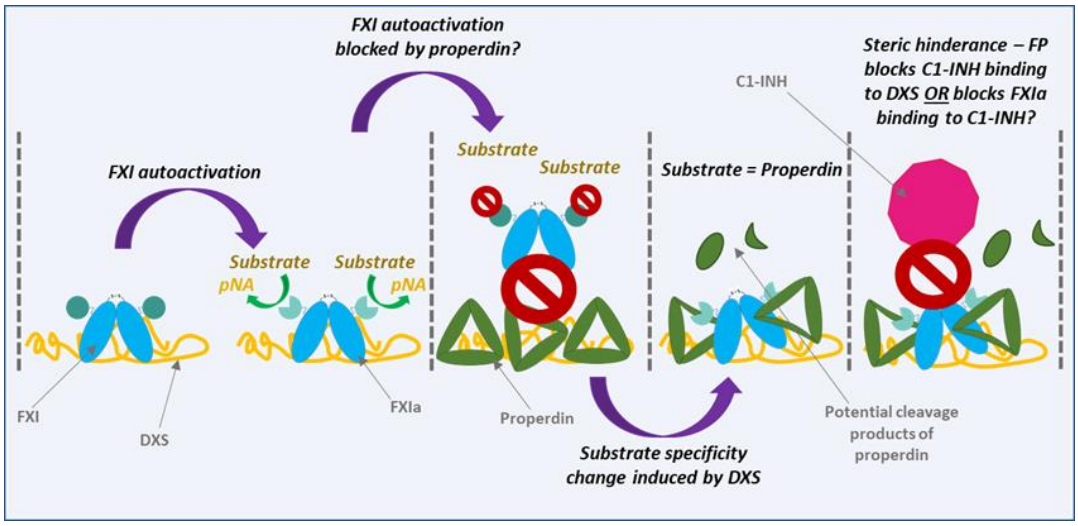


Figure 4.26 Schematic representation of potential interactions between FXI, DXS and FP.

FXI autoactivation by DXS appears to be “blocked” by FP, however it was determined that FXIa could cleave FP in the presence of DXS. It is more likely that FP undergoes conformational changes when bound to DXS making it more amenable to cleavage by FXIa. In the presence of C1-INH, FP may be blocking the interaction of DXS and the inhibitor when at low concentrations, demonstrated when this “blocking” effect is prevented at higher concentrations of DXS.

4.14 Considerations for Future Work

As this is a complicated mechanism to investigate there is still much left to explore. The interaction between FP and FXI is novel, and however it is still not clear whether this interaction would still take place in the presence of the physiological cofactor, HK. Thus, it would be important to investigate the binding interactions using SPR, to determine whether the FXI(a):HK complex would still bind to FP. It would be useful to back this up using ELISA style assays, whereby FP is immobilised on the plate surface, and purified FXI and FXI & HK is incubated to determine the effect of HK. The complexes could be detected by a polyclonal FXI antibody. These assays can also be used for plasma binding experiments where plasma is incubated over the FP surface. This would help to determine the physiological relevance of the interaction. It is also highly likely that a surface is necessary for the two proteins to interact. In the SPR experiments, FP was immobilised to a dextran surface via amine coupling. This may influence the interaction. It may also be useful to perform chromogenic assays to determine how FP may affect the activation of FXI by thrombin, as this is also greatly enhanced in the presence of anionic surfaces.

As FP is cleaved in the presence of DXS_{500kDa} and FXI(a), it is not known whether these cleavage products have a physiological effect. It would be interesting to determine if these cleavage products are fragments, or whether it leads to a different conformation of FP. If fragments of FP are found, it may be that these fragments affect opsonisation of pathogens and cell migration, as is seen with generation of C3a and C5a. It would be important to determine what peptides are within the fragments, which would help to elucidate potential functional mechanisms of the fragments. Affimers or antibodies could be used for detection of fragments on the surfaces on pathogens and for purification of fragments. It may be possible to generate the fragments, allowing them to be purified and used in functional experiments including cell migration assays and platelet aggregation assays. To

elucidate the effect on FP caused by the presence of DXS_{500kDa}, it would be important to investigate structural changes using methods such as circular dichroism. This method would be able to define changes in the secondary structure of FP (α-helices and β-sheets), monitoring conformational changes, including folding properties, during binding interactions with surfaces and other proteins. It can also determine the tertiary structure by measuring the ellipticity of the aromatic amino acids (phenylalanine, tryptophan and tyrosine) (Greenfield, 2006, Kelly et al., 2005), which may be more insightful, as surface interactions do not always lead to secondary structural changes (Engel et al., 2002).

Further insight into the kinetics of FXIa in the presence of HK and towards natural substrates may be beneficial to characterise the effects of surfaces FXIa substrate specificity. It is also a priority to perform autoactivation assays of FXII and PK to determine if FP affects the interactions between these zymogens and relevant surfaces for comparison to the FXI autoactivation experiments. This would help to support the hypothesis that FP interferes with surface interactions, however if this is not case it may suggest that the interaction is FXI-specific, and the FXI/FP binding study may be more physiologically relevant than anticipated.

It may be useful to determine the location of the binding site for FP on FXI(a). Properdin binds with higher affinity to FXIa, suggesting that FP binds in the active site or in proximity. An investigation into the differences in binding of FP to FXI, FXIa and ½FXIa may reveal novel binding mechanisms.

Assays to induce NETosis could reveal a physiological relevance, as FP is released by neutrophils upon stimulation and this could lead to cleavage of FP in the presence of anionic surfaces such as DNA and histones. This may also expose a function for the FP fragments. These assays could be analysed using fluorescence microscopy to determine colocalisation of the two proteins. Flow cytometry may be used to

determine whether FP and FXI bind at the surface of blood cells, including platelets, in close proximity.

As the mechanism of action of FP on FXI appears to be charge dependent, it would also be reasonable to hypothesise that FP may influence the activation of FXII in the presence of anionic surfaces. If there is an effect, it would be likely that FXII activation may be decreased and thus may reveal a protective role of FP in thrombus formation.

As FP can form non-physiological aggregates that can induce complement activation, and potentially activate proteins sensitive to this such as FXII, it is important to consider that within the purified samples these “active” forms of FP may exist. This could be examined using size-exclusion chromatography and the preparations could then be used in similar assays to ensure that physiological dimers, trimers and tetramers are being used.

Chapter 5 The Effect of AP C3 Convertase Components on Clot Formation

5.1 Introduction

While the crosstalk between complement and coagulation will likely impact on the structure and function of the blood clot, there is currently limited research on the effect of complement on clot formation and structure.

The effect of plasma proteins on clot structure has been of interest for many years. Complement proteins have been explored in clot formation and structure, including MASP-1 (Hess et al., 2012) and central component C3 (Howes et al., 2012). It was demonstrated that C3 increased clot density and prolonged lysis times whereas clots containing MASP-1 were less dense. Macrae et al. (2020) have also demonstrated that inhibition of C5 using eculizumab alters clot structure through reducing fibrinogen levels, fibre thickness and clot density suggesting an important role of complement activation in clot formation (Macrae et al., 2020). Therefore, providing validation to explore the effect of FP on clot structure, clotting time and thrombin generation.

Thrombotic disorders often arise due to the dysregulation of coagulation and inflammation pathways and are major pathological contributors to a wide variety of diseases, including cancer and atherosclerosis. Thrombus formation can lead to many disorders including myocardial infarction, and thromboembolic disorders such as pulmonary embolism and ischaemic stroke (Foley and Conway, 2016). Both venous and arterial thrombosis are associated with a high mortality rate and a plethora of research has been performed and is currently being conducted to allow insight into the mechanisms behind thrombus formation and the associated diseases (Foley and Conway, 2016).

Clot structure plays an important role in thrombus formation and its stability in terms of mechanical properties and resistance to fibrinolysis. Insoluble fibrin is formed via thrombin cleavage of fibrinogen, which then creates a mesh network to entrap blood cells and occlude injury sites to arrest bleeding. Many studies have investigated the

importance of fibrin clot structure in thrombosis and haemostasis, exploring clot elasticity, fibre length and polymer formation, and clot density outlined in a review by Undas & Ariëns (Undas and Ariëns, 2011).

Abnormal fibrin clot formation can lead to thrombotic clot formation, which results in thinner, more tightly packed fibrin polymers culminating in a denser, less permeable clot which is less accessible to fibrinolytic factors and therefore slower to dissipate via fibrinolysis. This is in part due to a slower rate of plasmin production as well as a slower rate of fibrin degradation by plasmin (Gabriel et al., 1992). This thesis has delineated that there is a potential impact on the intrinsic coagulation cascade from the AP of complement, as it has already been determined that FP modulates surface interactions of FXI(a). However, the downstream effects are still not certain. Turbidity & lysis assays are a key starting point to determine how the formation of a clot may be affected under specific conditions and can be run as both purified and plasma-based assays.

5.2 Purified Turbidity and Lysis

Purified turbidity & lysis was initially employed to determine how FP affects fibrin generation and potentially clot density.

Fibrin production can be measured using a purified turbidity assay (Wolberg and Campbell, 2008). Thrombin and $CaCl_2$ are added to purified fibrinogen. As fibrin is produced and protofibrils form fibres through lateral aggregation there is an increase in absorbance as insoluble fibrin increases the turbidity of the reaction. The final absorbance values relate to fibrin fibre thickness, where thicker fibres result in a higher absorbance value, and thinner fibres are represented by a lower maximum absorbance value. Turbidity and lysis will delineate general characteristics of fibrin polymerisation rate and overall density, but not detailed fibrin structure (Wolberg et al., 2002).

Thrombin concentrations directly affect fibre thickness and clot density. Higher concentrations of thrombin result in denser clots composed of thinner fibrin fibres, whereas low concentrations of thrombin culminate in clots that show higher permeability and thicker fibres. This results in varied physical properties of the clot and affects clot dissolution by fibrinolysis (Shah et al., 1985).

The first turbidity assay (Figure 5.1) involved incubation of 0.5 mg/mL IF1-purified fibrinogen with 0.1 U/mL thrombin in the presence and absence of physiological concentrations of FP (0, 5 and 25 µg/mL), 7.5 mM $CaCl_2$ and 3.75 mM $MgCl_2$ were included to allow sufficient thrombin activity. For turbidity & lysis, 500 nM plasminogen and 6.25 ng/mL tPA were added to reactions (Figure 5.2).

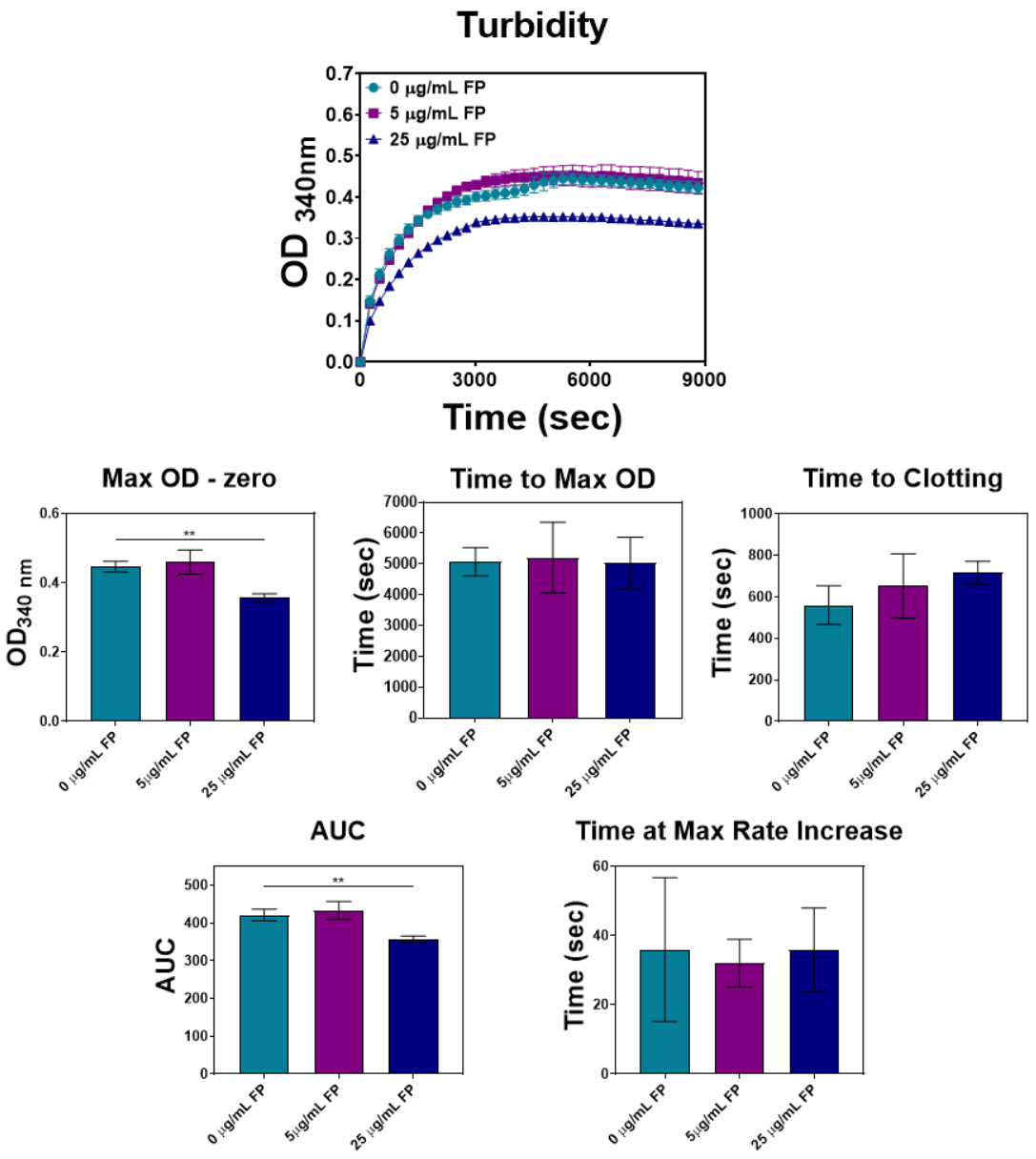


Figure 5.1 A high physiological concentration of FP leads to a decreased maximum absorbance and area under the curve in a purified turbidity assay.

