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The Regulatory Activities of recombinant Properdin and TSP1 on Platelet and Complement activation

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By

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Statement of originality

This accompanying thesis submitted for the degree of PhD entitled (Thrombospondin-1 and Properdin synergistically promote the activation of platelets, while they antagonise each other in the regulation of complement activation) is based on work conducted by the author at the University of Leicester mainly during the period between May 2011 and May 2014.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other University.

Abstract

The complement system provides a first line defence against microbial pathogens and cooperates with platelet activation of haemostasis/thrombosis system in a physiologically meaningful way by forming a physiological barrier to prevent blood loss following vascular injury.

Due to chondroitin sulphate (CS) secretion from activated platelets, some pattern recognition molecules, i.e. MBL, CL-11 and ficolins of the lectin pathway (LP), and C1q of the classical pathway (CP) can drive complement activation on the surface of activated platelets.

Properdin is, a positive regulator of the AP, is entirely composed of thrombospondin-like domains (TSR) sharing a high degree of identity with TSR Type-I repeat of Thrombospondin-1 (TSP1). TSP1 is an adhesive homo-trimeric glycoprotein that is released during platelet activation. The structural similarities between properdin and TSP1 are striking.

The current result showed a highly significant difference of P-selectin expression level which was observed in washed activated human platelets triggered by 10µg/ml (w/v) of highly oligomerised properdin using ELISA in comparison with other known agonists, including TSP1. Platelet activation resulted in α-thrombin release as shown by ELISA as well as thrombin generation monitored using platelet microparticles (PMPs). My work demonstrated for the first time that highly oligomerised properdin can bind to intact platelets while the binding of the physiologically occurring lower grade properdin oligomers; i.e. dimers, trimers and tetramers, to resting platelets was not observed. These lower grade oligomers only bind to activated platelets. That demonstrated the capability of highly oligomerised properdin to promote platelet adhesion. The activation was suggested through binding directly to platelet receptors or indirectly through supporting extracellular matrix (ECM) in sub-endothelial layer. The binding was observed with Collagen Type-I, von Willebrand factor (vWF) and Fibronectin (Fn), also, with atherogenic particles such as Low-density lipoprotein (LDL), cholesterol and triglycerides.

The crosstalk between activated platelets and the complement system was investigated by screening for a possible regulatory role of TSP1 during complement activation. 10µg/ml (w/v) of TSP1 were found to significantly down-regulate complement activation using C3 and C4 deposition assays under alternative pathway or lectin pathway specific assay conditions. While the down-regulation of lectin pathway functional activity was due to direct TSP1 binding to mannan and Chondroitin sulphate (CS) on the activator surface competing for the binding of MBL and/or CL-11, TSP1 was shown to inhibit the alternative pathway through direct binding to factor B (fB) and/or to C3 with surprisingly high binding affinity. TSP1 binding led to a competitive inhibition of properdin functional activity. These results suggest that TSP1 acts as a negative regulator which significantly shortens the half-life of the C3 and C5 convertase complexes of the

AP. Therefore, native TSP1 may act as a negative fluid-phase regulatory component of complement activation to protect activated-platelets from overshooting complement activation.
TSP1 is believed to influence the colonisation and dissemination of microbial pathogens such as *Streptococcus pneumoniae*. My results demonstrate that TSP1 release may in part protect *S.pneumoniae* (D39) from complement mediated killing, as demonstrated in a serum bactericidal assay (SBA). That may suggest that for colonisation/dissemination, TSP1 may help the pathogen to escape from elimination by the complement system.

In conclusion, my results propose a so far unknown physiological role of TSP1 as a negative regulator of the alternative pathway activation route of complement by shorting the half-life of the LP and/or AP C3 and C5 convertases and as such may provide a new example of synergisms and antagonisms between the platelet and the complement system.

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Abbreviations

ACD	Acid Citrate Dextrose
ADP	Adenosine 5’-diphosphate
ALK-phosphatase	Alkaline phosphatase
AP	Alternative pathway
APC	Antigenic presenting cells
ATP	Adenosine-tri-phosphate
BBS	Barbital buffer saline
bp	base pair
BHI	Brain heart infusion broth
BALF	Bronchoalveolar lavage fluid
BSA	Bovine serum albumin
cAMP	cyclic adenosine 3’,5’ mono-phosphate
CBD	Cell-binding domain
CD	Cluster differentiation
CP	Classical pathway
CS	Chondroitin sulphate
CL-11	Collectin-11
CRD	Carbohydrate recognition domains
CR	Complement receptor
C1-INH	C1-inhibitor
C4bp	C4 binding protein
CHO	Chinese hamster ovary
CFU	Colony formation unit
cDNA	Complementary deoxy-nucleic acid
DMSO	Dimethyl sulfoxide
dNTP	Deoxy-nucleotide triphosphate mixture
DAF	Decay-accelerating factor
DMEM	Dulbecco’s Modified Eagle Medium
dH_2O	Distal water
ECMs	Extracellular Matrix’s
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme Linked-Immunosorbent Assay
Et Br	Ethidium bromide

FBS	Fetal Bovine Serum
fD	Factor D
Fg	Fibrinogen
fH	Factor H
fI	Factor I
Fn	Fibronectin
GalNAc	*N*-acetylgalactosamine
GlcNAc	*N*-acetylglucosamine
MurNAc	*N*-acetylmuramic acid
GAG	glycosaminoglycan
GP	Glycoprotein
HRP	horse raddish peroxidase
HBS	HEPES buffer saline
HBD	Heparin Binding Domain
HEK	Human Embryonic Kidney cell
HPLC	High Performance Liquid Chromatography
HSA	Human Serum Albumin
HUS	Haemolytic Uraemic Syndrome
IIP	Idiopathic Interstitial Pneumonia
Ig	Immunoglobulin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IL	Interleukin
Kbp	Kilo-base pair
kDa	Kilo-Dalton
LDL	Low-Density Lipoprotein
LP	Lectin Pathway
LPS	Lipopolysaccharides
LTA	Lipoteichoic acid
MAC	Membrane attack complex
MASP	Mannan associated serine protease
MBL	Mannan binding lectin
MCP	Membrane cofactor protein
MPs	Microparticles
NSIP	Non-Specific Interstitial Pneumonia
OD	Optical density
PAMPs	Pathogen associated molecular patterns

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	Peptidoglycan
PKA	Protein kinase-A
PNH	Paroxysmal Nocturnal Haemoglobinuria
PAD	Peripheral Arterial Disease
p-NPP	p-Nitrophenyl phosphate
PRP	Platelet Rich Plasma
PPP	Platelet Poor Plasma
PMPs	Platelet Microparticles
PRRs	Pattern recognition receptors
Prostaglandin E1	PGE1
PS	Phosphatidylserine
RBCs	Red blood cells
rpm	Revolutions per minute
RT	Room Temperature
SBA	Serum bactericidal assay
SDS-PAGE	Sodium Dodecylsulfat-poly-acrylamide gel electrophoresis
TAE	Tris-acetate-EDTA
TBS	Tris-buffer saline
TEMED	N,N,N',N'-tetramethyl ethylene diamine
TEV	Tobacco Etch Virus Protease
TF	Tissue factor
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TSP	Thrombospondin
TSR	Thrombospondin-like repeats domain
TC	Tissue culture
TCR	T-cell receptor
TCC	Terminal complement complex
TxA_2	Thromboxane A_2
UIP	Usual Interstitial Pneumonia
V	Volt
VLDL	very-low-density lipoprotein
vWF	Von Willebrand factor

Chapter 1
(Introduction)

1. Introduction

1.1. The Cross-talk between the Haemostasis and Complement Systems

The haemostasis system is an essential life support system which fulfils the important physiological function to stop blood loss by formation of blood clots in vascular injury sites through two convergent systems, i.e. platelet activation and the coagulation pathways. In contrast, the complement system is involved in the innate immune system that is considered to be the first line of defence and provides essential protection from invading microbial pathogens (and non-self-cells) including bacteria, viruses and fungi, etc. Complement also plays an important role in the clearance of apoptotic host cells serving as a scavenger system. These systems cooperate together in order to operate the regulation process either by interference or synergism in both physiological conditions or abnormal situations (uncontrolled events). For example, where thrombosis is initiated in coronary artery disease, or in atherosclerosis disease, the complement system would be triggered through certain complement components attached on the surface of activated platelets due to the exposure of chondroitin sulfate (CS) (Hamad et al., 2008). In other words, some complement components would accelerate the thrombotic events by cross-talking, either with direct contact with platelets or indirectly with coagulation pathways. Moreover, the activated complement system would drag down the inflammatory cells; leukocytes, monocytes and macrophages, to the site of vessel injury in order to accumulate on the platelet plug (Verschoor and Langer, 2013, Li et al., 2012). Platelets expose many receptors such as Toll-like receptors (TLRs) that may potentially connect a link between innate immunity and thrombosis. Through the interaction of the pattern recognition molecules like TLRs with lipopolysaccharide (LPS) from bacterial pathogens, that would activate the platelets and subsequently lead to triggering platelet-neutrophils activation leading to kill the bacteria by degranulation (Clark et al., 2007), and also allow the initiation of the complement system on the microbial surfaces.
Moreover, platelets store multiple anti-inflammatory molecules and cytokines like transforming growth factor-β (TGF-β) and thrombospondin-1 (TSP1) (Li et al., 2012).

TSP1 is a glycoprotein secreted from different cells, and especially from activated platelets. TSP1 is essential for triggering platelet activation and subsequent blood loss

(Bonnefoy et al., 2008). On the other hand, Properdin is a positive regulator of the alternative pathway (AP) of the complement system. Properdin was found to promote the clearance and subsequent protection from different microbial pathogens like *Neisseria meningitidis* and *Streptococcus pneumoniae* (Ali et al., 2014). These two components from different systems will be illustrated from the point of cross-talking and interference between the complement system and platelet activation. Remarkably both components I worked with -TSP1 and properdin- share stringent structural homology between each other as both contain or are composed of thrombospondin-like repeats domain (TSR-repeats). My study investigates a novel link between haemostasis/thrombosis and the complement system. This link connects two essential biological activities required to limit the damage following blood vessel injury, i.e. the prevention of blood loss and the protection from infection.