Purified turbidity assay of 0.5 mg/mL IF-1 purified fibrinogen, 0.1 U/mL thrombin, FP (0-25 µg/mL), 7.5 mM $CaCl_2$ and 3.75 mM $MgCl_2$. Absorbance of the solution was measured every 12 seconds at a wavelength of 340 nm for 2.5 hours at 37°C with intermittent shaking. Baseline subtraction was performed by subtracting the first reading from all data points. Data represent triplicates run in one assay, mean ± SEM. *OD = optical density, AUC = area under the curve.*

25 µg/mL FP significantly reduced the max OD ($p \leq 0.01$) however time to max OD was not affected. The time to clotting (time to 50% max OD) was not significantly affected by the presence of FP, however there was a trend that the clotting time increased with the increasing concentrations of FP. The AUC was significantly reduced ($p \leq 0.01$) in the presence of 25 µg/mL FP, due to the decrease in max OD. This may suggest alterations to fibrin clot formation. Specifically, the reduction in absorbance signifies the FP may be affecting the density of the clot and the thickness of the fibrin fibres. Thrombin cleavage of fibrinogen may be affected, resulting in a denser fibrin clot with thinner fibres. Time at max rate increase was not affected by FP, suggesting the rate of fibrin clot formation was not affected.

Purified turbidity & lysis was then performed to determine if the significant changes in Max OD and AUC in the presence of fibrinolytic components (Figure 5.2).

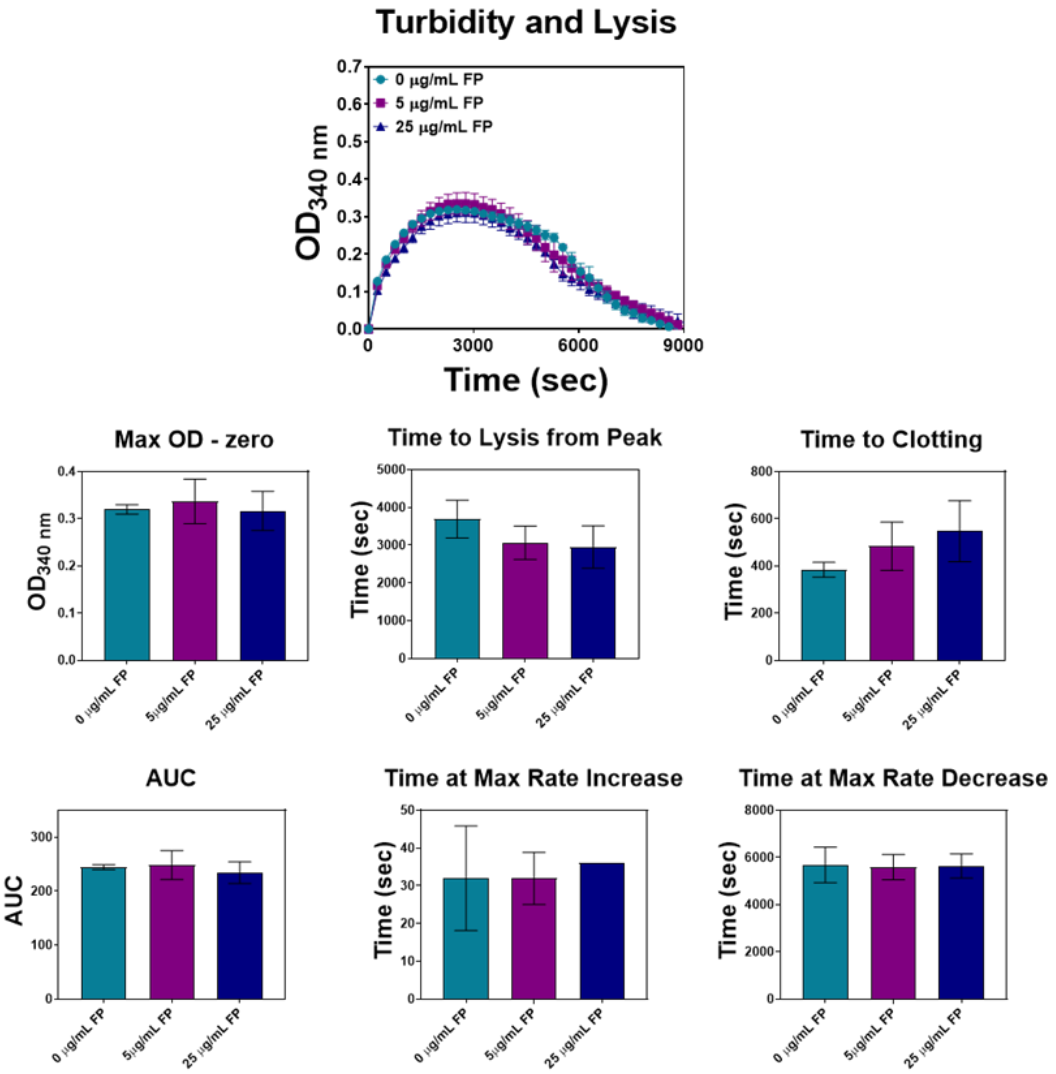


Figure 5.2 FP leads to an increased time to clot formation and decreased lysis time from peak in a purified turbidity and lysis assay.

Purified turbidity & lysis assay of 0.5 mg/mL IF1-purifed fibrinogen, 0.1 U/mL thrombin, FP (0-25 µg/mL), 6.25 ng/mL tPA, 500 nM plasminogen, 7.5 mM $CaCl_2$ and 3.75 mM $MgCl_2$. Absorbance of the solution was measured every 12 seconds at a wavelength of 340 nm for 2.5 hours at 37°C with intermittent shaking. Baseline subtraction was performed by subtracting the first reading from all data points. Data represent triplicates from one assay, mean ± SEM. Other parameters were measured but were not included for simplicity as they did not show any trends or differences. *OD = optical density, AUC = area under the curve.*

Properdin does not significantly affect purified turbidity & lysis (Figure 5.2). In the presence of FP, max OD and AUC are not affected, neither is the time at max rate increase or time at max rate decrease. The time to clotting is slower in the presence of FP and the time to lysis from the peak of the clot formation is faster (with 25 µg/ml FP in comparison to 0 µg/ml FP), suggesting a mild trend towards faster clot lysis in the presence of FP. This effect of slower clot formation and faster clot lysis also appears to be a dose dependent trend.

The significantly decreased max OD and AUC seen in the turbidity assay is not observed in the turbidity & lysis assay. However, the trend remains the same, and FP has an increasing effect on time to clotting.

As FP has a high affinity for the C3 convertase of the AP, the C3 convertase components (200 µg/mL C3b, 25 µg/mL FP & 200 µg/mL FB) were then introduced to a turbidity assay to determine if these effects were ruled out in the presence of C3b and FB, known physiological binders of FP (Figure 5.3).

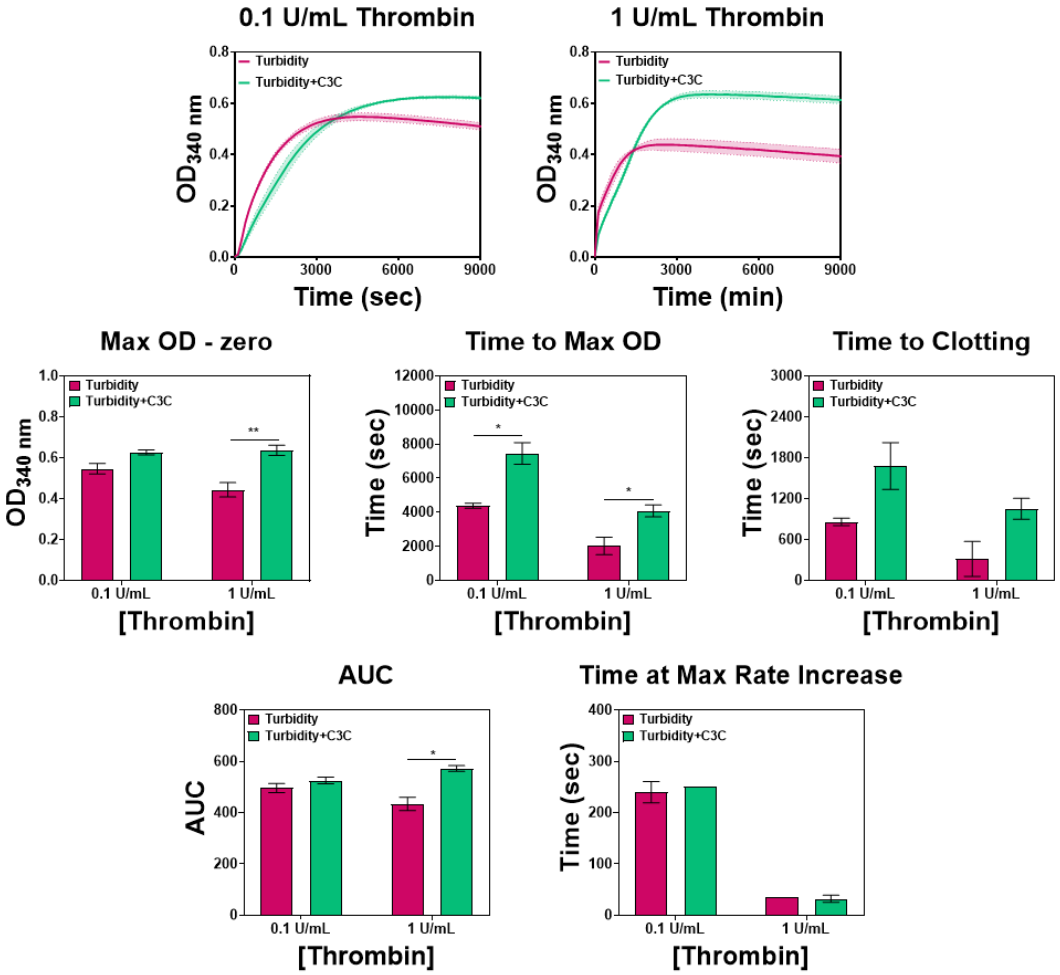


Figure 5.3 C3 convertase components lead to potential structural changes in purified fibrin clots and affect time to clot formation without affecting maximum rate of clot formation.

Purified turbidity of 0.5 mg/mL IF1-purified fibrinogen, 0.1 and 1 U/mL thrombin, C3 convertase components (C3C), 7.5 mM $CaCl_2$ and 3.75 mM $MgCl_2$. Absorbance of the solution was measured every 12 seconds at an optical density of 340 nm at 37°C with intermittent shaking. Baseline subtraction was performed by subtracting the first reading from all data points. C3 convertase components = C3b, FB and FP. Data represent triplicates from one assay, mean ± SEM. *OD = optical density, AUC = area under the curve.*

200 µg/mL FB and 25 µg/mL FP were chosen as these are the physiological concentrations in plasma. 200 µg/mL C3b was used as the physiological concentration of C3 is much higher, thus this represents what may occur after cleavage of C3, assuming that not all C3 gets cleaved. When all three components of the C3 convertase (C3b, FB and FP) were present, the clotting profiles were affected at both concentrations of thrombin (0.1 and 1 U/ml). Max OD was increased in the presence of C3 convertase components; however, this was only significant ($p \leq 0.01$) when initiated with the higher concentration of thrombin (1 U/mL). The time to max OD was significantly increased ($p \leq 0.05$) in both concentrations of thrombin with the C3 convertase components, suggesting clot formation was slower. Time to clotting (time to 50% clot formation) was increased in the presence of the C3 convertase components, however this was not significant. The changes in the time to max OD and time to clotting may be partly explained by the observation that the C3 convertase components caused an increase in max OD. The AUC was higher in the presence of the C3 convertase components; however, this was only significant ($p \leq 0.05$) at the higher concentration of thrombin. The time at max rate increase was not modified by the C3 convertase components.

These data may suggest that thrombin could be interacting with the C3 convertase components, resulting in lowered thrombin activity directed towards fibrinogen. This could explain the difference in clot density (Max OD) observed. Thrombin may be cleaving FB which may elucidate the rate of clot formation and the changes in clot density. The effect of the C3 convertase components appears to be thrombin dependent in this instance, with the effect being more obvious with the higher concentration of thrombin.

5.3 NPP Turbidity and Lysis

To determine the how increased concentrations of FP may affect turbidity and clot lysis in a more physiological setting, turbidity & lysis assays were performed using NPP. Properdin circulates at a concentration of 4-25 µg/mL, however, evidence suggests a localised increase in complement components into the inflammatory environment, particularly FP as it is synthesised by a variety of different cell types, including neutrophils which are also involved in DIC (Cortes et al., 2013). The effect of this local release of FP on clotting has not yet been investigated, and it is important to understand the relationship between FP and haemostasis, as FP knockout mice demonstrated resistance to DIC.

To determine whether FP may affect clot formation in plasma, FP was added into NPP turbidity & lysis assays. The assays were initiated with a titration of thrombin. Reactions contained thrombin (0.001, 0.01 and 0.1 U/mL), FP (0, 5 and 25 µg/mL), 20% NPP, 6.25 ng/mL tPA and 7.5 mM $CaCl_2$ (Figure 5.4).

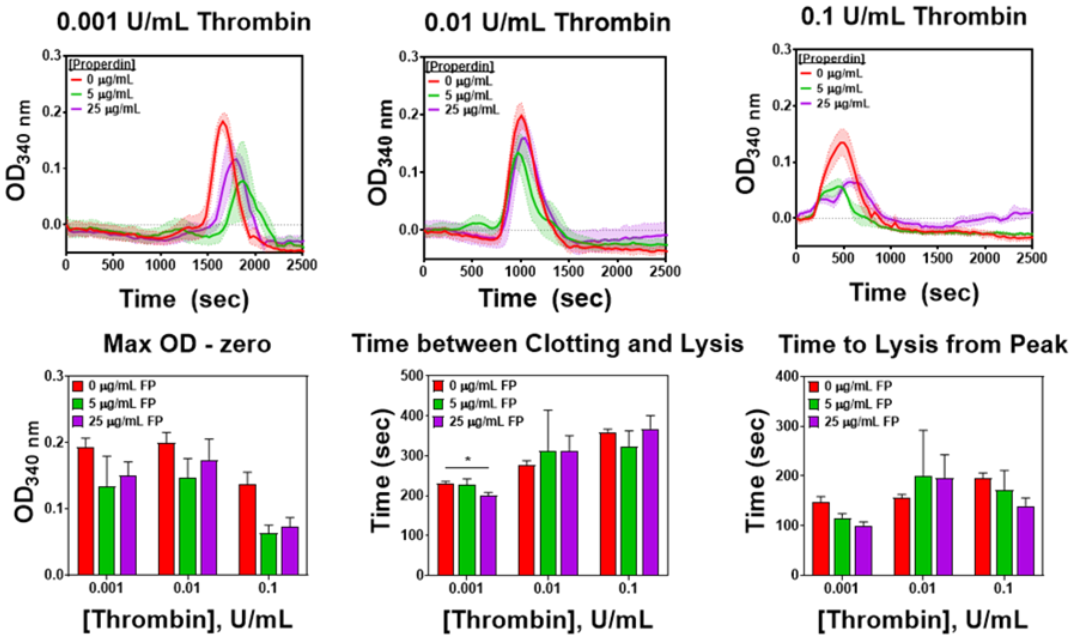


Figure 5.4 An investigation to determine how FP may affect plasma clot formation and fibrinolysis.

Turbidity & lysis assay of 20% NPP, 0-0.1 U/mL thrombin, 0-25 µg/mL FP, 6.25 ng/mL tPA and 7.5 mM $CaCl_2$. Absorbance of the solution was measured every 12 seconds at an optical density of 340 nm at 37°C with intermittent shaking. Baseline subtraction was performed by subtracting the first reading from all data points. Data represent triplicates from one assay, mean ± SEM. Other parameters were measured but were not included for simplicity as they did not show any trends or differences. *OD = optical density, AUC = area under the curve.*

An increased level of FP in NPP has a smaller effect than observed in a purified system, however the trend is similar in that the time to clot formation is delayed at low thrombin concentration. The time between clotting and lysis is significantly lower ($p \leq 0.05$) at the highest concentration of FP in the assay (25 µg/ml) initiated by the lowest concentration of thrombin (0.001 U/mL). Time to lysis from peak appears reduced under the same conditions, however this is not significant. Max OD was also affected however this was not significant or dose dependent.

To determine the effect of complement activation of clotting, plasma was incubated with zymosan A prior to running turbidity assays, initiated by a variety of different agonists (Figure 5.5).

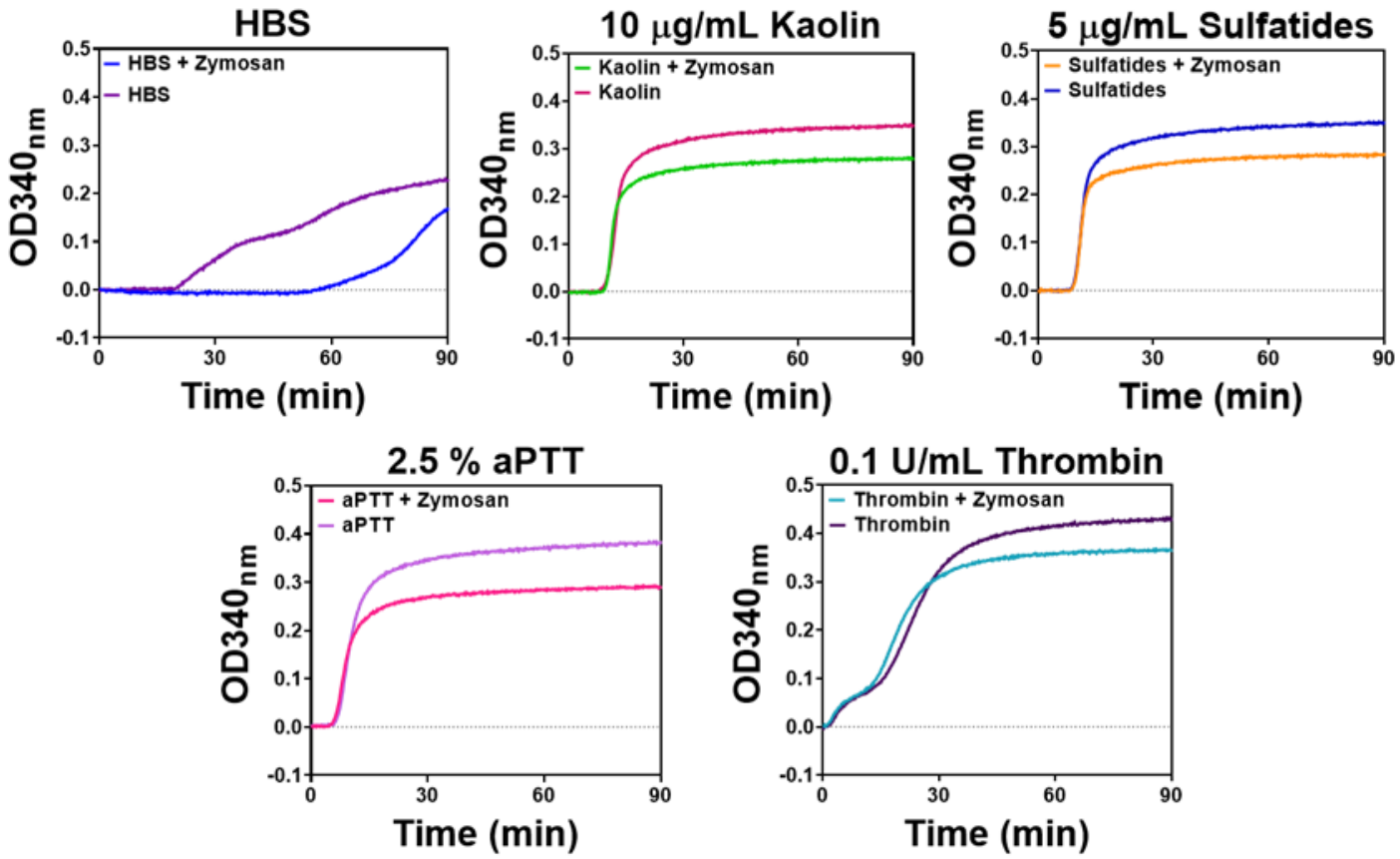


Figure 5.5 Activation of the complement pathway modulates fibrin structure determined by turbidity analyses in plasma.

Turbidity was performed in NPP in the presence and absence of 1% (w/v) zymosan A. NPP was incubated with zymosan A or vehicle for 1 hour prior to initiating coagulation by kaolin, sulfatides, aPTT or thrombin. Absorbance of the solution was measured every 12 seconds at an optical density of 340 nm at 37°C with intermittent shaking. Negative controls were included as HBS only ad GBS + zymosan A. data represent duplicates run in one assay, mean only.

Zymosan A alone appears to have a minor anticoagulant effect on NPP when no agonist is present. When turbidity was initiated by contact agonists kaolin, sulfatides and aPTT, this was overridden, and zymosan had no effect on time to clot formation. The maximum OD was lower in the presence of zymosan A which may be caused by the insolubility of zymosan A or modification to the structure of the clot. As the experiment was run in duplicate, rather than triplicate, the results should be taken cautiously as error bars are not present. It would be useful to repeat this, maybe with other surfaces such as LPS or PL included to determine the true effect of complement activation on blood coagulation. The C5a ELISA could also be used to determine the level of complement activation in the plasma.

There are limitations to turbidity & lysis assays in NPP systems. There are no blood cells involved and FP may be influencing interactions between a variety of blood cells such as neutrophils and monocytes and their interactions with platelets, however this has not been investigated in this thesis.

These data generated in plasma, combined with the data from purified studies, suggest that the effect of FP on turbidity and lysis may be thrombin dependent, therefore thrombin generation was subsequently explored.

As it appears that there may be some effect of FP and other components on thrombin, the next investigation was to determine the effect of FP on thrombin generated via both initiating pathways of the coagulation cascade.

5.4 Thrombin Generation

Thrombin generation in plasma is a widely used clinical test that allows insight into the complex interactions between coagulation proteases and their inhibitors, resulting in a more global overview of a patient's haemostatic status. Measuring thrombin generation over time is known as the endogenous thrombin potential (ETP) and is measured by cleavage of a chromogenic or fluorogenic substrate. Fluorogenic thrombin generation is preferred as it allows the measurements to be taken from platelet rich plasma (PRP) as well as platelet poor plasma (PPP) (van Veen et al., 2008).

The parameters that can be measured in fluorogenic thrombin generation are lag time, peak thrombin, time to peak and ETP (calculated as AUC). All thrombin generation parameters are calculated from a curve that represents the rate of substrate cleavage. Thrombin generation is calibrated using known standards to ensure reproducibility (Hemker et al., 2003).

Thrombin generation (TG) assays measure the rate of thrombin that is generated in a plasma system. A fluorogenic substrate is cleaved over time, and the maximum rate of thrombin activity can be indirectly inferred as the substrate is cleaved and the fluorescent product formed. TG can be initiated via the intrinsic (aPTT) or extrinsic (PPP-reagent comprised of TF with phospholipids) pathways of coagulation. A titration of FP was added to the TG assay to determine the downstream effects of FP in coagulation, by measuring the differences in thrombin generation (Figure 5.6).

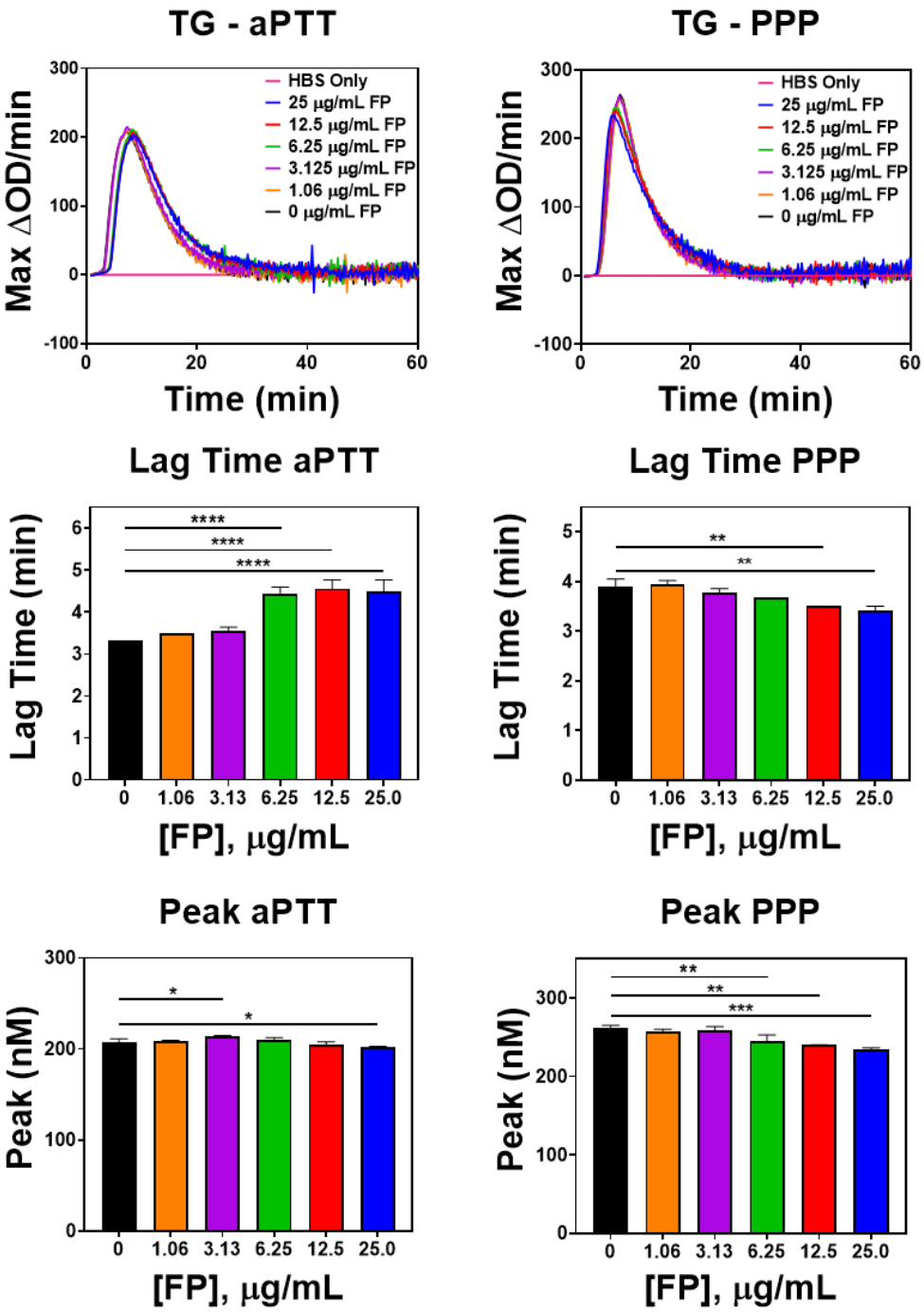


Figure 5.6 Lag time to thrombin generation is modulated by FP.