Before addressing the cross-talk (or interference) between the complement system and haemostasis system, it is crucial to introduce each system individually. This topic will be divided into two sub-topics; the haemostasis/thrombosis system and the complement system.

1.1.1 An Overview of the Immune System

The word 'immunity' originated from a Latin word, *immunitas*, that defines the main immune defense role initiated toward infectious diseases (Abbas et al., 2010). The immune system is categorised into two main branches regarding immune response; innate and adaptive immunity, though both branches are connected for synergy to achieve the defense against invading microbial pathogens, alongside with clearance of the cellular debris and abnormal cells. The innate immune system extends to include 'physical and chemical barriers' for example, skin and mucosal membrane surfaces in the respiratory tract, and gastrointestinal (GI) tract, designed to limit bacterial numbers by providing a non-suitable pH environment for growing germs, and membrane-bound proteins, secreting mucus or antimicrobial peptides (Gallo et al., 2002, Abbas et al., 2010) and also by exposing microbial pathogens to phagocytic cells through binding Pathogen-Associated Molecular Patterns (PAMPs), for example, lipopolysaccharides (LPS), the common patterns amongst Gram negative bacteria, lipoteichoic acid (LTAs), the common patterns amongst Gram positive bacteria, and mannan and zymozan among yeasts etc., for pathogen elimination (Janeway, 1989, Hoffmann et al., 1999, Goldsby, 2003, Medzhitov, 2007). The most immediate function of specialised immune cells is to phagocytose or engulf pathogens as described for polymorphonuclear cells (PMNCs) / neutrophils (granulocytes), macrophages, natural killer (NK) cells and dendritic cells (DCs). Encountering foreign bodies as the first line of defence (innate immunity) can involve pathogen recognition by pattern recognition receptors (PRRs) of phagocytes toward PAMPs. Non-self and self-surfaces can be distinguished via the PRRs such as Toll-like receptor as well as mannose and scavenger receptors on the surface of phagocytic cells through which PAMPs can be attached to specific binding sites within the PRRs (Roitt and Delves, 2001, Abbas et al., 2010). The response of the adaptive immune system for pathogenic invaders follows, but often is primed and depends on, the first-line of defence (i.e. innate immunity). In contrast, adaptive immunity requires the involvement of antigen presenting cells for antigen presentation as a key mechanism to stimulate the adaptive immune response, a process that may require several days, while the innate immune system can respond instantly. Since the adaptive immune system develops a specific immune response through cellular selection and adaptation; it is often referred to as acquired immunity, especially since this system maintains a memory function towards specific pathogens that allows a very potent and efficient secondary immune re-

sponse towards specific pathogens in repeat infections with the same or a very similar pathogen (Figure 1.1). While the specific activities of the innate immune response are triggered instantly and immediately without possessing memory functions for specific foreign antigens of pathogens, the kinetic activities of the adaptive immune response occur slowly during the primary infection with a specific pathogen, but due to the memory function of the adaptive immune response, upon secondary infection with a pathogen against which immunological memory has been established previously, the secondary immune response provides an effective and fulminant response that can lead to an effective clearance of a pathogen even before a full blown infection itself (Borghesi and Milcarek, 2007).

There are two effector arms categorised as essential branches for the adaptive immune system; cellular and humoral immunity (Schatz et al., 1992, Abbas et al., 2010). The humoral immunity is the system responsible for immune globulin production and release from B lymphocytes (B-cells) after encountering pathogens against which immunity has been established in a previous infection. The establishment of antibody mediated immunity requires a complex coordination between antibody presenting cells (APC) and specific T Helper cells. This adaptive immune response also requires the priming of innate immunity through the release of cytokines from different innate immune cells such as epithelial cells and phagocytes. Macrophages and other antigen presenting cells then trigger the adaptive immune response through the presentation of pathogen specific antigens to trigger antigen specific responses by B cells via B cell receptors and T cell receptors (Holmskov et al., 2003, Medzhitov, 2007). While the innate immune system is composed of physical and chemical barriers, etc., as stated previously, as well as of specific serum components, including complement proteins associated to form an effective first line of defence to protect the body even in absence of specific immunity and to prime and to allow a specific immune response to develop (Roitt and Delves, 2001, Abbas et al., 2010) (Figure 1.2).

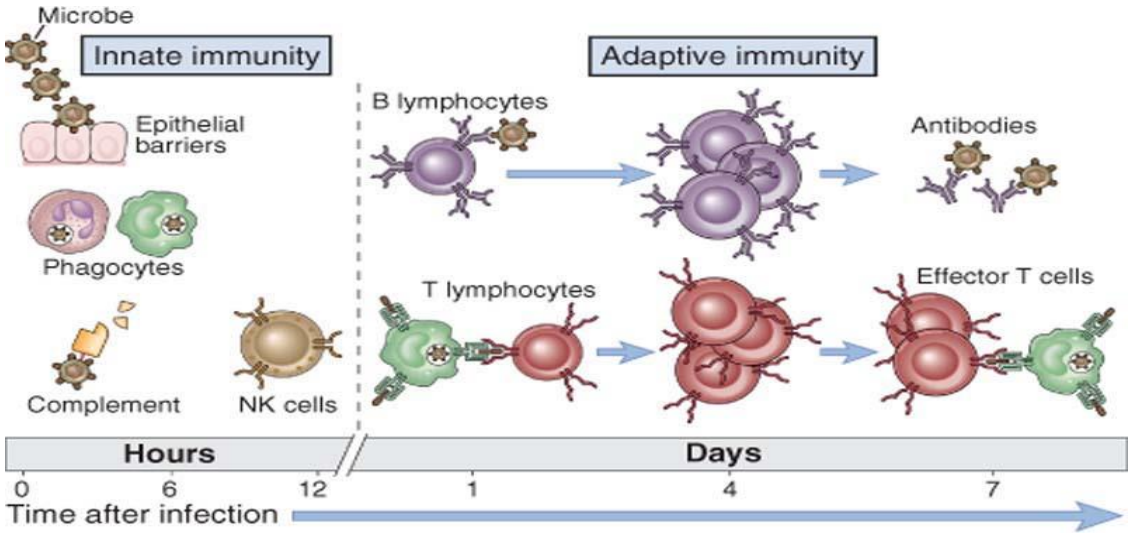

Figure 1.1 The differences between innate and adaptive immune response as illustrated by Abbas et al. (2010).

1.1.1.1. The Complement System

The complement system provides the most fundamental response of innate immunity to eliminate microbial pathogens either by providing protection as a critical component of innate immunity or acting as an effector system of antibody-mediated immunity (Fujita, 2002). The bacteriolytic activity of serum was first described more than 120 years ago by Jules Bordet and subsequent work uncovered the plasma or serum proteins that compose the complement system (Kaufmann et al., 2011). The terminology of 'complement' was coined by the discovery that this bacteriolytic activity of serum is lost through heat inactivation (incubating serum at 56°C for 1 hour), but can be recovered by adding back ("complement") a heat-sensitive component from fresh serum as described by Jules Bordet (Kaufmann, 2008). It was in fact Paul Ehrlich who suggested the name COMPLEMENT because its activity could be restored by adding back the heat-sensitive components to restore serum lytic activity in heat-inactivated serum (Kaufmann, 2008).

The complement system is composed of more than 32 components, both fluid phase and cell surface associated proteins, circulating either in blood plasma or acting as membrane associated receptors or regulators on cell membranes (both positive and negative regulators of activation), respectively, all of which are involved in the regulation and control of the complement system (Makrides, 1998). The activation of the complement system includes a cascade of enzymatic initiation steps, and is carefully controlled by a system of positive and negative regulators that mediate a physiological state of homoeostasis (Parker, 1992, Liszewski et al., 1996, Whaley and Schwaeble, 1997).

The soluble plasma resident complement components are often zymogens present in their pro-enzymatic form. Complement activation occurs as a carefully regulated cascade of events during which complement zymogens are converted into their enzymatically active form often forming multi-molecular enzyme complexes that subsequently cleave and activate other molecules within the system in a clearly defined order of events (Walport, 2001).

There are three activation pathways responsible for complement activation: the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP) (Fujita, 2002, Schwaeble et al., 2002, Thiel, 2007). Despite the differentiation of compositions and the different mechanisms of triggering activation of each pathway, all the pathways channel

into the activation of the third component of complement, C3 through C3 convertase complexes. C3 activation subsequently shifts the activity of the C3 convertase complexes to cleave C5, the 5th component of complement. With the cleavage of C5, the terminal activation cascade of complement is initiated where the C5 cleavage product C5b associates with C6, C7 and C8. C8 inserts the C5bC6C7C8 complex into the membrane of target cells and initiates the formation of the membrane attack complex (MAC) by acting as an anchor for the insertion of C9 polymers into the membrane of target cells to form a pore into the target cell membrane that can initiate the lysis of the target cell through osmolytic activity. (Figure 1.2). In addition during complement activation, small activation/cleavage products such as C3a and C5a act as potent anaphylatoxins by binding to the surface of specific C3a and C5a receptors that can trigger chemotaxis of leukocytes, degranulation of mast cells, neutrophils and basophils, relax smooth muscle contractions and raise vascular permeability (Kirschfink and Mollnes, 2003). Following the activation of C5, C5b anchors into the targeted surface to initiate the formation of C5b-9, the terminal complement complex (TCC) or the MAC resulting in bacterial lysis (Stover et al., 1999, Schneider et al., 2006). As a result of the activation of complement components, the inflammatory response is raised continuously resulting in the generation of toxic oxygen radicals and the secretion of arachidonic acid and cytokines (Kirschfink and Mollnes, 2003).

On the other hand, it is believed that the complement system and the haemostasis/thrombosis system converge at some points resulting in a physiological cross-talk. Arachidonic acid has long been established as a potent agonist to trigger platelet activation (Saggu et al., 2013) releasing TSP1 from intracellular stores, to further amplify the activation of platelets leading to the formation of thrombi.