FP increases the lag time of thrombin generated via the intrinsic pathway but decreases the lag time and the maximum rate of thrombin generated via the extrinsic pathway. Thrombin was generated by the addition of aPTT or PPP reagent. Thrombin generation was measured by cleavage of the fluorogenic substrate over 60 minutes.

The TG curves show subtle changes in both lag time and peak in the presence of FP when the assay was initiated by aPTT (Figure 5.6). Lag time was affected in a dose dependent manner and was significantly ($p \leq 0.001$) increased by concentrations of FP equal to and above 6.25 µg/mL. This supports previous data that suggested FXIa substrate specificity may change, and that FXIa may be cleaving FP when at a high concentration. The maximum rate of TG was also significantly affected ($p \leq 0.05$) at the highest concentration of FP (25 µg/mL) (Figure 5.6). These data, although subtle, suggested that at high concentrations of FP, thrombin is generated at a slower rate, which may be a downstream effect of the substrate specificity change of FXIa.

The TG initiated using PPP had a dose dependent decrease in lag time. This decrease in lag time was only significant at the two highest concentrations of FP ($p \leq 0.001$). The decreased lag time suggests that thrombin was generated faster in the presence of high concentrations of FP. The peak was reduced in a dose dependent manner, which may suggest that the max rate of thrombin generation was lowered in the presence of FP. This reduction was significant at concentrations of 6.25 µg/mL ($p \leq 0.01$), 12.5 µg/mL ($p \leq 0.01$) and 25 µg/mL ($p \leq 0.001$).

The results from the TG assays suggest a potential modulation of thrombin generation in the presence of FP at concentrations higher than would usually circulate in plasma, thus this effect may be relevant under inflammatory situations, where FP may be released locally by stimulated neutrophils.

To determine the effect of clot formation in whole blood, clotting times were subsequently evaluated using a coagulometer.

5.5 Coagulometer

Clinical tests have been standardised which measure the clotting time after an agonist to the intrinsic pathway (aPTT) and or an agonist to the extrinsic pathway (PT) are employed. The PT test is often used for evaluation of oral anticoagulant therapy (warfarin), and aPTT is often used to monitor unfractionated heparin (UFH) dosage during surgery. These tests can be used in a research setting to determine effects on whole blood (if the coagulometer employs a mechanical detection mechanism) and plasma time to clot formation.

The aPTT test is initiated by a reagent such as PTT automate, which contains silica and PL to induce contact activation via FXII, initiating the intrinsic pathway of coagulation. The PT agonist is comprised of thromboplastin and PL, to mimic TF release upon injury inducing the extrinsic pathway of coagulation. Due the agonists being used to induce the two initiating pathways of coagulation separately this method can be employed to evaluate deficiencies and dysregulation of clotting factors.

FP was titrated in both whole blood and in NPP, and clotting time was measured through initiation of coagulation by the intrinsic and extrinsic agonists mentioned above (Figure 5.7).

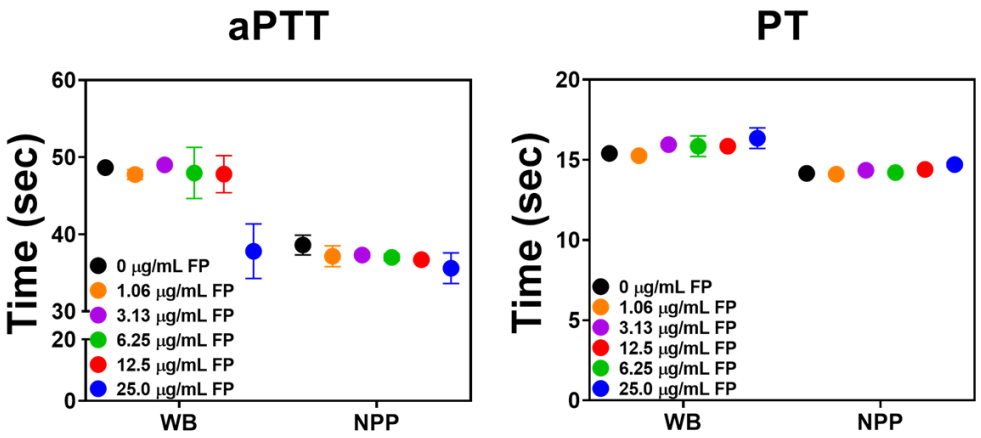


Figure 5.7 A high concentration of FP affects clotting time in aPTT in whole blood.

Clotting times were examined by initiating coagulation via the intrinsic pathway (aPTT) and the extrinsic pathway (PT), in whole blood (WB) and in normal-pooled platelet-poor plasma (NPP). Data shown as mean ± SD, n=2.

Coagulation initiated by aPTT displayed a decrease in clotting time with FP added at a final concentration of 25 µg/mL in whole blood, however in NPP, clotting times decreased suggesting a prothrombotic characteristic; these results are mild, and may lie within the CV of the assay. In the PT assay, the increase in FP slightly increased clotting times in both whole blood and NPP however again this likely lies within the CV of the assay.

These results combined suggest that when a high trigger to coagulation is employed there is little noticeable effect of FP on the coagulation cascade. However, it is most likely that an effect comes into play at low agonist concentrations or when low amounts of thrombin are generated. These studies require further investigation at lower doses of agonist in order to understand if FP plays a role and to further understand if a differing mode of action for the intrinsic pathway is apparent compared to the extrinsic pathway.

5.6 Discussion

As FP deficiency results in protection from DIC, it was important to determine if FP may affect clotting and clot structure. Clot structure can affect lysis times therefore studying the effect of FP on clotting and fibrinolysis could then help to understand the mechanism of FP in DIC.

Purified turbidity & lysis assays were first performed to determine if FP exhibited an effect on fibrin clot formation and the structural properties of the clot. It was determined that in turbidity alone, FP was having a modest prolongation effect on time to clot formation using thrombin as agonist, suggesting that FP may be reducing the activity of thrombin. In the turbidity & lysis assay it was observed that FP mildly reduced the time to lysis from peak which correlates with a faster clot lysis in the presence of FP. The reduction in maximum absorbance and AUC observed in the turbidity assay was not seen in the turbidity & lysis, suggesting that FP may have affected the clot formation in the turbidity only assay via interactions with thrombin. However, in the presence of dynamic on-going fibrinolysis this effect was masked. The presence of complement components was further investigated using a purified turbidity assay, utilising the proteins C3b, FP and FB which are all involved in the C3 convertase complex. In the presence of the C3 convertase components, the time to clotting was prolonged and the maximum absorbance was increased, suggesting a slower rate of clotting and a change in fibrin clot density, more likely to arise in a more open, less dense structure in the presence of C3 convertase components. As the time to clot formation was prolonged in the presence of C3 convertase components although not significant, is worth investigating further to determine if this is an effect on thrombin activity or fibrin clot formation. This can be explored using reptilase to delineate if the effect is due to fibrin formation or on the activity of thrombin. The increased time to clot formation is interesting as it suggests that there may be a modest inhibitory activity of one of the components of the C3 convertase, or

potentially that thrombin cleaves components of the C3 convertase pathway resulting in substrate competition for fibrinogen. It has previously been observed that thrombin has amidolytic activity towards several components of the complement cascade including C5 and C3 (Huber-Lang et al., 2006b).

The effects of FP on turbidity & lysis was further explored in a plasma system using NPP created *in-house*. In plasma, the effect of FP is not as clear. There still appears to be a trend towards prolongation of fibrinolysis with low thrombin suggesting that the effects seen in both purified and plasma systems may be dependent on thrombin levels. The effect of complement activation on clot formation was then explored by adding zymosan A to plasma, and then adding inducing clot formation with a variety of agonists. There is a trend that clot density changes in the presence of zymosan A, however this may be due to the turbidity of zymosan A itself as it is not soluble. Zymosan A also appeared to have a slight anticoagulant effect in the absence of a coagulation agonist, which may be due to activation of the complement pathway but this needs further research to understand the mechanism, and unfortunately, due to the closure of the laboratory due to COVID-19 it was not possible to explore this further.

Due to the concept that the effects observed may be dependent on thrombin, the assays were followed up by TG assays with a titration of FP. Thrombin was generated using agonists for both the extrinsic and intrinsic pathways of coagulation. During intrinsic TG the lag time was increased by FP in a dose dependent manner suggesting lower generation of thrombin or thrombin activity. In the extrinsic TG experiment, the peak thrombin generation was marginally reduced in a dose dependent manner by FP, however the lag time was decreased suggesting thrombin was generated faster. These differences are modest, however, within a pro-inflammatory environment these modest effects may lead to downstream consequences.

Clotting times in whole blood and NPP were subsequently investigated to determine how they may potentially correlate with the TG data. Agonists were used to induce coagulation via either the intrinsic or extrinsic pathways. In whole blood, the aPTT assay showed no difference in clotting times with the addition of FP, however in NPP clotting times decreased marginally, however, the difference could be within the CV of the assay. The PT assay demonstrated a slight increased clotting time in both whole blood and NPP which supports a more prothrombotic effect, however, again the differences are within the CV of the assay. These results would suggest that FP may not play a major role in the presence of strong agonists such as the PT and aPTT but may play a role under circumstances where there are mild activators of coagulation.

5.7 Considerations for Future work

The investigations into the effect of FP on clot formation and structure is limited. It would be useful to have access to FP deficient plasma (which is not commercially available) to determine how turbidity and lysis may be affected in the absence of FP. This would help to elucidate how FP is integrating in the coagulation cascade, and whether it would have a direct effect on clot structure. It may be possible to deplete plasma of FP, however the antibody purchased for this product was not sensitive enough to detect FP in NPP, and therefore it was not tested in depletion of FP from NPP. Further optimisation of an anti-FP monoclonal antibody is needed to explore this further and could also be used in Western blot and ELISAs if sensitive and specific enough.

One crucial element of this project is the access to a FP knockout mouse model. There is a collaboration in motion with a group at the University of Leicester to obtain these mice for experimental use however due to the current COVID-19 situation this has been put on hold. It would be interesting to investigate the effect that a lack of FP has on bleeding and clotting. Tail bleeding time assays could be performed, and blood and plasma could be obtained from the mice and analysed via a variety of clotting assays. Clot formation and lysis could be analysed using rotational thromboelastometry (ROTEM), which can measure clot stiffness, clotting times and fibrinolysis times. TG could be performed to determine how the lack of FP affects the clotting cascade and turbidity & lysis assays could be used to explore clot formation. A titration of FP back into the deficient plasma could be useful for comparison. The FP knockout mice could also be subjected to various injuries using $FeCl_3$ or laser induction of thrombosis to determine the effect of FP on thrombus formation in a model that differs from the localised Shwartzman reaction previously mentioned.

Chapter 6 General Discussion and Conclusions

6.1 Discussion

The first key aim of this thesis was to optimise a drug discovery strategy where a high volume of compounds could be screened, targeting the AP C3 convertase and the second key aim was to explore the crosstalk between inflammatory pathways and coagulation. Complement is a complex inflammatory pathway comprised of sequential activation of a cascade of enzymes which “complements” other immune responses including phagocytosis through opsonisation of pathogens and lysis of pathogenic cells.

Complement plays a crucial role in innate immunity, acting as the first line of defence against invading pathogens. The initiation of the catalytic cascades generates a terminal system in which pathogens are destroyed through phagocytosis and lysis due to the formation of proinflammatory molecules and protein complexes. The complement system has long been established and for many years was considered solely as an arm of the innate immune response, confining pathogens and preventing their dissemination. However recent developments have shown that the complement cascade is involved in adaptive immunity, influencing the processes of T- and B-cells.

The complement system has three pathways of activation. The CP is initiated via the C1 complex binding to the Fc region of IgG and IgM antibodies which are complement-fixing and bind to pathogenic surfaces. Autocatalysis of C1r and C1s leads to cleavage of C4 and C2, resulting in formation of the CP C3 convertase C4b2a at the pathogen surface, and the generation of the opsonin C3b and the anaphylatoxin C3a – the junction at which all activation pathways converge. The LP does not require antibodies for activation, and instead requires PRRs including MBL and ficolins to recognise non-self surfaces. These PRRs can bind to PAMPs found on the surfaces of pathogens and results in autocatalysis of MASPs, and ultimately generation of the C3 convertase C4b2a. Initiation of the AP employs a different mechanism of activation compared to CP and LP, and

requires a constant tick-over of hydrolysed C3, creating the C3b analogue C3(H_2O). C3b and C3(H_2O) both have an exposed internal thioester bond which allows covalent binding to hydroxyl groups on pathogenic surfaces, tagging the pathogen for phagocytosis. C3(H_2O) is quickly inhibited in the absence of a pathogenic surface. The exposure of the thioester bond also allows FB to bind to C3(H_2O) and the protease FD can cleave and activate FB generating the initial AP C3 convertase C3bBb. This complex is stabilised by the cofactor FP. C3b generated via this initial convertase can bind to surfaces in the vicinity of its generation and can also bind to FB resulting in an amplification loop. The AP is the only activation pathway with a positive regulator (FP); however, it has been speculated that this protein has a dual role and can act as an initiator of complement by promoting *de novo* C3 convertase assembly at microbial surfaces. This new mechanism has been supported by FP-knockout mouse models. Complement activation via all three pathways leads to initiation of the terminal pathway where C5 is cleaved recruiting immune cells and leading to MAC formation, resulting in the containment and destruction of pathogens. Complement may also be initiated by activated coagulation factors via cleavage of C3 and C5 by PKa and thrombin – an important link between complement and coagulation.

Coagulation is another complex and dynamic catalytic cascade, involving proteolytic activation of zymogens, and incorporation of cells into a fibrin network generating a clot in order to arrest bleeding at sites of trauma. Coagulation is initiated via two separate pathways. The extrinsic pathway is activated upon tissue injury, and involves the exposure of subendothelial TF to the blood where it comes in to contact with FVII ultimately leading to the junction at which the intrinsic and extrinsic pathways meet, the conversion of FX to FXa (common pathway). The intrinsic pathway is initiated via contact activation of FXII which leads to a reciprocal activation mechanism where PK is

converted to PKa which can cleave FXII. This will initiate the intrinsic pathway via FXI activation and subsequent FIX cleavage, and activation of the KKS will result in cleavage of HK and the release of BK. The common pathway begins with FX activation by the extrinsic and intrinsic tenase complexes at the phospholipid surface where FXa in conjunction with its cofactor FV converts prothrombin to thrombin ultimately resulting in the generation of a fibrin clot. The coagulation cascade has many inhibitors including the SERPINs AT-III and C1-INH. Ions also play an important role in the coagulation cascade, with $CaCl_2$ acting as a cofactor for many proteases including thrombin and FX, and $ZnCl_2$ playing a vital role in contact activation, mediating HK binding and FXII activity.

Complement and coagulation have many known intercommunications and are both implicated in thromboinflammatory disorders. This has recently become an important topic of discussion and the COVID-19 pandemic has been a vital area of research. Infection by this novel coronavirus has led to over 10 million cases worldwide and over 500,000 deaths. The pathology of this virus is varied, ranging from asymptomatic to fatal. In the worst cases of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), patients present with septic shock and severe organ dysfunction, as SARS-CoV-2 enters cells via the angiotensin-converting enzyme (ACE) 2 receptors, manifesting in hypoxaemia and dyspnoea resulting in pneumonia (Lu et al., 2020). Coagulopathy of COVID-19 is diagnosed by detecting increased D-dimer, fibrinogen and testing for clotting abnormalities (Iba et al., 2020). These tests are also used for the diagnosis of DIC (Iba et al., 2019). There are currently several treatments for SARS-CoV-2 infection in clinical trials, some of which are targeting inflammatory mechanisms including C5 and IL-6 (Tu et al., 2020). Growing evidence for complement implications in the pathology of COVID-19 suggests that dysregulation of complement activation is a major driver for thromboinflammtion via TMA and NETosis. Therapeutic targets are being studied for the

treatment of COVID-19 at the levels of C3 and, further downstream, C5. A recent study suggests C3 may result in broader therapeutic control, as C3a generation is attenuated, as well as MAC formation and FB consumption (Mastellos et al., 2020). This supports the small molecule study in this thesis.

There exists the potential to target complement as a therapeutic for what are often described as a thrombotic disorders such as DIC which has been supported by the lack of thrombosis observed when FP knockout mice are exposed to the localised Shwartzman reaction; however further work is needed to characterise how FP directly affects thrombosis. The complement and coagulation systems are intricately linked. They are descended from a common ancestor with similar mechanisms that share the same regulators, including C1-INH and the protein C pathway via thrombomodulin (Conway, 2012). There exists a reciprocal mode of activation between the two cascades, where coagulation enzymes can lead to complement end products (Huber-Lang et al., 2006a) and activation of complement catalysts can result in fibrin clot formation (Gulla et al., 2010). The intrinsic pathway of coagulation is closely linked with inflammatory processes, with FXII modulating pathways outside of coagulation. Factor XIIa can lead to *in vitro* complement activation via the CP of complement. Factor XII can bind to pathogen surfaces, will activate in the presence of bacterial polyP and can become activated during neutrophil activation, all of which lead to initiation of the KKS and release of the vasoactive peptide, BK resulting in inflammatory activation mechanisms. Factor XI is also implicated in inflammatory pathways, particularly those involved in the innate immune response. Factor XI can directly bind to neutrophil surfaces via its active site and may have the ability prevent chemotaxis of PMN leukocytes (Henderson et al., 1994, Itakura et al., 2011). The role of FXI in sepsis is controversial, with some research suggesting a protective role in pneumonia-derived sepsis independent of FXII, and

others implying that inhibition of FXI with a monoclonal antibody improved survival rates during microbial sepsis induced by CLP. Amara et al. (2010) also suggested the potential for FXIa to cleave C3 and C5 of the complement system however whether this results in physiological complement activity remains unclear (Amara et al., 2010). The KKS is also associated with the complement system via activation of zymogens or through common receptors on vascular endothelial cells. There are data to suggest that MASP-1 can activate FXIII (Hess et al., 2012, Krarup et al., 2008) and that FXIIIa can integrate C3 into the fibrin clot (Howes et al., 2012). Thrombin plays a vital role in haemostasis, cleaving fibrinogen to fibrin. Thrombin can be produced either via cleavage by the prothrombinase complex or by MASP-2. Thrombin may be able to cleave C3 and C5 (Amara et al., 2010) and may lead to complement activity independent of C3 however the physiological relevance of this has been disputed (Keshari et al., 2017a). Thrombin can also mediate complement inhibition via PARs resulting in reduced inhibition of the C3 convertase by FI (Markiewski et al., 2008).

The crosstalk between the two cascades can become dysregulated in cases of hyper thromboinflammatory states, which can lead to the consumption of important factors, cofactors and regulators. This can result in a thrombotic phenotype with mass inflammation and can be fatal. The severe thrombosis and consumptive characteristic can eventually lead to haemorrhage, and the battle to treat such disorders is time dependent. DIC is a thromboinflammatory complication which arises from a variety of conditions including sepsis and trauma. Coagulation and the inflammatory host response become dysregulated and a cytokine storm leads to endothelial dysfunction (Tisoncik et al., 2012). Micro-thrombi which are highly resistant to fibrinolysis form in the small vessels, leading to a phenotype of thrombosis with severe haemorrhagic events. There is no specific treatment for this devastating condition, only guideline protocols provided

by the BCSH, ISTH and JAAM. It has previously been shown that a deficiency of FP is protective against DIC when using the localised Shwartzman reaction. The AP is a target of interest, as it involves an amplification loop of activation and is the only pathway that involves a positive regulator. The AP is implicated in a variety of disorders including PNH, aHUS and AMD (Mastellos et al., 2019). As FP is a key positive regulator of the AP of complement a hypothesis was generated; small molecules can be used to inhibit the AP C3 convertase.

This is not the first time that complement has been targeted for treatment of thrombotic disorders. C5 is inhibited using eculizumab for the treatment of TMA as a result of disorders such as aHUS, PNH and DIC. However, care must be taken when inhibiting complement as it is required to opsonise pathogens for clearance and aids in their destruction, thus the risk of death may increase (Gavriilaki et al., 2019). This is where time may be the limiting factor of treatment of thrombotic disorders, to ensure complement inhibition is used once any infection is under control. This may also involve prophylactic antibiotics to reduce the risk of new infections taking hold.

In this study, a screening process was optimised to scan a library of compounds that may be utilised as inhibitors for the AP C3 convertase. Small molecules were used as they are low molecular weight and can be manipulated via structure, function and relationship studies in order to increase efficacy. Although there are limitations to using small molecules as therapeutics, such as their selectivity, this may also be used as an advantage to ensure low instances of off-target effects. The modifications that can be performed can increase their binding affinity, however it can lead to a low dissociation of the molecule from the target resulting in undesirable side effects caused by ongoing target modifications.

SPR was used for the initial screen as this is a high throughput method which provides data in real-time and generates values to determine affinity constants. Specificity of binding partners can be evaluated by immobilising different proteins on the sensor surface. Large libraries of small molecules can be screened in a short amount of time to allow the compounds to be “ranked” and eliminated as required. The Pioneer platform used in the thesis is advantageous over other platforms, such as BIAcore, as it automatically titrates the analyte (in this case the compound) over the ligands, creating the affinity constants without the need for a manual titration. This is beneficial; not only is this less time consuming, it also uses less of compound. The Pioneer system utilises Taylor dispersion to titrate the analyte over the surface by forces of pressure where fluid mechanics causes molecules to diffuse at increasing rates through the flow channel.

The binding characteristics of the AP C3 convertase were first explored and it was shown that FP enhanced the binding of FB to C3b by an order of magnitude, which is supported by the literature (Hourcade, 2006). It was then determined whether the compounds supplied interfered with the binding of the C3 convertase complex, by immobilising C3b at the sensor surface, and injecting FB and FP as analytes as the positive control; compounds were mixed with FB and FP to determine the effect of the compounds on complex formation Using this method, over one hundred compounds were reduced to twelve compounds of interest. These twelve compounds were then further characterised using SPR by changing the orientation of the complex; FP was immobilised to the sensor surface and FB and C3b were injected with and without small molecules. Once this was investigated, a binding study was performed to determine the affinity constants for the compounds to C3b and to FP separately to ensure specificity of the compounds. All twelve showed binding to both C3b and FP which was promising because this was the target area of interest. The complex was then separated to further characterise their

specificity and it was observed that the compounds had the most effect on the interaction between either the entire complex, or on the interaction between C3b and FP. The compounds were then further explored in more physiological assays and were screened using ELISAs where complement was activated in serum and/or plasma. These assays are inconclusive thus far as there may be off target effects and other binding partners for the compounds. However, based on the data generated so far it would appear the use of plasma rather than serum for activating the complement pathway may provide better results, as it may limit pre-activation of complement from the coagulation cascade and provide better sensitivity for the screening of compounds. This is yet to be explored due to limitations incurred by the COVID-19 induced closure of the laboratory, in my last 6 months of PhD study. The availability of the small molecules employed were limited because they were part of a library and many were no longer available to repurchase. However, although the original compounds were depleted, their analogues are available to be rescreened using the initial SPR assays. After the identification of HITs, further, more detailed characterisation would be required to determine IC50 values in the plasma-based assays. This would be useful to further progress this study as the ELISAs performed in this study used fixed single concentrations of the compounds. There is a requirement for plasma deficient of individual complement components for research purposes, although these are not accessible at this time, as they would help to determine the specificity of compounds.

This study discovered a novel interaction between the intrinsic pathway of coagulation and the AP of complement. A direct interaction was determined between FXI(a) and FP with functional consequences. An SPR screen was performed injecting coagulation factors over C3b and FP. Factor XI was shown to bind with high affinity to both C3b and FP, and an assay was performed to determine the kinetics of FXIa, which also bound to

both with high affinity. This interaction was further explored with a variety of chromogenic assays to characterise the autoactivation of FXI, the enzyme kinetics of FXIa, the activation of FXI by FXIIa, the substrate specificity of FXIa and the inhibition of FXIa by C1-INH. Many of these assays involved the use of GAGs. GAGs are important in many biological mechanisms and are involved in complement and coagulation. Autoactivation of some coagulation zymogens can be initiated by the negative charge of GAGs, however they also play a role in the regulation of contact activation via enhancing the inhibition of FXI by C1-INH and AT-III (Pike et al., 2005, Wuillemin et al., 1997, Wuillemin et al., 1996). GAGs also provide sites for localisation of complement components with microbes. It is known that FP has GAG binding properties, thus this is an interesting area to research as it is a likely junction of coagulation-complement intercommunication.

It was found that FP affected the amidolytic activity of FXIa towards the chromogenic substrate S-2288. It was initially thought that FP was inhibiting the autoactivation of FXI to FXIa by effectively chelating the negatively charged agonist. Further assays determined that FP did not affect FXIa activity when incubated on its own, and a negatively charged surface was needed to elicit an effect. The presence of DXS_{500kDa}, a synthetic highly sulfated GAG commonly used in biochemical assays, affected amidolytic activity of FXIa, reducing the V_{max} and k_{cat} but increasing the K_m. In the first instance this was viewed as an inhibitor, as it effectively inhibited FXIa cleavage of the chromogenic substrate, however it was later determined the decrease in k_{cat} and increase in K_m suggested a substrate specificity change away from the chromogenic substrate. This was evaluated using SDS-PAGE, and it was observed that autoactivated FXI and FXIa could, in fact, cleave FP in the presence of DXS_{500kDa}. To our knowledge, this is the first time that cleavage of FP has been witnessed. This was further characterised by exploring whether FXIa could still cleave FIX, its natural substrate, in the presence of

both FP and DXS_{500kDa}. It was found that FXIa could cleave both FP and FIX when incubated with DXS_{500kDa}. It was apparent that the phenomenon of FP being cleaved was only occurring in the presence of DXS_{500kDa} and thus different surfaces were explored, however FP cleavage products did not appear with other surfaces. There was a potential that cleavage products were visible when incubated with sulfatides however this is yet to be further explored; this is an interesting avenue to take as sulfatide binding properties of FP are well known, as is the involvement of sulfatides in contact activation. Activation of FXII by FXI was investigated, and there was a decrease in activated FXI activity towards the chromogenic substrate when FXII was activated via aPTT reagent or by sulfatides, however in the presence of HK, FXI activation by FXII was not affected by FP. This may suggest that FP is interacting with FXI via the same binding site but with lower affinity, and it may also be that HK has a higher affinity for the surface when compared to FP. These data suggest that FP interacting with FXI(a) may not have downstream effects in the coagulation cascade, which also supports our data for the inhibitor study.

The data suggest a surface mediated interaction between FP and FXI(a). It has already been defined that the inhibition of FXI by C1-INH is modulated and enhanced by GAGs, thus an assay was optimised to determine the effect of FP on the inhibition of FXI by C1-INH in the presence of DXS_{500kDa}. It was determined in our assays that C1-INH was not able to inhibit FXIa efficiently without DXS_{500kDa}, and when DXS_{500kDa} was present at low concentrations, FP could prevent FXIa inhibition. When the concentration of DXS_{500kDa} was increased the effect of FP was not as potent, supporting the hypothesis that this is a surface mediated interaction, and that at the higher concentration of DXS_{500kDa}, FP could not saturate the negatively charged surface. The assay was also run in the presence of HSS, which did increase the inhibitory activity of C1-INH, but not to the same extent as DXS_{500kDa}. This was also prevented by the presence of FP.