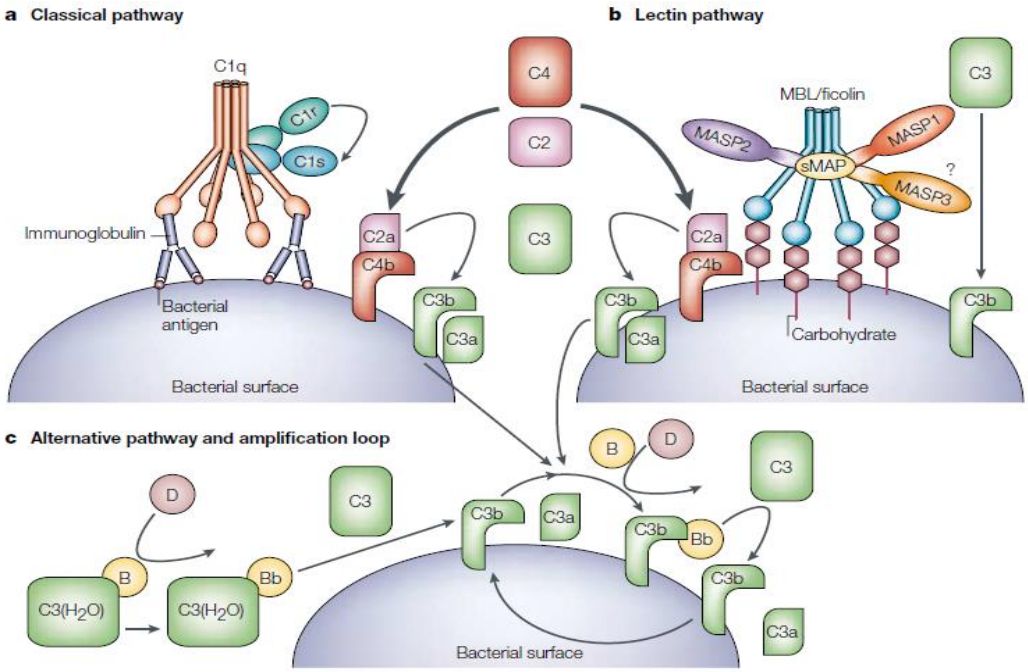

Figure 1.2 A simplified diagram of the three complement pathways: the classical (CP), the lectin (LP) and the alternative pathway (AP) as illustrated by Abbas et al. (2007).

1.1.1.1.1. Classical Pathway (CP)

The activation of classical pathway (CP) depends on the binding of the multi-molecular C1 complex (composed of the multimeric recognition subcomponent C1q which forms a functional complex with the hetero-tetrameric serine protease complex C1s-C1r-C1r-C1s) to either bacterial surface components, or to the FC region of IgG/IgM antibody-antigen complex (Boes et al., 1998, Yuste et al., 2006). The critical role of the classical pathway activation complex C1 in both the innate and the adaptive immune response has been established many decades ago (Arlaud et al., 2002). The molecular weight of the total C1 complex is approximately 790kDa. The C1 complex is composed of the following components: the recognition molecule C1q, and two pro-enzymatic zymogens C1r and C1s, which form a hetero-tetrameric complex -C1s:C1r:C1r:C1s- that sits within the hexameric, bunch of tulip shaped macromolecule C1q (Cooper, 1985). The recognition subcomponent C1q is composed of six identical subunits, each formed of three different polypeptide chains C1qA, C1qB, C1qC. The six C1q subunits are held together via the collagenous stalk (Figure 1.3) (Arlaud et al., 2002). Once C1q binds to

complement activators, auto-activation occurs within the pro-enzymatic zymogens C1s-C1r-C1r-C1s induced by a conformational change which occurs for the collagenous stalk of C1q. The serial auto-activation of CP serine proteases first occurs to C1r, which activates C1s by which the complement cascade is triggered through firstly cleaving C4 by C1s into C4b and C4a. C4b covalently binds to the bacterial surfaces through its thi-oester group (Gadjeva et al., 1998), leading to bind C2, whereas C4a is an unbound fragment which functions as an anaphylatoxin promoting the response of leukocytes to the infected site (Wallis et al., 2007). After binding C2 to C4b on the bacterial surface, C1s cleaves C2 into C2a and C2b. C2a is the important part which binds to C4b, forming the complex of C3 convertase C4bC2a of CP. The C3 convertase cleaves C3 into C3a and C3b. Similar to C4a, C3a is an anaphylatoxin which acts as a powerful chemotaxic agent while the larger cleavage fragment C3b is anchored into the targeted surfaces by acting as an opsonin molecule on the surface of pathogens to be engulfed by phagocytes through binding of C3b and iC3b to phagocyte resident C3 receptors (Figure 1.2). Once C3b binds in close proximity to the C3 convertase (C4bC2a), this C3 convertase complex shifts its specificity from cleaving C3 to cleaving C5, i.e. develops C5 convertase activity and activates C5 to release the potent anaphylatoxic cleavage product C5a and the larger fragment C5b. The latest fragment (C5b) initiates the formation of the Membrane Attack Complex by binding to the terminal pathway components C6, C7 and C8 which anchors into the pathogen surfaces, resulting in the formation and insertion of a poly-C9 pore into the target membrane and induction of osmolysis of the bacterial pathogen (Vorup-Jensen et al., 2000, Arlaud et al., 2002).

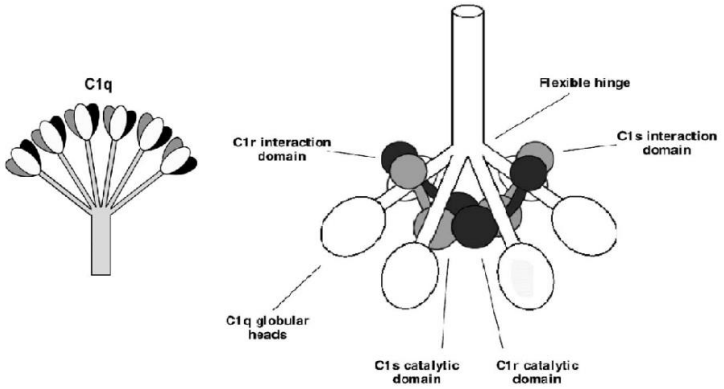

Figure 1.3 Structure of the C1 complex containing C1q and the serine proteases C1r and C1s of the CP of the complement system. It shows three globular heads representing three polypeptides for each subunit of C1q. It also shows homo-dimers of C1r and C1s intercalate with the C1q molecule. Adapted from (Arlaud et al., 2002).

1.1.1.1.2. Lectin Pathway (LP)

Lectin pathway (LP) is involved in innate immune defence against pathogenic microorganisms via an antibody-independent mechanism (Turner, 1996). The LP is composed of several different multimeric pattern recognition molecules that recognise and bind to PAMPs (mostly microbial polysaccharides/carbohydrates on bacterial pathogen surfaces or acetylated surface proteins on oxygen-deprived or apoptotic cells). These recognition molecules differ in binding affinity and selectivity toward the initiator activation. Mannose-binding lectin (MBL), ficolins and CL-11 are all involved in the recognition process of LP activation by which complement cascade is triggered by MBL-associated serine proteases (MASPs) and mainly by MASP-2 (Gadjeva et al., 2001, Matsushita and Fujita, 2002, Schwaeble et al., 2002, Schwaeble et al., 2011), while MASP-1 and MASP-3 were recently shown to play a role in the activation of the alternative pathway (AP). The LP activation is initiated through binding the pattern recognition molecules to microbial carbohydrate residue pattern which brings these activation complexes together in close proximity to each other which facilitates the cleavage of MASPs loaded on these complexes as homo- or hetero-dimers. MASP-2 has a critical role in the activation of the LP since only MASP-2 can cleave complement C4, an activity that is critical for the formation of the LP C3 convertase (C4bC2a). Since MASP-1 can only cleave C2, but not C4, MASP-2 deficient mice are lectin pathway deficient (Schwaeble et al., 2011). Moreover, MASP-2 truncates the C4-bound C2 resulting in the formation of C3 convertase complex of LP (C4bC2a) (Figure 1.6). The activation cascade of LP leads to activation of the terminal complement cascade by initiating the C5 convertase (C4bC2a(C3b)n), as the same complex as the CP C3 or C5 convertase complexes (Thiel et al., 2000, Schwaeble et al., 2002).

1.1.1.1.2.1 The Complexes of the Lectin Pathway

1.1.1.1.2.1.1. MBL, CL-11 & Ficolins

The pattern recognition molecules and their subcomponents of LP are Mannan-Binding Lectin (MBL) and ficolins, and the MBL-associated serine proteinases MASP-1, MASP-2 and MASP-3 and also include non-enzymatic 19kDa protein (known as Map19 or sMAP) (Stover et al., 1999, Takahashi et al., 1999).

MBL and CL-11 are members of the collectin family, characterised by a N-terminal collagen like domain and a C-terminal C-type lectin domain that mediates carbohydrate binding in a Ca^{+2} dependent manner (Brouwer et al., 2008, Hansen et al., 2010).

The normal concentrations of MBL in plasma vary between healthy volunteers from 0.1-10μg/ml, while the level can be raised up about threefold more than the normal concentration in case of infection response (Brouwer et al., 2006, Frakking et al., 2007, Pagowska-Klimek and Cedzynski, 2014). MBL is formed in multiple forms of higher oligomers (trimers, tetramers and hexamers) in plasma (Wallis et al., 2005). Each single monomer of oligomerised MBL consists of three identical polypeptide chains which are composed of N-terminal cysteine-rich region, collagen-like region and neck region followed by carbohydrate recognition domain (CRD) (Figure 1.4).

The derivatives of carbohydrates mannose and N-acetyl-glucosamine (GlcNAc) assist as ligands for MBL, while other derivatives cannot bind or have low affinity for MBL. Whereas, it has been shown that MBL could trigger LP activation through binding to IgM immune complexes (McMullen et al., 2006). However, the role of MBL has been suggested to act as an opsonic molecule by many reports, independent of complement to facilitate direct phagocytosis through leukocytes (Kuhlman et al., 1989, Polotsky et al., 1997, Ogden et al., 2001, Jack et al., 2005). Therefore, the MBL has been proposed for many microorganisms to be an opsono-phagocytic molecule and also may accelerate the phagocytosis process by professional phagocytes (Jack et al., 2005, Jack et al., 2001, Shiratsuchi et al., 2008, van Asbeck et al., 2008). Besides, ficolins are likely to perform the same function of connecting the apoptotic cells and microorganisms with phagocytic cells via collectin receptors (Figure 1.5) (Turner, 2003, Matsushita, 2010).