FXI has several known physiological substrates, including FIX, FX and pro-chemerin. Factor IX chromogenic assays are not sensitive, and it was decided to explore the effect of FP on the FXIa cleavage of FX using a chromogenic assay, involving PL and $CaCl_2$. In the absence of PL, FP had no effect on the activation of FX by FXIa, however when PL were present, the activation of FX towards the chromogenic substrate S-2765 was lowered. This may be due to the substrate specificity change in FXI towards FP, however this was not visible when using SDS-PAGE. It may also be due to FP sequestering the PL, preventing FX from binding to the PL micelle surface thus reducing FXa activity. Properdin may also be preventing efficient FX activation via interactions with the Gla domains on FX, necessary for binding to $CaCl_2$ and PL surfaces.

There are novel substrates of contact activation being explored and it has recently re-emerged that PKa can cleave FIX in plasma with downstream effects (Noubouossie et al., 2020, Visser et al., 2020). Factor XIa can cleave pro-chemerin which generate a potent chemoattractant, chemerin, suggesting an important role of FXI in inflammatory processes (Ge et al., 2018). Factor XIa can also cleave HK, resulting in the release of BK (Scott et al., 1985, Gailani and Broze, 1991). Factor XIa can cleave prothrombin, however the physiological relevance of this is still unclear and requires further investigation as the cleavage product does not act like thrombin, and shows no activity towards fibrinogen (Matafonov et al., 2013a).

As FXIa is described as a promiscuous trypsin-like serine protease, data generated *in vitro* must be viewed with caution. It is vital that experiments are performed *in vivo* and *ex vivo*, using plasma experiments to determine the physiological relevance of findings. The main limitation of this study has been the access to FP deficient plasma and FP knockout mice. It would be beneficial to determine the effect of FP deficiency on bleeding, clotting and thrombosis. Further work that may support this study would be to

acquire FXI deficient plasma and explore complement activation in the absence of FXI, using the ELISAs from the small molecule study, and haemolytic assays could also be utilised. Structural evaluation of FP when interacting with surfaces would also be interesting, as well as the determination of relative binding sites on FXI and FP – i.e. does FP interact with FXI via the same site as HK?

Further to this, purified and plasma clot formation and TG were evaluated. FP appeared to have an effect on purified clot density, observed in turbidimetric analysis, however when T&L was performed, this effect was lost under the dynamic process of clot formation and fibrinolysis. When purified turbidity assays were performed in the presence of all three components of the AP C3 convertase, there was a marked reduction in time to clot formation, and an increase in maximum OD. There may be several reasons for this, thrombin may be cleaving FB – though there is no literature to support this, or the complex is binding to fibrinogen and reducing the cleavage by thrombin. The max OD is higher in the presence of the components of the complex suggesting that complement activation affects clot density, towards a more open porous structure. Due to this change in clot formation, the effect of complement was further explored by incubating plasma with a complement agonist which resulted in a decrease in maximum OD in the presence of the agonist. However, this data may be ruled as inconclusive due to the insolubility of zymosan A, which may affect the turbidity of the solution itself. The control with no coagulation agonist showed a prolonged time to clotting suggesting a potential regulatory mechanism involving complement activation, or involving the zymosan A.

FP affected TG in different ways depending on the agonist. Initiating with aPTT reagent resulted in a reduction in lag time, that was dependent on the concentration of FP, and TG induced by the addition of PT resulted in a decrease in the peak of thrombin

generation, also dependent on the concentration of FP. The effect on TG by addition FP is modest, though it is statistically significant. It signifies that a localised increase of FP in proinflammatory environment may affect thrombin generation and may have downstream effects, though this is to be further explored. FP was also added to whole blood and plasma and clotting times were evaluated using a coagulometer. Again, the results are modest and are not significantly different to the CV of the assay. However there appears to be a trend that FP decreases the clotting time during contact activation but increases clotting time in activation via the extrinsic pathway suggesting that FP may interfere at different levels of the coagulation cascade.

There is clearly a need for novel therapeutic targets in thromboinflammatory disorders. Many thrombotic disorders including DIC, PNH and aHUS are complement driven, thus a complement therapeutic is of interest. It is difficult to determine the best target, as the mechanisms of action remain unclear and need further investigation. The studies performed in his thesis go hand in hand, with the development of an inhibitor targeting the AP C3 convertase being useful to determine how this may affect other processes, including coagulation and clot formation.

There remain many novel mechanisms to be discovered and explored. This thesis has highlighted the importance of a rigorous routine for drug discovery to ensure that novel therapeutics do not have undesirable off target effects, however these studies are difficult to perform when the literature is contradictory and not all mechanisms are fully understood.

This study supports that FXI(a) may be implicated in inflammatory mechanisms, and thus encourages research into the effect of novel FXIa inhibitors and the effect these may have on conditions unrelated to a thrombotic phenotype. This thesis has raised more

questions than it has answered, mainly due to the limitations previously mentioned. This is the first report that FP can be cleaved and further insight into the function of these cleavage products must be elucidated. Further work must be performed using more physiological surfaces to determine the clinical relevance of these findings, and disease models of thrombosis and sepsis may prove useful tools to further evaluate this interaction. Further work must be performed to determine if FP and FXI(a) interact in the presence of natural cofactors, such as C3b and HK, and it is highly important to optimise plasma-based assays to determine the relevance.

These findings are important, highlighting the need for further research into the crosstalk between complement and coagulation helping to improve our understanding of thromboinflammatory disorders which may lead to the exposure of novel therapeutic targets.

6.2 Conclusions

Several conclusions can be drawn from this thesis:

1. Small molecule screening requires optimisation of high throughput assays, including purified and plasma-based systems to ensure potency and the likelihood of off-target effects.
2. ELISAs may not be specific enough for screening compounds at the beginning of a drug discovery study.
3. Structural insight is useful for determining interactions between small molecules and their targets.
4. FXI(a) and FP interact, and this is modulated by negatively charged surfaces.
5. FXIa can cleave FP in the presence of DXS_{500kDa}, resulting in novel cleavage products, however this does not appear to affect FXIa cleavage of FIX.
6. FP may affect the activation of FXI by FXIIa in the absence of HK and may bind to FXI(a) via the same binding site.
7. The AP C3 convertase may impact on clot structure and function.
8. The mechanisms by which the AP C3 convertase influences clot structure may include interactions with thrombin and/or fibrin(ogen).

This thesis has set in motion the development of a screening process for small molecules targeting the AP C3 convertase, however the process can be modified for other therapeutic targets. It has explored novel crosstalk pathways between complement and coagulation and has revealed an exciting novel interaction between FXI(a) from the intrinsic pathway of coagulation and FP of the AP of complement. The data presented in this thesis demonstrate the importance of understanding the intercommunication between coagulation and inflammation, in order to develop novel therapeutics with limited side effects. This thesis has shown that our knowledge of complement and coagulation crosstalk is still very limited and must remain an area of interest for future research.

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Appendices

Appendix 1 Patient Information Sheet

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Role of different components on fibrin clot structure and function

Volunteer Information Sheet

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information carefully and discuss it if you wish. Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Purpose of the study
People at risk of cardiovascular problems, e.g. heart attack, can have differences in their components (proteins) of the blood clotting system. These differences can be beneficial, detrimental, or not have any effect. We hope to understand the contribution of these differences to cardiovascular problems, and thus aid future drug development against these diseases.

Why have I been chosen?
You have been chosen because you are between 18 and 70 years old and are generally healthy.

Do I have to take part?
It is up to you whether or not to take part. If you do decide to participate, you will be given this information sheet to keep, asked to sign a consent form, may be asked to give telephone or email address (so we can contact you again asking if you would be willing to provide subsequent blood donations), and may be asked to complete a questionnaire detailing basic information. Completing any part of this questionnaire is voluntary. You do not have to provide any information if you do not want to. Your answers will be treated in confidence and will not be identifiable. We will hold your information securely in accordance with the Data Protection Act (1998). Your name and contact details (if you choose to provide these) will be kept solely on the consent form. Your volunteer ID code will be the only link between your name and contact details, and any stored samples and questionnaires. If you do decide to take part, you are still free to withdraw at any time and without giving a reason. If you do decide to participate, your blood sample will either:

- be tested within 6 hours of being taken and any remaining discarded after 6 hours
- separated into different components (e.g. plasma, cells, proteins) and either tested on the same day or stored for future use

If you do decide to participate, it is accepted that your sample is a gift and may be retained for use in future ethically approved research.

What will happen to me if I take part?
The study will take approximately 15 minutes of your time on one or more occasions when you will be asked to come to the LIGHT laboratories in the University of Leeds where you will see one of our research nurses or Dr Hethershaw. Up to 54 mls of blood (which is the equivalent of about 4 tablespoons) will be taken from a vein on the inside of your arm. Additionally, you may be asked to complete a questionnaire detailing basic information.

What will happen to any samples I give?
The blood sample provided will be labelled with your volunteer ID code only so that you can not be identified. The whole blood will then either:

- be used as is and tested on the same day
- separated into different components (e.g. plasma, cells, proteins) and either tested on the same day or stored for future use

Standard blood clotting assays will be used to test the effects of the different components of blood clotting system with your sample. Any stored samples will also be labelled with your volunteer ID code as the only identifier and so you can not be identified.

What are the possible disadvantages and risks of taking part?
Giving a blood sample may be uncomfortable and there is a slight risk of bruising from the needle piercing the vein to take the blood.

What is there is a problem?
If you have a concern about any aspect of this study, you should contact one of the researchers who will do their best to answer your questions. Our contact numbers are at the end of this information sheet. If you are harmed by taking part in this study, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for this.

Will my taking part in this study be kept confidential?
All information which is collected about you during the course of this research will be kept strictly confidential.

Contact Details
If you require any further information please contact us on the telephone numbers below. Thank-you for reading this information sheet and for considering participating in the research.

Dr Emma Hethershaw
0113 343 7743
e.hethershaw@leeds.ac.uk

Julie Bailey – Research Nurse
0113 343 7702

Appendix 2 Patient Consent Form

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Consent Form
Role of different components on fibrin clot structure and function
Blood Donation

Volunteer ID code:
Date sample taken: **Date test(s) performed:**
Date sample discarded:

Please initial box

1. I confirm that I have read and understood the volunteer information sheet for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without any legal rights being affected. ☐
3. I agree that my sample is a gift and may be retained for use in future ethically approved research. ☐
4. I understand that any samples and questionnaires will be labelled with my volunteer code only so that I can not be identified from them. ☐

Name of volunteer Date Signature

Contact details (optional)

Name of person taking consent Date Signature

Appendix 3 ab193695 Complement C5a Human ELISA Kit

Version 3 Last updated 21 March 2018

ab193695 Complement C5a Human ELISA Kit

For the quantitative measurement of Human C5a in serum, plasma and cell culture supernatants.

This product is for research use only and is not intended for diagnostic use.

Table of Contents

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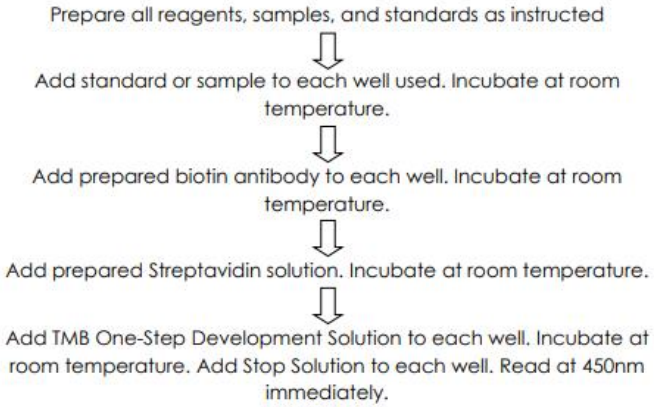
1. Overview

Abcam's Complement C5a Human ELISA Kit (ab193695) is an *in vitro* enzyme-linked immunosorbent assay for the quantitative measurement of Human C5A in serum, plasma and cell culture supernatants.

This assay employs an antibody specific for Human C5a coated on a 96-well plate. Standards and samples are pipetted into the wells and the immobilized antibody captures C5a present in the samples. The wells are washed and biotinylated anti-Human C5a antibody is added. After washing away any unbound biotinylated antibody, an HRP-conjugated streptavidin is pipetted to the wells. After incubation, the wells are again washed, followed by the addition of a TMB substrate solution to the wells. Color will develop in proportion to the amount of C5a bound in each well. Addition of the Stop Solution will change the color from blue to yellow, and the intensity of the color is measured at 450 nm.

2. Protocol Summary



3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at -20°C immediately upon receipt. Avoid multiple freeze-thaw cycles. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage Condition
Pre-coated C5a microplate (12 x 8 well strips)	96 wells	-20°C
20X Wash Buffer Concentrate	25 mL	-20°C
Human C5a Standard	2 vials	-20°C
Assay Diluent A	30 mL	-20°C
5X Assay Diluent B	15 mL	-20°C
Detection Antibody C5a (biotinylated anti-Human C5a)	2 vials	-20°C
200X HRP-Streptavidin concentrate	200 µL	-20°C
TMB One-Step Substrate Reagent	12 mL	-20°C
Stop Solution	8 mL	-20°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring absorbance at 450 nm.
- Precision pipettes to deliver 2 µL to 1 mL volumes.
- Adjustable 1-25 mL pipettes for reagent preparation.
- 100 mL and 1 liter graduated cylinders.
- Absorbent paper.
- Distilled or deionized water.
- Log-log graph paper or computer and software for ELISA data analysis.
- Tubes to prepare standard or sample dilutions.

8. Technical Hints

- This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.
- Samples which generate values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Completely aspirate all solutions and buffers during wash steps.
- When preparing your standards, it is critical to briefly centrifuge the vial first. The powder may adhere to the cap and not be included in the standard solution resulting in an incorrect concentration. Be sure to dissolve the powder thoroughly when reconstituting. After adding Assay Diluent to the vial, we recommend inverting the tube a few times, then flick the tube a few times, and centrifuge briefly; repeat this procedure 3-4 times. This is an effective technique for thorough mixing of the standard without using excessive mechanical force.
- Do not vortex the standard during reconstitution, as this will destabilize the protein.
- Once your standard has been reconstituted, it should be used right away or else frozen for later use.
- Keep the standard dilutions on ice during preparation, but the ELISA procedure should be done at room temperature.
- Be sure to discard the working standard dilutions after use – they do not store well.

9. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells.
- Prepare only as much reagent as is needed on the day of the experiment.

9.1 1X Assay Diluent B

5X Assay Diluent B should be diluted 5-fold with deionized or distilled water before use.

9.2 1X Wash Solution

If the 20X Wash Concentrate contains visible crystals, equilibrate to room temperature and mix gently until dissolved. Dilute 20 mL of 20X Wash Buffer Concentrate into 380 mL deionized or distilled water to yield 400 mL of 1X Wash Buffer.

9.3 Detection Antibody C5a (biotinylated anti-Human C5a)

Briefly centrifuge the Detection Antibody vial before use. Add 100 µL of 1X Assay Diluent B into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days). The detection antibody concentrate should be diluted 80-fold with 1X Assay Diluent B and used in Assay Procedure.

9.4 1X HRP-Streptavidin Solution

Briefly centrifuge the 200X HRP-Streptavidin concentrate vial and pipette up and down to mix gently before use. The 200X HRP-Streptavidin concentrate should be diluted 200-fold with 1X Assay Diluent B.

For example: Briefly centrifuge the vial and pipette up and down to mix gently. Add 50 µL of HRP-Streptavidin concentrate into a tube with 10 mL 1X Assay Diluent B to prepare a 1X HRP-Streptavidin solution (do not store the diluted solution for next day use). Mix well.

10. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Prepare serially diluted standards immediately prior to use.
- Standard (recombinant protein) should be stored at -20°C or -80°C (recommended at -80°C) after reconstitution.

10.1 Briefly centrifuge the vial of Human C5a Standard and then add 400 µL Assay Diluent A (for serum/plasma samples) or 1X Assay Diluent B (for cell culture supernatants) into the Human C5a Standard vial to prepare a 50 ng/mL **Stock Standard**. Mix thoroughly but gently.
10.2 Label tubes #1-8.
10.3 Prepare the 2000 pg/mL **Standard #1** by adding 40 µL Stock Standard into tube #1 along with 960 µL Assay Diluent A or 1x Assay Diluent B. Mix thoroughly but gently.
10.4 Add 300 µL Assay Diluent A or 1x Assay Diluent B into tubes 2-8.
10.5 Prepare **Standard #2** by adding 300 µL Standard #1 to tube #2. Mix thoroughly but gently.
10.6 Prepare **Standard #3** by adding 300 µL from Standard #2 to tube #3. Mix thoroughly but gently.
10.7 Using the table below as a guide, prepare further serial dilutions.
10.8 Standard #8 contains no protein and is the Blank control.

Standard #	Volume to dilute (µL)	Volume Diluent (µL)	Starting Conc. (pg/mL)	Final Conc. (pg/mL)
1	-	40	50,000	2,000
2	300 µL Standard #1	300	2,000	1,000
3	300 µL Standard #2	300	1,000	500
4	300 µL Standard #3	300	500	250
5	300 µL Standard #4	300	250	125
6	300 µL Standard #5	300	125	62.50
7	300 µL Standard #6	300	62.50	31.25
8	-	300	0	0

11. Sample Preparation

General Sample Information:

- If your samples need to be diluted, Assay Diluent A should be used for dilution of serum/plasma samples. 1X Assay Diluent B should be used for dilution of culture supernatants.
- Suggested dilution for normal serum/plasma: 40-400-fold.
- Please note that levels of the target protein may vary between different specimens. Optimal dilution factors for each sample must be determined by the investigator.

12. Plate Preparation

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused well strips should be returned to the plate packet and stored at 4°C.
- For each assay performed, a minimum of 2 wells must be used as blanks, omitting primary antibody from well additions.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Well effects have not been observed with this assay. Contents of each well can be recorded on the template sheet included in the Resources section.

13. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.

13.1 Add 100 µL of each standard (see Standard Preparations, section) and sample into appropriate wells. Cover plate and incubate for 2.5 hours at room temperature or overnight at 4°C with gentle shaking.

13.2 Discard the solution and wash 4 times with 1X Wash Buffer. Wash by filling each well with 300 µL 1X Wash Buffer using a multi-channel pipette or automatic plate washer. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it by tapping gently against clean paper towels.

13.3 Add 100 µL of the prepared biotinylated Human C5a Detection Antibody (see Reagent Preparation section) to each well. Incubate for 1 hour at room temperature with gentle shaking.

13.4 Discard the solution. Repeat the wash as in step 13.2.

13.5 Add 100 µL of prepared 1X HRP-Streptavidin solution (see Reagent Preparation section) to each well. Incubate for 45 minutes at room temperature with gentle shaking

13.6 Discard the solution. Repeat the wash as in step 13.2.

13.7 Add 100 µL of TMB One-Step Substrate Reagent to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.

13.8 Add 50 µL of Stop Solution to each well. Read at 450 nm immediately.

14. Calculations

Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the average zero standard optical density. Plot the standard curve on log-log graph paper, with standard concentration on the x-axis and absorbance on the y-axis. Draw the best-fit straight line through the standard points.

15. Typical Data

Typical standard curve – data provided **for demonstration purposes only**. A new standard curve must be generated for each assay performed.

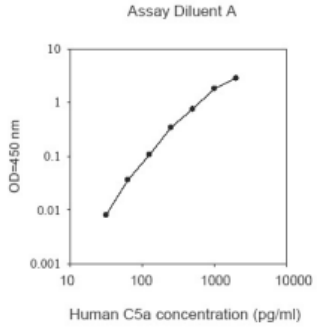


Figure 1. Example of typical human C5a standard curve using Assay Diluent A. The standard curve was prepared as described in Section 10.

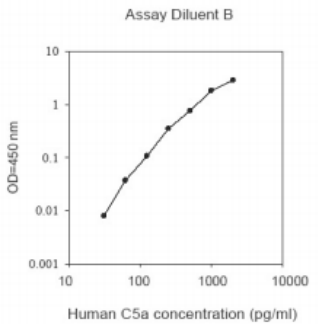


Figure 2. Example of typical human C5a standard curve using 1X Assay Diluent B. The standard curve was prepared as described in Section 10.

16. Typical Sample Values

SENSITIVITY –

The minimum detectable dose of C5a is 31 pg/mL.

RECOVERY –

Recovery was determined by spiking various levels of Human C5a into Human serum, plasma and cell culture media. Mean recoveries are as follows:

Sample Type	Average % Recovery	Range (%)
Serum	127.1	122-138
Plasma	122.5	114-130
Cell culture media	92.43	70-104

LINEARITY OF DILUTION –

Serum Dilution	Average % Expected Value	Range (%)
1:2	105.0	97-113
1:4	98.36	93-104

Plasma Dilution	Average % Expected Value	Range (%)
1:2	100.4	92-108
1:4	92.01	84-100

Cell Culture Media Dilution	Average % Expected Value	Range (%)
1:2	104.7	97-113
1:4	103.1	95-111

PRECISION –

	Intra-Assay	Inter-Assay
CV (%)	<10%	<12%

17. Assay Specificity

The antibodies used within this ELISA kit detect Human C5a.

The antibodies do not cross-react with rhC3a, rha2-macroglobulin, rmC5a, or rmC5d.

Please contact our Technical Support team for more information.

18. Troubleshooting

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standards dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store the reconstituted protein at -80°C, all other assay components 4°C. Keep substrate solution protected from light.

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Appendix 4 WEISLAB® Complement System Screen

Instruction

WIESLAB® Complement system Screen

Enzyme immunoassay for assessment of
Complement functional activity

Break apart microtitration strips (4x8x3) 96 wells
Store the kit at +2-8° C
Store the positive control at -20° C
For in vitro diagnostic use only

CE

Document No. LABEL-DOC-0029, 2.0
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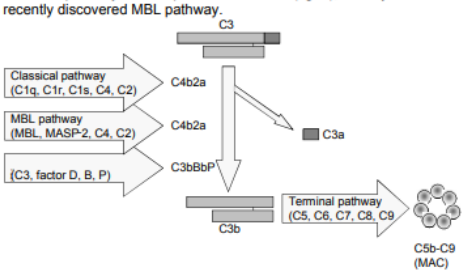
WIESLAB® Complement system Screen

REF COMPL 300 IVD Σ 96

INTENDED USE
The Wieslab® Complement system Screenkit is an enzyme immunoassay for the qualitative determination of functional classical, MBL and alternative complement pathways in human serum.
FOR *IN VITRO* DIAGNOSTIC USE.

Summary and explanation
The complement system plays an essential role in chronic, autoimmune and infectious disease. There are three pathways of complement activation (fig. 1), namely the classical, the alternative and the recently discovered MBL pathway.



Impaired complement activity causes humans to become susceptible to repetitive fulminant or severe infections and may contribute to development of autoimmune disease. Inappropriate activation of complement contributes to chronic inflammation and tissue injury.

Principle of the Wieslab® Complement assay
The Wieslab® Complement assay combines principles of the hemolytic assay for complement activation with the use of labelled antibodies specific for neoantigen produced as a result of complement activation. The amount of neoantigen generated is proportional to the functional activity of complement pathways.

The wells of the microtitre strips are coated with specific activators of the classical, or the MBL, or the alternative pathway. Patient serum is diluted in diluent containing specific blocker to ensure that only the respective pathway is activated. During the incubation of the diluted patient serum in the wells, complement is activated by the specific coating.

The wells are then washed and C5b-9 is detected with a specific alkaline phosphatase labelled antibody to the neoantigen expressed during MAC formation.

After a further washing step, detection of specific antibodies is obtained by incubation with alkaline phosphatase substrate solution. The amount of complement activation correlates with the colour intensity and is measured in terms of absorbance (optical density (OD)).

Warnings and precautions
- For *in vitro* diagnostic use.
- The human serum components used in the preparation of the controls in the kit have been tested for the presence of antibodies to human immunodeficiency virus 1 & 2 (HIV 1&2), hepatitis C (HCV) as well as hepatitis B surface antigen by FDA approved methods and found negative. Because no test methods can offer complete assurance that HIV, HCV, hepatitis B virus, or other infectious agents are absent, specimens and human-based reagents should be handled as if capable of transmitting infectious agents.
- The Centers for Disease Control and Prevention and National Institutes of Health recommended that potentially infectious agents be handled at the Biosafety Level 2.

- All solutions contain ProClin 300 as a preservative. Never pipette by mouth or allow reagents or patient sample to come into contact with skin. Reagents containing ProClin may be irritating. Avoid contact with skin and eyes. In case of contact, flush with plenty of water.
- Material safety data sheets for all hazardous components contained in this kit are available on request from Svar Life Science.

BUF	WASH	30X
DIL		
CONJ		

CONTROL	-
SUBS	pNPP

Warning

Contains ProClin 300:
Reaction mass of: 5-chloro-2-methyl-4-isothiazolin-3-one [EC no. 247-500-7] and 2-methyl-4-isothiazolin-3-one [EC no. 220-239-6] (3:1)

H317:	May cause an allergic skin reaction.
P264:	Wash hands thoroughly after handling.
P280:	Wear protective gloves/protective clothing/eye protection/face protection.
P302+352:	IF ON SKIN: Wash with plenty of soap and water.
P333+313:	If skin irritation or rash occurs: Get medical advice/attention.

Specimen collection
Blood samples are to be collected using aseptic venipuncture technique and serum obtained using standard procedures. A minimum of 5 mL of whole blood is recommended. Allow blood to clot in serum tubes, for 60-65 minutes at room temperature (20-25° C). Centrifuge blood samples and transfer cell-free serum to a clean tube. **Sera must be handled properly to prevent *in vitro* complement activation.** Sera should be frozen at -70° C or lower in tightly sealed tubes for extended storage or for transport on dry ice. Samples should not be frozen and thawed more than once. Avoid using sera which are icteric, lipemic and hemolyzed. Heat-inactivated sera can not be used. Plasma can not be used. The NCCLS provides recommendations for storing blood specimens, (Approved Standard-Procedures for the Handling and Processing of Blood Specimens, H18A, 1990).

Kit components and storage of reagents
- One frame with break apart wells (4x8x3) sealed in a foil pack with a desiccation sachet. 4 blue coloured strips for classical pathway (CP), coated with human IgM. 4 green coloured strips for MBL pathway (MP), coated with mannan. 4 red coloured strips for alternative pathway (AP), coated with LPS.
- 10 ml Diluent CP (Dil CP), labelled blue.
- 10 ml Diluent MP (Dil MP), labelled green.
- 10 ml Diluent AP (Dil AP), labelled red.
- 13 ml conjugate containing alkaline phosphatase-labelled antibodies to C5b-9 (blue colour).
- 13 ml Substrate solution ready to use.
- 30 ml wash solution 30x concentrated.
- 0,2 ml negative control (NC) containing human serum (to be diluted as for a patient serum sample).
- 0,2 ml positive control (PC) containing freezed dried human serum, see "Reconstitution of positive control", below.

All reagents in the kit are ready for use except washing solution and controls. The reagents should be stored at 2-8° C except the positive control. **The positive control should be stored at -20° C.**

Materials or equipment required but not provided
- Microplate reader with filter 405 nm.
- Precision pipettes with disposable tips.
- Washer for strips, absorbent tissue, tubes and a timer.

PROCEDURE
Remove only the number of wells needed for testing, resealing the aluminium package carefully. Let all solutions equilibrate to room temperature (20-25° C) before analysis.

Preparation of washing solution
In case salt crystals are observed in the vial with concentrated wash solution, place the vial at 37°C water bath until the crystals have dissolved before dilution of wash solution. Dilute 30 ml of the 30x concentrated wash solution in 870 ml distilled water. When stored at 2-8° C, the diluted wash solution is stable until the date of expiration of the kit.