Despite the fact that in the events of thrombosis/haemostasis where platelets take part in the activation, the activated platelets release chondroitin sulphate (CS), which has been shown to bind to MBL, by which MBL may act as an opsonic molecule to be engulfed by phagocytes.

However, ficolins are part of pattern recognition molecules that bind GlcNAc of carbohydrate residues on the pathogenic surface, similar to MBL (Wallis, 2007). In addition, it binds to gram positive bacteria through lipoteichoic acids (Endo et al., 2005, Lynch et al., 2004). There are two types of human ficolins; L-ficolin and H-ficolin. The acetylated sugar represented on the pathogenic surfaces is carbohydrate residual ligand for

ficolins that initiate LP activation. In addition to ficolins types, M-ficolin is found in humans where is precisely associated with the neutrophil and monocyte cell wall, which presumably functions in phagocytosis as phagocytic receptor (Matsushita and Fujita, 2001). Like MBL, ficolins are high oligomer forms which consist of multimers of homo-trimer subunits. Each subunit consists of three identical polypeptide chains that contain N-terminal cysteine-rich region, collagen-like domain and neck region followed by fibrinogen-like carbohydrate recognition domain (FBG). The three polypeptide chains are connected together through the collagen-like domain (Figure 1.4). However, MASPs associate with both ficolins and MBL via collagen-like domain (Roos et al., 2001).

A new collectin has been discovered recently, called collectin-11 (CL-11) which was found in circulated plasma with association of both MASP-1 and MASP-3 (Keshi et al., 2006). It was also found to attach to D-mannose and L-fucose on different microbial surfaces, thereby, it may be involved with pattern recognition molecules of LP (Hansen et al., 2010).

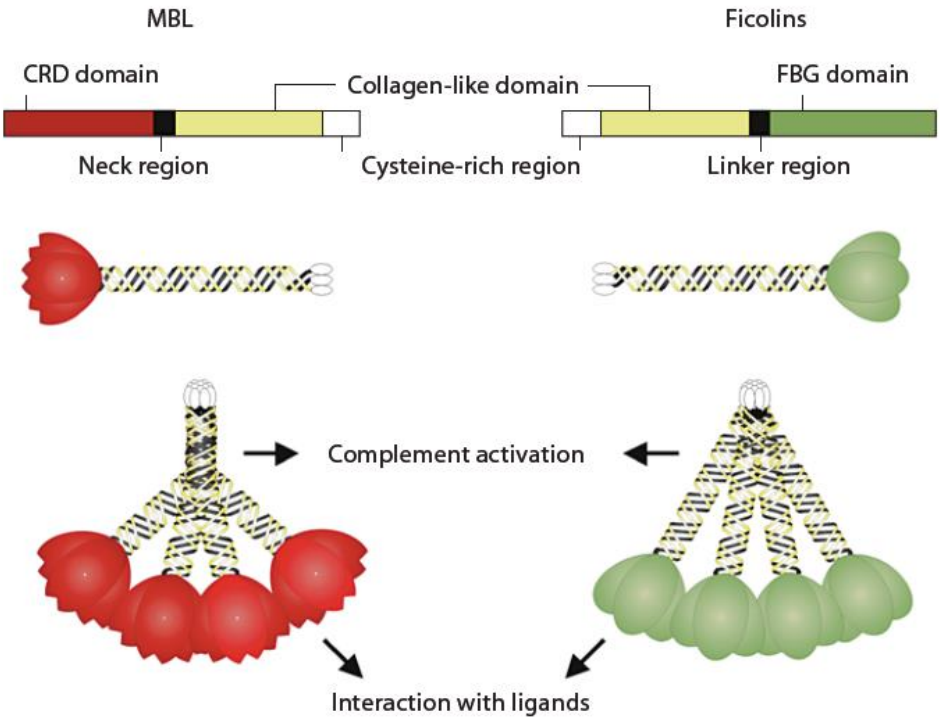

Figure 1.4 The oligomer molecules and domain structures of MBL and ficolins (Garred et al., 2010).

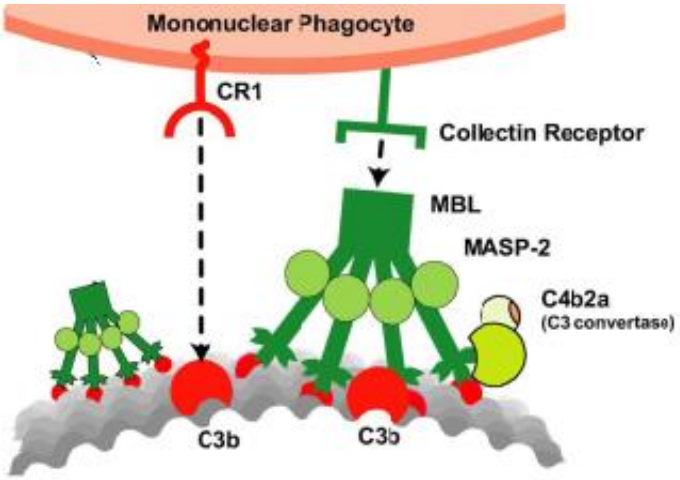

Figure 1.5 The independent complement function of MBL through enhancing the killing of targeted surfaces by mononuclear phagocyte cells via collectin receptor and also dependently through CR1 receptor for C3b deposited molecules of the LP (Turner, 2003).

1.1.1.1.2.1.2. MBL-associated Serine Proteases (MASPs)

The serine proteases form complexes with ficolins and MBL. The associated serine proteases are MASP-1, MASP-2 and MASP-3 (Stover et al., 2003). Moreover, there is also another protein synthesized by alternative splicing of the *MASP-2* gene. The former protein is associated with MBL and ficolins, the same as MASPs. The alternative splicing of the gene *MASP-2* formed a truncated fragment known as Map19 (MBL-associated plasma protein of 19kDa) or sMAp19 (small MBL-associated protein) (Stover et al., 1999, Matsushita et al., 2000, Thielens et al., 2001).

MASP-1 was explored by Teizo Fujita's group (2002) who named it MASP, followed by discovering MASP-2. Therefore, the first MASP was named MASP-1, as a result of the first discovery being followed by MASP-2 and then MASP-3 (Fujita, 2002, Hajela et al., 2002). Unlike the serine proteases of CP, MASP-2 is able to trigger the complement activation of LP without need for MASP-1 and MASP-3 (Thiel et al., 1997, Vorup-Jensen et al., 2000, Rossi et al., 2001, Schwaeble et al., 2011, Ali et al., 2012). MASP-3 has been found to be an alternative splicing from MASP-1 (Dahl et al., 2001).

The proteolytic process of MASP-2 to C4 allows the C4b to bind to the targeted surfaces and bind to C2 which will be truncated by MASP-1, while MASP-3 seems to have no proteolytic action toward C4 and C2 (Figure 1.6) (Zundel et al., 2004).

However, MASP-1 has recently been shown to convert the pro-enzymatic form factor D of AP into an active form to be, then, able to cleave C3-bound factor B into Bb and Ba (Takahashi et al., 2010). Whereas, MASP-3 has recently been shown to initiate the activation of AP through cleaving C3b-bound factor B on pathogenic surface that are incubated with recombinant MASP-3 which then formed a complex with recombinant MBL *in vitro* (Figure 1.6) (Iwaki et al., 2011).

On the other hand, it has been observed that MASP-1 possesses thrombin-like activity, and interferes with other substrates in the coagulation cascade by cleaving factor XIII (fibrin stabilizing factor) as well as fibrinogen (Fg) *in vitro* (Hajela et al., 2002, Zundel et al., 2004). As a result of Fg cleavage process by MASP-1, the fibrinopeptide B would act as a chemotactic factor on neutrophils, fibroblasts and monocytes surfaces (Hajela et al., 2002). Consequently, the inflammatory cells are likely engaged in the process of haemostasis/thrombosis indirectly by MASP-1 on the surface of activated platelets,

where MBL and other recognition molecules of LP might associate and interfere for the activation process.

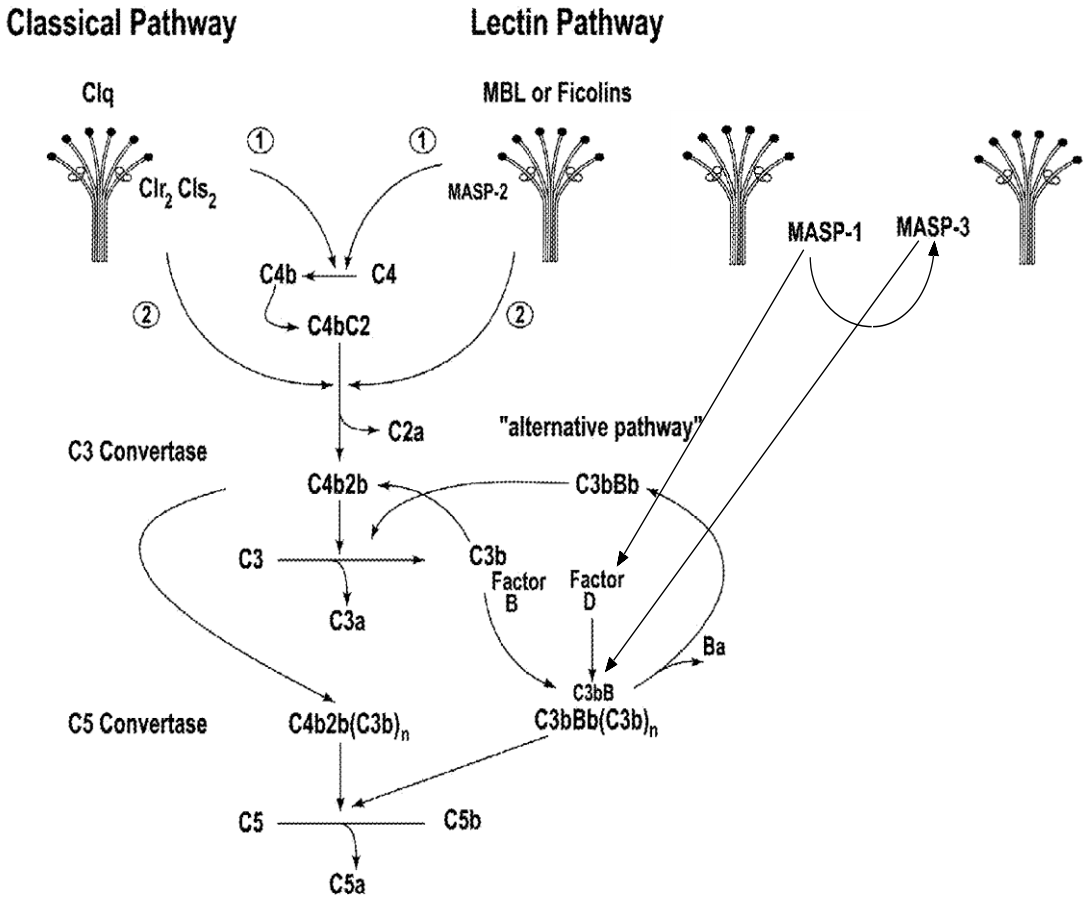

Figure 1.6 A simplified diagram of the Lectin pathway activation complexes and also the initiation of alternative pathway activation. (Kindly provided by Professor W Schwaeble, University of Leicester, UK.) (Demopulos et al., 2015).

1.1.1.1.3. Alternative Pathway (AP)

The alternative pathway (AP) was first described in the 1950s and is considered to be the phylogenetically old complement activation pathway. The AP is composed of the following components: Complement C3, Factor B (fB), Factor D (fD) and Properdin (Matsumoto et al., 1997). The activation of the AP is independent of immunoglobulins and pattern recognition molecules which initiate activation of the other complement pathways (Walsh et al., 2005). The initiation of the AP starts slowly and spontaneously, as stated in current textbooks, through the hydrolysis of C3 into C3(H_2O) (Roitt and Delves, 2001, Abbas et al., 2010). A recently published hypothesis that postulated that properdin could act as a pattern recognition molecule of AP, is based on the observation that properdin deposition can be detected on the surface of certain microbes and on N-acetylated structures like GlcNAc as well as on the surface apoptotic cells and necrotic cells. This hypothesis postulated that properdin may initiate the formation of the C3/C5 convertase of AP (Kemper et al., 2010). The recent discovery, however, showing that properdin fails to bind to microbial surfaces in absence of complement C3 casts some doubt over the validity of this hypothesis, since the failure of properdin to bind to microbial surfaces in C3 deficient serum implies that properdin is more likely to bind to already formed C3/C5 convertase complexes attached to the AP activating surface via the covalent bond of the reactive site generated by C3 cleavage (Harboe et al., 2012). Therefore, the issue of what initiates AP activation is still unresolved. The continuous cleavage process of C3 molecule of AP is called a tick-over mechanism that generates C3b (Quin et al., 2005, Frank, 2010). The hydrolysed C3(H_2O) binds fB forming a zymogen complex (C3(H_2O)B) and then initiates the formation of C3 convertase of AP (C3(H_2O)Bb) by cleaving C3-bound fB into Ba and Bb by fD. MASP-1 was recently shown to convert pro-fD into its enzymatically active form (Takahashi et al., 2010). Once the C3 convertase of the AP is formed, the further cleavage of C3 releases C3b that initiates the formation of further C3 convertases resulting in a highly efficient amplification loop of complement activation (Figure 1.7) (Schwaeble and Reid, 1999b). The cleavage product of C3b either attaches to the surface of bacterial pathogens acting as an opsonin to enhance the phagocytosis, or channels into the formation of further C3 convertases by binding to fB (Thurman and Holers, 2006). Subsequently, the C5 convertase of the AP is generated through the binding of multiple C3b molecules in close proximity of the C3 convertase complex (C3bBb or C3(H_2O)Bb) to form the C5 con-

vertase of the AP (i.e. C3bBb(C3b)n also termed (C3b)nBb) (Pettigrew et al., 2009). The major difference between the C3 convertases of the AP and the LP and the CP pathways is that the C3 convertase of the LP and CP is formed of the cleavage products of the fourth (C4) and the second (C2) complement component, resulting in an enzyme complex called C4bC2a. Like the C3 convertase of the AP. the C3 convertase of the LP and CP switches its substrate specificity from C3 to C5 upon binding of multiple C3b molecules in close proximity to form the LP and CP C5 convertase complex C4bC2a(C3b)n (Frank, 2010).

Properdin is an important regulatory component of the AP. Properdin acts as a positive regulator of the AP by stabilising the C3 convertase and C5 convertase complexes on activating surfaces forming C3bBbP and C3bBbP(C3b)n (Schwaeble and Reid, 1999b, Kemper and Hourcade, 2008). Properdin is produced by T cells and mononuclear phagocytes, and its expression can be modified by cytokines (Wirthmueller et al., 1997). Upon C5 cleavage the released C5a activation fragment is released into the fluid phase, and acts as a potent chemoattractant for neutrophils which bear C5a receptors on their surface and release properdin by degranulation from the secretory granules (Figure 1.7) (Schwaeble and Reid, 1999b).

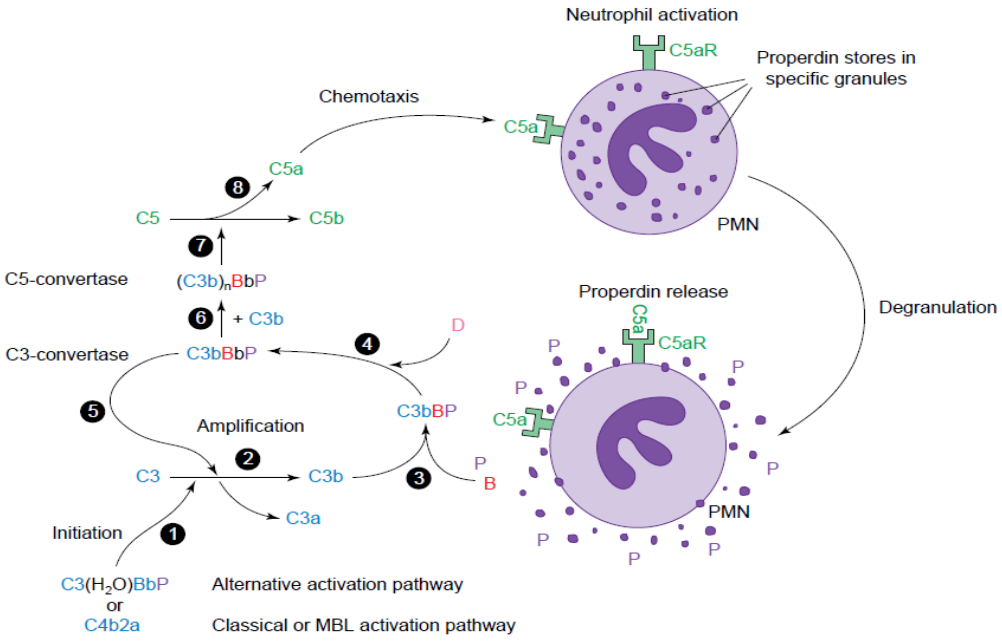

Figure 1.7 A simplified diagram representing the amplification loop of the AP by C3/C5 convertase formation, and properdin release by polymorphonuclear cells (PMNs) via C5a (Schwaeble and Reid, 1999b).

1.1.1.1.3.1. Properdin

Human properdin gene is comprised of 10 exons, positioned on the short arm of human X-chromosome from Xp11.23-Xp11.3 (Coleman et al., 1991, Nolan et al., 1992, Nolan and Reid, 1993).

The normal concentration of properdin in circulating plasma varies from 4-25µg/ml (Nolan and Reid, 1993). Properdin is found in plasma as a homo-oligomer, mainly dimers, trimers and tetramers, in a natural dispersal ratio of 20:54:26, monomers are not found in serum (Smith et al., 1984, Schwaeble and Reid, 1999b). It has been reported that the complement activation consumed first of all the properdin tetrameric form and then the properdin trimeric form and last, the properdin dimeric form, by which properdin forms have sizes consistent with their specific activities (Pangburn, 1989). It has been established that the tetrameric form is ten times more active than the dimeric form with respect to binding to C3b and fB, and also protects the convertase complex from the action of negative regulatory protein factor I by blocking the access of the essential factor I cofactors factor H, MCP and C4bp and decay accelerating factor DAF (Fearon and Austen, 1975, Medicus et al., 1976, DiScipio, 1981, Farries et al., 1988a, Pangburn, 1989, Schwaeble and Reid, 1999b).

It has been determined that the binding site for properdin on C3 molecules is between the amino acid residues 1402-1435 that localise in the α-chain (Daoudaki et al., 1988) by overlapping studies using peptide inhibition experiments (Grossberger et al., 1989, Becherer et al., 1990). Furthermore, the interaction of Properdin with fB (DiScipio, 1981) has been found to be likely to be located on both Ba and Bb (Farries et al., 1988a).

Properdin has been characterised by Higgins et al. (1995) by generating mutant recombinant forms deficient of individual thrombospondin type-I like repeats (TSRs). This work has shown that the lack of TSR5 abolishes the ability of properdin to bind C3b and sulfatides, while the absence of TSR4 does not prevent the binding to C3b or sulfatide, but prevents the stabilization of the C3 convertase (C3bBb) complex. In absence of TSR3 mutagenised recombinant properdin is still able to stabilise the complex and allow the binding to C3b and sulfatide as well as the formation of properdin oligomers (dimers, trimers and tetramers). Interestingly, the lack of TSR6 resulted in the loss of oligomerisation (Higgins et al., 1995). The N-linked carbohydrates of properdin are not

required for polymerization or complex stabilization (Farries and Atkinson, 1989, Higgins et al., 1995). Therefore, Higgins's results have concluded the importance of TSR5 in binding to C3b and sulfatide, and also the importance of TSR4 in the complex stabilization (Figure 1.8) (Higgins et al., 1995).

However, there are other, higher, oligomer forms of properdin, like pentamers and hexamers, etc., but some researchers believe that the oligomer forms are artifacts and formed during the purification process or thawing repeating cycles due to presuming properdin contain amorphous aggregates (Farries et al., 1988a, Farries et al., 1987), while other researchers suggest that the multiple and higher forms exist in plasma in inter-convertible native and activated forms (Gotze and Muller-Eberhard, 1974, Chapitis and Lepow, 1976, Medicus et al., 1976, Medicus et al., 1980, Smith et al., 1984) especially, or even during, inflammation at the infected sites (activation condition) (Ruddy and Purkall, 1987).