Reconstitution of positive control
Gently tap down all lyophilized material to the bottom of the vial and remove the cap. Immediately add 200 µl of distilled water directly to the lyophilized material. Replace the cap. Allow the vial to stand on ice for 5 minutes and then gently shake or vortex occasionally until completely dissolved. Dilute the reconstituted control in the same way as a patient serum sample. The reconstituted control can be stored for up to 4 hours prior to use if kept at 2-8° C or on ice. It can be frozen at –70° C and thawed once.

Serum
Partially thaw frozen sera by briefly placing in a 37°C water bath with gentle mixing. After partially thawing immediately place the tubes in an ice bath and leave on ice until completely thawed. Mix briefly on a vortex mixer.

Dilution of serum
Classical Pathway (CP): Dilute the serum 1/101 with Diluent CP, blue label, (500 µl Diluent + 5 µl serum). The diluted serum can be left at room temperature for a maximum of 60 minutes before analysis.

MBL Pathway (MP): Dilute the serum 1/101 with Diluent MP, green label, (500 µl Diluent + 5 µl serum). The diluted serum must be left at room temperature for >15 minutes before analysis, but not more than 60 minutes.

Alternative Pathway (AP): Dilute the serum 1/18 with Diluent AP, red label, (340 µl Diluent + 20 µl serum). The diluted serum can be left at room temperature for a maximum of 60 minutes before analysis.

Incubation of samples
Pipet 100 µl/well in duplicate of Diluent (Dil) as a blank, positive control (PC), negative control (NC) and diluted patient's serum (P) for each pathway according to the diagram. Incubate for 60-70 minutes at +37° C with lid.
Please note that no incubation should be performed under CO_2 atmosphere. If a CO_2 cabinet is used, make sure that the CO_2 supply is disconnected/off.

	Classical Pathway				MBL Pathway				Alternative Pathway			
	1	2	3	4	5	6	7	8	9	10	11	12
A	Dil CP	P2			Dil MP	P2			Dil AP	P2		
B	Dil CP	P2			Dil MP	P2			Dil AP	P2		
C	PC	etc			PC	etc			PC	etc		
D	PC				PC				PC			
E	NC				NC				NC			
F	NC				NC				NC			
G	P1				P1				P1			
H	P1				P1				P1			

After serum incubation
Empty the wells and wash 3 times with 300 µl washing solution, filling and emptying the wells each time. After the last wash, empty the wells by tapping the strip on an absorbent tissue.

Adding conjugate
Add 100 µl conjugate to each well. Incubate for 30 minutes at room temperature (+20-25° C).

After conjugate incubation
Wash 3 times as before.
Adding substrate solution
Add 100 µl substrate solution to each well, incubate for 30 minutes at room temperature (+20-25° C). Read the absorbance at 405 nm on a microplate reader. (5 mM EDTA can be used as stop solution, 100 µl/well. Read the absorbance of the wells within 60 minutes.)

Calculation of result
Subtract the absorbance of the Blank (Diluent) (for each pathway) from the NC, PC and the samples. The absorbance of the positive control should be >1 and the negative control absorbance < 0.2.
The negative and positive controls can be used in a semiquantiative way to calculate complement activity. Calculate the mean OD405nm values for the sample, PC and NC and calculate the % complement activity as follows: (Sample-NC)/(PC-NC)x100. The negative and positive controls are intended to monitor for substantial reagent failure. The positive control will not ensure precision at the assay cut-off. It is recommended that each laboratory establish its own reference level and cutoff value for deficiencies.
A negative result i.e. deficiency, should always be verified by testing a new sample to ensure that no in vitro complement activation has taken place.

Limitations
The individual patient's complement level can not be used as a measure of disease severity, as it may vary from patient to patient. Thus, it is difficult to obtain an absolute standardisation of results.
The test should not be relied upon as the sole basis of decisions on clinical therapy, but should be used in combination with clinical symptoms and the results of other available tests. Therapy should not be started on basis of the complement assay result. Initiation or changes in treatment should not be based on changes in complement levels alone, but rather on careful clinical observation.

Expected results
In vitro activation of the complement sequence leads to the consumption of complement components which, in turn, leads to a decrease in their concentration. Thus, the determination of complement proteins or complement activity is used to indicate whether the complement system has been activated by an immunologic and/or pathogenic mechanism. Both functional and immunochemical complement measurements are used to evaluate patients when a complement-activating disease is suspected or an inherited deficiency is possible. The level of complement activity evaluated by functional assays such as Wieslab® Complement kit takes into account the rate of synthesis, degradation, and consumption of the components and provides a measure of the integrity of the pathways as opposed to immunochemical methods which specifically measure the concentration of various complement components. When decreased levels of complement components or complement function are found, a deficiency or an ongoing, immunologic process, leading to increased breakdown of components and depression of complement levels is considered by clinicians. Increased complement levels are usually a nonspecific expression of an acute phase response.
The combination of the three Wieslab® assays for complement can be helpful for detection of complement deficiencies as shown in the table below:

Classical pathway	MBL pathway	Alternative pathway	Possible deficiency
Positive	Positive	Positive	None
Negative	Positive	Positive	C1q, C1r, C1s
Positive	Positive	Negative	Properdin, Factor B,D
Positive	Negative	Positive	MBL, MASP2
Negative	Negative	Negative	C3, C5,C6,C7,C8,C9
Negative	Negative	Positive	C4, C2 or combination

Performance characteristics
120 sera from blood donors were tested in the three assays and the normal reference range was calculated. The values were expressed in % of the positive control. See Figure 1 and Table 1. In the CP assay no blood donor was below 40 %. In the MP assay 23 samples were below 10 % and they had MBL values (established in a separate assay) below 500 ng/ml and in the AP assay no blood donor was

below 10 %. Please note that true deficient MP, i.e. an activity of <10%, may be found in a normal population at a frequency of 20-30%.
Figure 1.

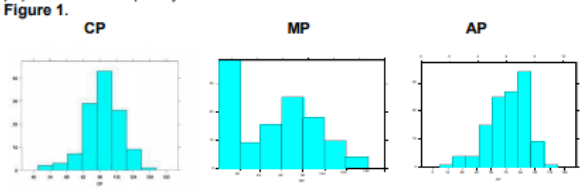


Table 1.

	n	Mean (%)	±2SD (%)	Median (%)
Classical pathway	120	99	69-129	100
MBL pathway	120	49	0-125	56
Alternative pathway	120	71	30-113	73

Table 2
Sera with known complement deficiencies were tested in the assays and the following results were obtained. All deficient sera were detected in the assay and gave values below 5 %.

Deficiency	C1q	C2	C3	C4	C5	C6	C7	C8	C9	P	H	I
Number of patients	3	11	1	1	2	1	2	2	1	9	1	2
Number of deficient sera detected	3	11	1	1	2	1	2	2	1	9	1	2

Table 3. Inter-assay precision was determined by testing three samples in duplicate. Results were obtained for six different runs.

Sample	Mean value %	SD	CV %
CP P1	98	4.3	4
CP P2	92	3.9	4
CP P3	21	1.7	8
MP P1	91	3.3	4
MP P2	37	4.0	11
MP P3	16	2.3	15
AP P1	48	5.1	11
AP P2	89	8.0	9
AP P3	16	3.1	20

Table 4. Intra-assay precision was determined by testing one sample in 40 wells.

Assay	Mean value %	SD	CV %
CP	85	2.9	3
MP	74	3.9	5
AP	83	5.7	7

Troubleshooting

Problem	Possible causes	Solution
Control values out of range	Incorrect temperature, timing or pipetting, reagents not mixed	Check that the time and temperature was correct. Repeat test.
	Cross contamination of controls	Pipette carefully.
	Optical pathway not clean.	Check for dirt or air-bubbles in the wells. Wipe plate bottom and reread.
	Positive control not properly dissolved.	Check the positive control dissolve a new.
	Mixup of plates or reagents from another pathway. Improper dilution.	Repeat test.
All test results negative	One or more reagents not added, or added in wrong sequence.	Recheck procedure. Check for unused reagents. Repeat test.
	Antigen coated plate inactive.	Check for obvious moisture in unused wells. Wipe plate bottom and reread.
	Serum inactive.	Dilute new samples.
	Mixup of plates or reagents from another pathway.	Repeat test.
All test results yellow.	Contaminated buffers or reagents.	Check all solutions for turbidity.
	Washing solution contaminated.	Use clean container. Check quality of water used to prepare solution.
	Improper dilution of serum.	Repeat test.
Poor precision.	Pipette delivery CV >5% or samples not mixed.	Check calibration of pipette. Use reproducible technique. Avoid airbubbles in pipette tip.
	Serum or reagents not mixed sufficiently or not equilibrated to room temperature.	Mix all reagents gently but thoroughly and equilibrate to room temperature.
	Reagent addition taking too long, inconsistency in timing intervals.	Develop consistent uniform technique and use multi-tip device or autodispenser to decrease time.
	Optical pathway not clean.	Check for airbubbles in the wells. Wipe plate bottom and reread.
	Washing not consistent, trapped bubbles, washing solution left in the wells.	Check that all wells are filled and aspirated uniformly. Dispense liquid above level of reagent in the well. After last wash, empty the wells by tapping the strip on an absorbent tissue.

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Explanation of symbols. L'explication de symboles. La explicación de símbolos. Erklärung der Symbole. La spiegazione di simboli. Explicação dos símbolos. Forklaring til symboler. Symbolforklaring. Förklaringar till symboler.

LOT	Batch code. Numéro de lot. Número de lote. Chargen-Nummer. Numero di lotto. Código do lote. Partinummer. Lot nummer.Satsnummer.
REF	Catalogue number. Référence catalogue. Número de catálogo. Katalog-Nummer. Numero di catalogo. Número catalogo. Katalognummer.
	Use-by date. Date de péremption. Fecha de caducidad. Verfallsdatum. . La data di scadenza. Prazo de validade. Udløbsdato. Utløpsdato. Använd före.
	Temperature limit. Seuils de températures. Rango de temperature. Temperaturbereich. Limitazioni di temperatura. Limite de temperatura. Opbevaringstemperatur. Oppbevares ved. Förvaringstemperatur.
	Biological risk. Risque biologique. Riesgo biológico. Biologische Gefährdung. Rishio biologico. Risco biológico. Biologisk risk.
	Consult instructions for use. Lire le mode d'emploi. Consulte las instrucciones de uso. Gebrauchsanweisung beachten. Leggere le istruzioni per l'uso. Consultar as instruções de utilização. Se brugsanvisning. Se bruksanvisningen. Läs instruktionsmanualen.
IVD	In vitro diagnostic medical device. Dispositif médical de diagnostic in vitro. Producto sanitario para diagnóstico in vitro. In-vitro-Diagnostikum. Dispositivo medico-diagnostico in vitro. Dispositivos médicos para diagnóstico *in vitro*. In Vitro medisinsk diagnoseutstyr. In vitro diagnostik medicinsk utrustning.
	Warning. Attention. Atensión. Achtung. Attenzione. Atenção. Advarsel. Advarsel. Varning.
	Manufacturer. Fabricant. Fabricante. Hersteller. Produttore. Fabricante. Fabrikant. Producent. Produsent. Tillverkare.
Σ 96	Contains sufficient for 96 tests. Contenu suffisant pour 96 tests. Contenido suficiente para 96 pruebas. Inhalt ausreichend für 96 Tests. Contenuto sufficiente per 96 test. Indeholder tilstrækkelig for 96 test. Inneholder tilstrekkelig for 96 test. Innehåller tillräckligt för 96 test.
CE	Conformity to 98/79/EC on In Vitro Diagnostic Medical Device Directive. Conformément à la directive européenne 98/79/CE relative aux dispositifs médicaux de diagnostic in vitro. La conformidad con la Directiva 98/79/CE sobre productos sanitarios para diagnóstico in vitro. Konform mit Richtlinie 98/79/EG zu In-vitro-Diagnostika. Conformità alla direttiva 98/79/CE relativa ai dispositivi medico-diagnostici in vitro. Medicinsk udstyr til in vitro-diagnostik, i overensstemmelse med Europa-Parlamentets og Rådets direktiv 98/79/EF. Medisinsk utstyr i samsvar med EU in vitro diagnostic directive 98/79/EF. Överensstämmer med direktiv 98/79/EG för medicintekniska produkter.

Ag	Antigen. Antigène. Antigeno, Antigen. L'antigene. Antígeno. Antigen. Antigen. Antigen.
DIL	Diluent. Diluant. Diluyente. Probenverdünnungspuffer. Il diluente. Diluente. Diluent. Fortynning. Spädningsbuffert.
CONJ	Conjugate. Conjugué. Conjugado. Konjugat. Conjugato. Conjugado. Konjugat. Konjugat.
BUF WASH 30X	Wash solution 30x conc. Solution lavage conc. 30x. Solución de lavado conc. 30x. Waschpuffer 30x konz. Soluzione di lavaggio 30x conc. Solução de lavagem conc. 30 vezes. Vaskebuffert 30x konc. Vaskeløsning 30x kons. Tvättbuffert 30x konc.
SUBS pNPP	Substrate pNPP. pNPP Substrat. Sustrato pNPP. Substrat pNPP. Substrato pNPP. Substrato pNPP. Substrat pNPP.
CONTROL -	Negative control. Contrôle négatif. Control negativo. Negativkontrolle. Controllo negativo. Controlo negativo. Negativ control. Negativ kontroll. Negativ kontroll.
CONTROL + LYO	Lyophilized positive control. Contrôle positif lyophilizé. Control positivo liofilizado. Lyophilisierte Positivkontrolle. Controllo positivo liofilizzato. Controlo positivo liofilizado. Frysetørret positiv kontrol. Lyofilisert positiv kontroll. Frystorkad positiv kontroll.