However, it has been shown that by observing a weak interaction of native properdin with cell-bound C3b in physiological ionic strength condition, the interaction was significantly raised in the contribution of fB by five-folds increase with the complex cell-bound C3bBb (Farries et al., 1988b, Farries et al., 1988a). Native properdin fails to interact with C3 or C3i in the fluid-phase, whereas it shows weak interaction to C3 derivatives; C3b, iC3b and C3c, in fluid-phase at low ionic strength. The constant C3 levels in serum take advantage of the incapability of native properdin to consume C3 in serum without using activator surfaces (Farries et al., 1988a, Gotze et al., 1977).

Despite the absence of properdin monomers in plasma, monomers can be generated under low pH, i.e. acidic conditions. Therefore, it has been suggested that for the polymerisation is dependant on ionic conditions (Smith et al., 1984, Pangburn, 1989). After treatment under acidic conditions, the monomeric forms were permitted to reform into oligomers again a process that again achieved the very same ratio between dimers, trimers and tetramers (26 : 54 : 20) found in serum (Smith et al., 1984). It has therefore been suggested that the nature of forming oligomers is likely to occur naturally after biosynthesis (Nolan and Reid, 1993).

Properdin presents as a mixture of cyclic polymers and oligomers by head-to-tail interactions of a single-chain glycoprotein. The properdin molecule consists of N-terminal region followed by six distinct tandemly repeated motifs related to Type-I repeat (TSR) with a length of approximately 60 amino acids, which were identified in thrombospon-

din (TSP), followed by C-terminal region with a molecular weight of approximately 53kDa (Figure 1.8) (Lawler and Hynes, 1986, Goundis and Reid, 1988, Goundis et al., 1988, Schwaeble and Reid, 1999a).

Precisely, the molecular weight of human properdin monomeric un-glycosylated form is 48,949 Da, referring to 442 amino acid residues of polypeptide chains which was calculated from the cDNA-derived amino acid sequence (Nolan et al., 1991). By presuming the total carbohydrates within the monomeric form consist of 9.8% (w/w) which is composed of 3.8% hexose, 3.8% sialic acid, 1.5% hexosamine and 0.8% fucose (Minta and Lepow, 1974), hence, the estimated molecular weight of the human properdin monomeric glycosylated form is 53,246 Da (Nolan and Reid, 1990).

The degree of sequence identity between the TSR motifs of properdin and TSP1 overall is in the region of approximately 47% (Goundis and Reid, 1988). This suggests that TSP1 may potentially interact with components of the complement system, while properdin may fulfil so far unknown roles during platelet activation, respectively. For instance, the homology of TSR repeats on both TSP1 and properdin with regard to amino sequence, for example, CSVTCG which is responsible for platelet activation in TSP1, and subsequently leads to haemostasis/thrombosis activation events (Bonnefoy et al., 2008). Moreover, the similarity of TSR motifs are presented in the terminal complement components C6, C7, C8 and C9 (DiScipio and Hugli, 1989, Stanley et al., 1985) and also shows similarity with malaria parasite protein TRAP (Goundis and Reid, 1988).

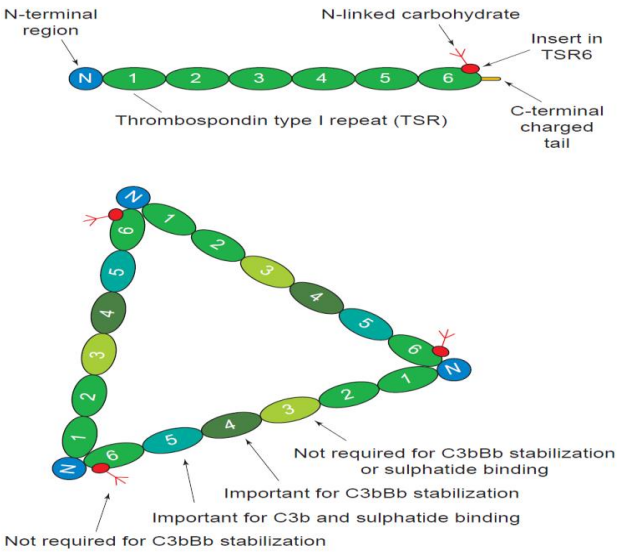

Figure 1.8 A model of Properdin monomer and trimer cyclic forms. Adapted from (Schwaeble and Reid, 1999a).

1.1.1.1.4. Membrane Attack Complex (MAC)

As a result of C3 convertase formation by all three complement pathways either forming C4bC2a or C3bBb, the C3 convertases would shift the substrate specificity of cleaving C3 molecule into C5 substrate by cleaving C5 via attaching another C3 into the C3 convertases. Therefore, convertases would become C4bC2a(C3b)n for CP and LP, or C3bBb(C3)n for AP. After cleaving C5 into C5b and C5a, C5b anchors onto the targeted surfaces and enhances the terminal pathway components C6, C7 and C8 to bind and attach with C5b on the targeted surfaces in order to form the C5b-C6C7C8 complex of the terminal pathway. Consequently, the complex serves in the polymerisation of 10-16 molecules of C9 anchored into the lipid bilayer of cell targeted surfaces either bacterial surfaces (Podack et al., 1982), or activated platelets through the phospholipids (PS) between the two leaflets of the platelet bilayer exposed during activation into the surface (Lhermusier et al., 2011).

The process of the polymerisation of C9 molecules initiates the formation of a hole structure called MAC (Figure 1.9) leading to the membrane to lose critical functions by permitting water to influx into the cell, which lastly leads to cell lysis (Podack et al., 1982).

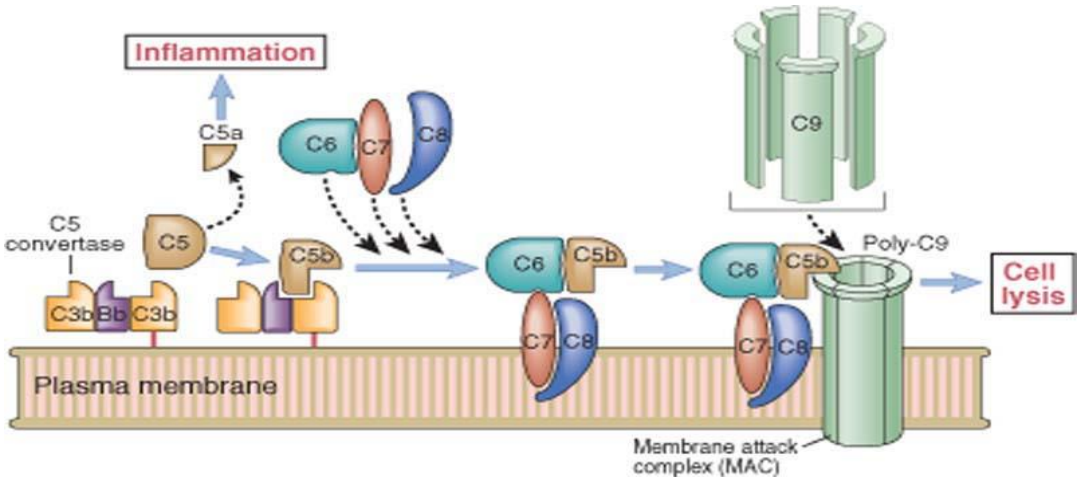

Figure 1.9 A simplified diagram of the terminal pathway of the complement system forming the membrane attack complex (MAC) as illustrated by Abbas et al. (2007).

1.1.1.1.5. The Biological Functions of the Complement System

There are many biological activities involved by triggering complement activation, for instance: enhance triggering pro-inflammatory response, bacterial opsonisation, and also play a role in lysing bacteria via MAC formation (Walport, 2001). The protection from invader pathogens or even eliminating apoptotic cells occurs through three major mechanisms;

i. Opsonisation assists the uptake of pathogens or apoptotic cells by phagocytic cells. The process of opsonisation is mediated by C3b or iC3b, which is an inactive derivative product of C3b cleavage, and C4b, but is considered a lesser opsonic molecule (Mevorach et al., 1998). Phagocytosis occurs through firstly binding C3b onto the targeted surfaces, then leading to stimulate leukocytes for engulfing process via linking the targeted cells to leukocytes through complement-receptor-type-1 (CR1), type-3 (CR3) and type-4 (CR4) (Aoyagi et al., 2005). Moreover, MBL and ficolins are likely to stimulate the uptake process through collectin receptors (Figure 1.5) (Jack et al., 2001). In addition, C1q attaches to immune complex on apoptotic cells mediating cellular clearance by macrophages (Taylor et al., 2000). Erythrocytes (RBCs), which expose CR1, assist in immune complexes clearance via attaching C3b on targeted surfaces and by acting as transporters to liver and spleen, where the residential macrophages clear the complexes (Manderson et al., 2004).
ii. Complement chemotaxis anaphylatoxin is one of complement system mediators that drag inflammatory/immune cells to site of the injured/infected area. The small fragments C4a, C3a and C5a act as anaphylatoxins that acts as mediators and stimulators for inflammatory cells to infected/injured sites, through promoting vessels permeability and develop the inflammatory exudates that may help on leukocytes extravasation to the inflammatory site. Anaphylatoxins stimulate mast cells to release histamine by degranulation (Nataf et al., 1999). C5a and other anaphylatoxins induce interleukin-1 (IL-1) and tumour necrosis factor (TNF) (Schindler et al., 1990). C5a and C3a are capable of inducing endothelial cells in expression and production macrophage inflammatory protein-2 (MIP-2) and monocyte chemotactant protein-1 (MCP-1) in mice (Laudes et al., 2002). In addition to the phagocytosis process, monocytes and granulocytes up-regulate CR3 receptor on their surfaces by the induction reaction ability of C5a toward C5a receptor on their surfaces. That would help for phagocytosis by targeting C3b-bound cells (Sprong et al., 2004).
iii. To form MAC for direct killing through making holes on targeted surfaces, leading to water influx and ions through osmotic gradient leading finally to cell lysis.

1.1.1.1.6. Complement System Regulation

The complement system is controlled by self-host cells because excessive amounts of complement activation are considered harmful for living tissues around the complement activation site. Therefore, complement activation is controlled by proteins characterised into two categories; fluid-phase regulatory and membrane-bound regulatory proteins (Kirschfink and Mollnes, 2003).

1.1.1.1.6.1. Complement Fluid-phase Regulators

There are numbers of regulator proteins circulating in plasma. These regulators are C4bp, C1-INH, fH, vitronectin (S-protein), clusterin and SCPN. The function of each one will be defined and illustrated.

The acute phase serum protein C4 binding protein (C4bp) is an essential regulator of the complement system in fluid-phase (Blom et al., 2004). The main role of C4bp is to inhibit C3 convertase formation in both CP and LP by binding to C4b and then prevent C2a binding. Moreover, C4bp regulates C4b into inactive derivatives like iC4b, C4c and C4d, by acting as a cofactor for factor I mediated cleavage of C4b (Jurianz et al., 1999).

C1 inhibitor (C1-INH) is involved in serpin superfamily that inhibits serine proteases. The main role of C1-INH is to form complexes with the serine proteases of the CP; C1r and C1s, to block the spontaneous activation of the pro-enzymes by forming C1-INH-C1r-C1s-C1-INH complex (Harpel and Cooper, 1975). Furthermore, C1-INH was found to erase the complex C1q-C1r-C1s of CP from targeted surfaces leading to prevent further complement activation (Chen and Boackle, 1998, Prada et al., 1998). Moreover, C1-INH negatively regulates LP activity via interaction with MASP-2 (Kerr et al., 2008, Matsushita et al., 2000, Petersen et al., 2000).

Factor H (fH) is one of the soluble plasma regulators that regulate AP and is also found in all body fluids (Zipfel et al., 1999). FH binds to C3b subsequently to prevent the formation of C3 and C5 convertases of AP (Pangburn et al., 2000). Moreover, fH acts to prevent C3 convertase in two ways; first of all, it speeds up the decay of C3 convertase

by holding C3b and erasing it from the complex, and the second way is that by acting as a cofactor for factor I mediated cleavage of C3b into derivatives (iC3b, C3c and C3dg) (Turnberg and Botto, 2003).

Vitronectin (S-protein) and clusterin are the regulators targeting the terminal pathway activation. Vitronectin is an extracellular matrix protein and also an adhesive glycoprotein present in plasma and in the sub-endothelial layer (extracellular matrix) (Asch and Podack, 1990). Both the inhibitors (Vitronectin and clusterin) function to prevent the MAC formation by binding to C5b-C7 complex and subsequently leading to block the interaction of C8 and C9 in host cell membrane (Jenne and Tschopp, 1989) (Figure 1.10).

1.1.1.1.6.2. Complement Membrane-bound Regulators

In addition to complement regulators, host-cells including platelets can control complement activity on their surfaces by membrane-bound receptors or regulator proteins. Complement receptors (CRs), protectin (CD59), membrane co-factor of factor I mediated proteolysis such as membrane cofactor protein (MCP) or CD46, and decay accelerating factor (DAF) are examples of complement membrane-bound regulators.

There are four classes of complement receptors that all bind to C3b. These receptors called CR1 (CD35), CR2 (CD21), CR3 (CD11b/CD18) and CR4 (CD11c/CD18) (Leslie and Hansen, 2001). Many cells like erythrocytes and lymphocytes express CR1. CR1 binds to C3b and C4b of complement components leading to prevent C3 convertase formation of AP, LP and CP by acting as a cofactor for factor I-mediated degradation, while, CR2, is expressed on most human cells like epithelial cells, T-cells, B-cells and also platelets, binds to C3b and C3b derivatives including hydrolysed C3(H_2O). C4b and C3b are degraded into inactive derivatives like C4c and C4d, and like iC3b, C3c and C3dg, respectively (Leslie and Hansen, 2001).

CR3 and CR4 are expressed in most inflammatory cells, such as, neutrophils, eosinophils, basophils and monocytes/macrophages, and also both are expressed on platelets. Both regulators prevent the formation of C3 convertase by binding to C3b and iC3b (Leslie and Hansen, 2001).

As a membrane-bound regulator, MCP mediates the degradation of C3b as a co-factor for factor I mediated conversion of C3b and C4b into their haemolytically inactive

cleavage products (Whaley and Schwaeble, 1997). Moreover, DAF is expressed on the surface of erythrocytes, endothelial cells, and inflammatory cells including monocytes, neutrophils and T-cells, and also presented on platelets. DAF promotes the dissociation of C3 and C5 convertases (Medof et al., 1984, Medof et al., 1987). CD59 (protectin) is a membrane-bound regulator for the terminal pathway. CD59 binds to C8 and C9 by forming complex to prevent C9 polymerisation, which in turn leads to disturbing the MAC formation (Rollins et al., 1991). The C1q receptors: cC1qr, C1qRp and gC1qR, are presented on varieties of cell surfaces including platelets. They all bind to C1q components with different binding specificity leading to inhibiting C1 complex formation (C1q-C1r2-C1s2) (van den Berg et al., 1998, Leslie and Hansen, 2001) (Figure 1.10).

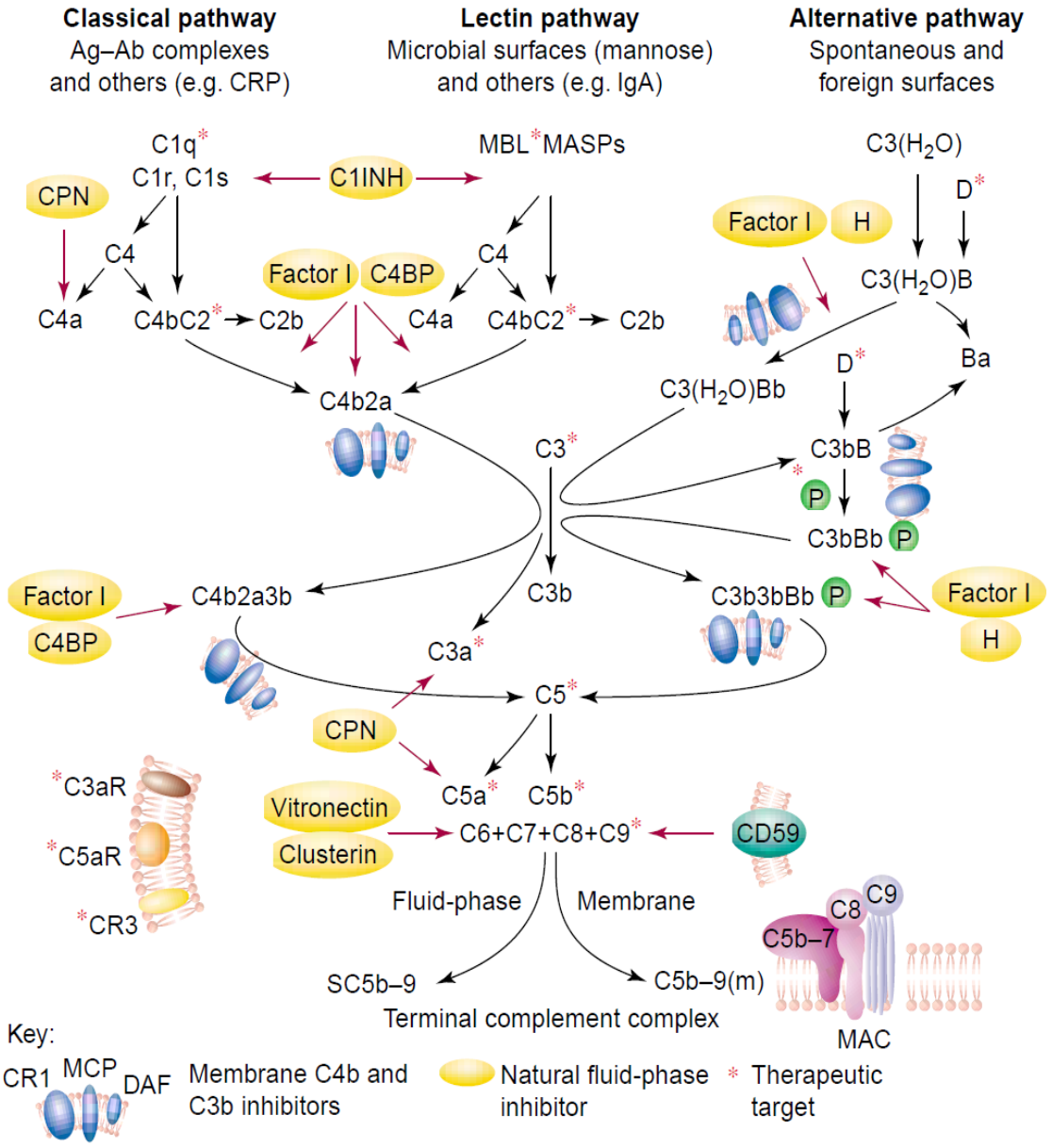

Figure 1.10 A simplified diagram of the complement regulatory proteins including fluid-phase and membrane bound regulating the activation of complement pathways (Mollnes et al., 2002).

1.1.2. An Overview of Haemostasis/Thrombosis

The haemostasis system is a major component of the body's homeostasis composed of several effector mechanisms and pathways: i.e. vascular endothelium, platelets, coagulant proteins, anticoagulant proteins, and fibrinolytic proteins, which coordinate together the limitation of blood-loss after tissue injury by forming platelet plugs which depend on fibrin mesh clot formation (Figure 1.11). The haemostasis/thrombosis system is an essential life support system to protect the vascular system from life-threatening blood loss after vascular injury. The difference between haemostasis and thrombosis is about the ability to retain thrombotic events in the local vicinity of damage (Pasi, 1999). It has long been established that platelets play an essential role in the healing process of vascular injury by forming haemostatic plug in order to stop blood loss (Holmsen, 1989). Nevertheless, the response of platelets at the site of vessel injury is induced by exposing the extracellular matrix proteins (ECMs) from the sub-endothelial layer surface. ECMs not only cause platelet adherence but also adhere onto artificial surfaces, such as catheters, artificial heart valves, vascular grafts, haemodialysis membranes and oxygenators, leading to platelet adhesion (Rao, 1999, Feuerstein and Sheppard, 1993). The ECMs also indirectly promote the wound-healing process through platelets by inducing the release of chemokines and growth factors from platelets granules (Blair and Flaumenhaft, 2009). Thrombospondin-1 (TSP1) is one of the ECMs. TSP1 is expressed in the sub-endothelial layer and secreted by platelets. It has been well established that the interaction of TSP1 with circulating platelets is through the TSR repeats domain and also via the N- and C-terminal domains. The TSR domains of properdin have been first described by Goundis and Reid (1988) and were shown to share about 47% of structural similarity in identical amino acid positions with the domains of TSP1 (Goundis and Reid, 1988). Therefore the purpose of my project was to characterise whether and to what extent these striking similarities between properdin and TSP1 are reflected by functional similarities between these key components of the complement and the haemostasis system.

As much as I am curious to what extent TSP1 may have a regulatory activity of complement activation, I was curious to see whether or not properdin may express any functional activity on platelet activation.

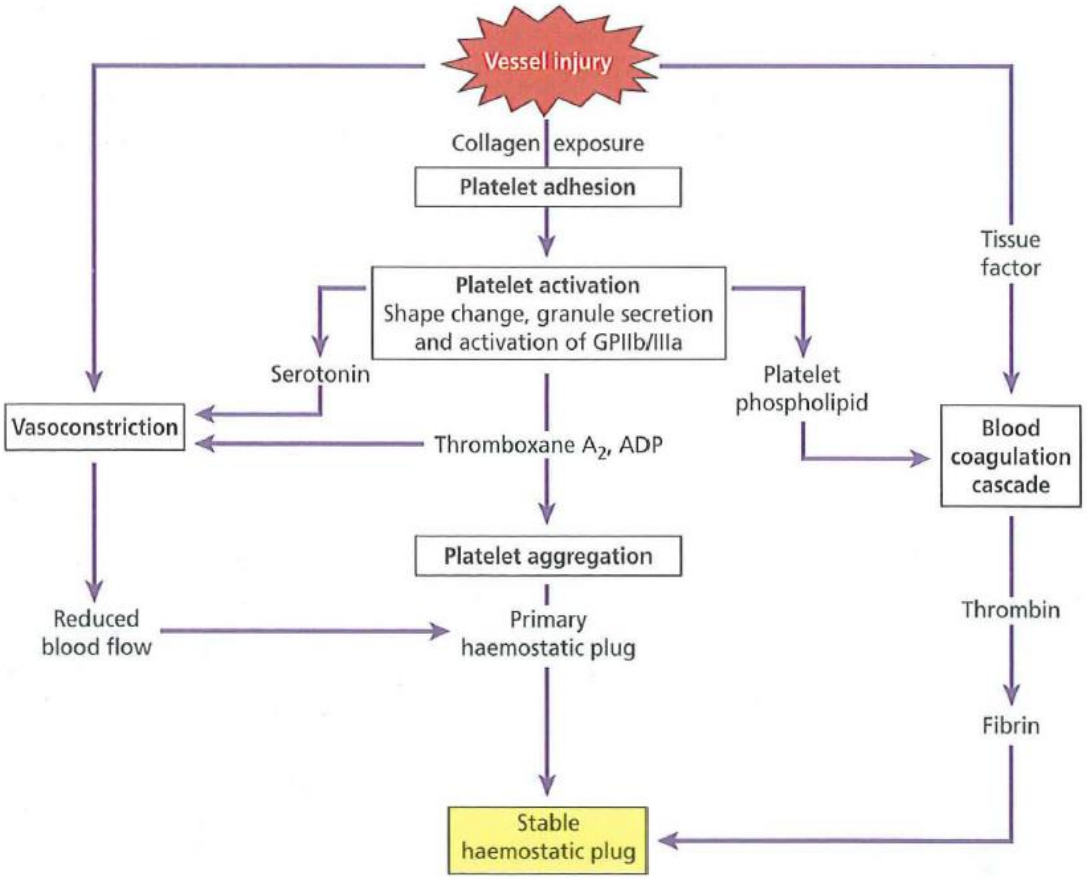

Figure 1.11. A schematic overview shows the strategy of promoting the formation of a haemostatic plug by coordination between many compositions of the haemostasis system (Hoffbrand et al., 2006).

1.1.2.1 Blood Platelets

In 1780, undefined blood particles were first described by Hewson, and later described in more detail in several reports in the middle of 19th century (Gazzaniga and Ottini, 2001). In 1865, the blood platelets were first described morphologically, while in 1882, it was shown that platelets are involved in haemostasis (Ribatti and Crivellato, 2007, Brewer, 2006).

1.1.2.1.1 Platelets Biology, Structure and Function

The residential megakaryocytes in bone marrow release platelets to the bloodstream mostly by fragmenting their cytoplasm containing platelet-alpha and dense granules, membrane glycoproteins, and lysosomes which all are surrounded by cell wall shed from the mother cell (Pasi, 1999, George, 2000). The production of platelets is also initiated in the bloodstream and the lungs as long as the mother cell migrates from bone marrow to other tissues (Behnke and Forer, 1998, Italiano and Hartwig, 2007). However, the precise mechanism of producing platelets is not understood (Italiano and Hartwig, 2007). It has been proposed that platelet production occurs under the tight control of thrombopoietin (Kaushansky, 1999). However, it has been recently shown that driving human megakaryocytes to high shear rates subsequently leads to accelerated platelet production by which possibly the presence of megakaryocytes in blood microvessels is controlled by the force rate of blood circulation (Dunois-Larde et al., 2009).

As derivative from megakaryocytes, platelets comprise messenger RNA (mRNA) and translation machinery for protein production, even though, they lack a proper nucleus with genomic DNA (Healy et al., 2006, Weyrich et al., 2009).

The intact platelet has a discoid shape with a diameter from 2-5μm and a thickness of about 0.5-0.7μm (White, 2007). The lifespan of intact circulating platelets is between 7-10 days in peripheral blood, and then transported later to spleen and liver where the degradation process is initiated (Hoffbrand et al., 2001, Cramer, 2002). Under physiological conditions, number of platelets in daily is approximately $1x10^{11}$ in adults to keep up normal circulating platelets levels in blood balance between $150\text{-}400x10^{9}$/L, whereas, a third of resting platelets are stored in spleen to raise levels of circulating platelets up to more than 10-fold as when thrombotic events demand (Kaushansky, 2009).

As stated previously, circulating platelet plays a crucial role in haemostatic plug formation by adhering into the exposed ECM such as collagen, TSP1 and vWF, following vessel injury, and causing platelet activation within seconds and subsequently forming a primary shield containing adhered and aggregated platelets, to preclude the continuous blood loss, acting as a physical barrier (Jurk and Kehrel, 2005, Jackson, 2007, Varga-Szabo et al., 2008, Eriksson, 2008). That process of platelet adhesion and activation leads to a conversion process for platelet shape by elongation and pseudopod formation through extension of platelet plasma membrane (Figure 1.12) (Fox, 1993). These pseudopods facilitate sub-endothelial surfaces adhesion (George, 2000). As a result of plate-

let adhesion, platelets release components, such as adenosine diphosphate (ADP), serotonin, thromboxane-A_2 (TxA_2), TSP1, vWF, collagen, fibronectin (Fn) and fibrinogen (Fg), and also release calcium ions (Ca^{+2}) to reinforce platelet aggregation and also to activate some Ca^{+2} dependent proteins like TSP1, etc., from their dense and alpha granules. The released components bind in turn onto activated platelet receptors causing further platelet activation, and play a role as chemotactic components to attract other circulating platelets to the vascular injury (Figure 1.11) (Woulfe, 2005). Moreover, activated platelet expresses an essential receptor GPIIb/IIIa (αIIbβ3-integrin) for platelet activation and aggregation via fibrinogen binding to link other platelets by making bridge (Figure 1.13) (Jurk and Kehrel, 2005). Unfortunately, physiological and pathophysiological sites cannot be distinguished by platelets, hence, platelets are very sensitive in activation (Sachs and Nieswandt, 2007, Turitto et al., 1985).

The second plug formation is caused by the activation of coagulation pathways producing fibrin mesh formation that provides rigid permanent plug. The trigger of coagulation pathways occurs by the negative charge of phosphatidylserine (PS) that is exposed by activated platelets, and by tissue factor (TF) that is exposed on the damaged vascular sites and PMPs (Monroe and Hoffman, 2006).

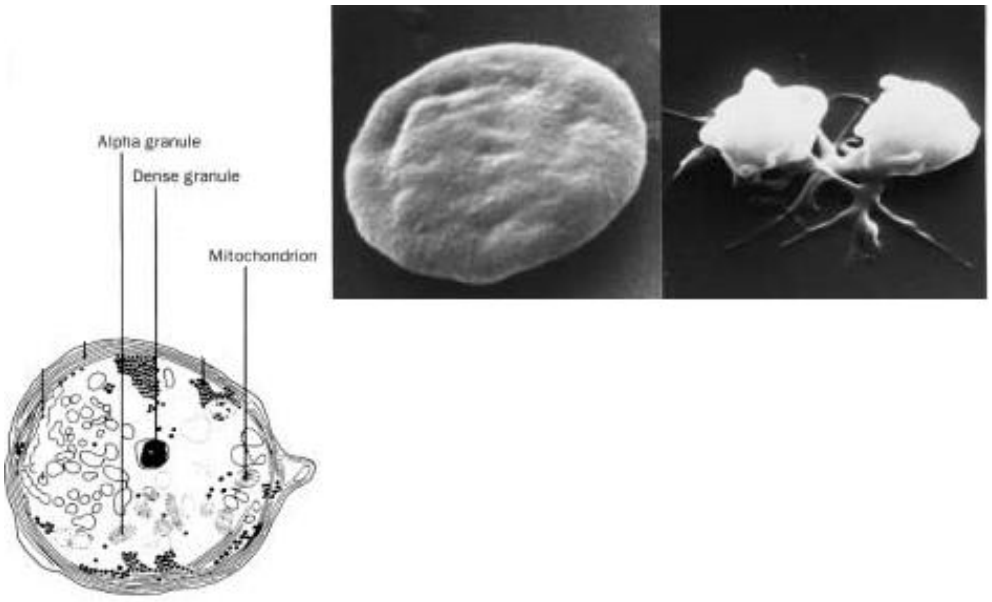

Figure 1.12. Drawing of normal platelet contents and the difference in shape between resting and activated platelets is observed by electron micrograph. Top left; showing scanning the disc shape of resting platelet by electron micrograph. Top right; showing scanning the long pseudopodia of the activated platelet (George, 2000).

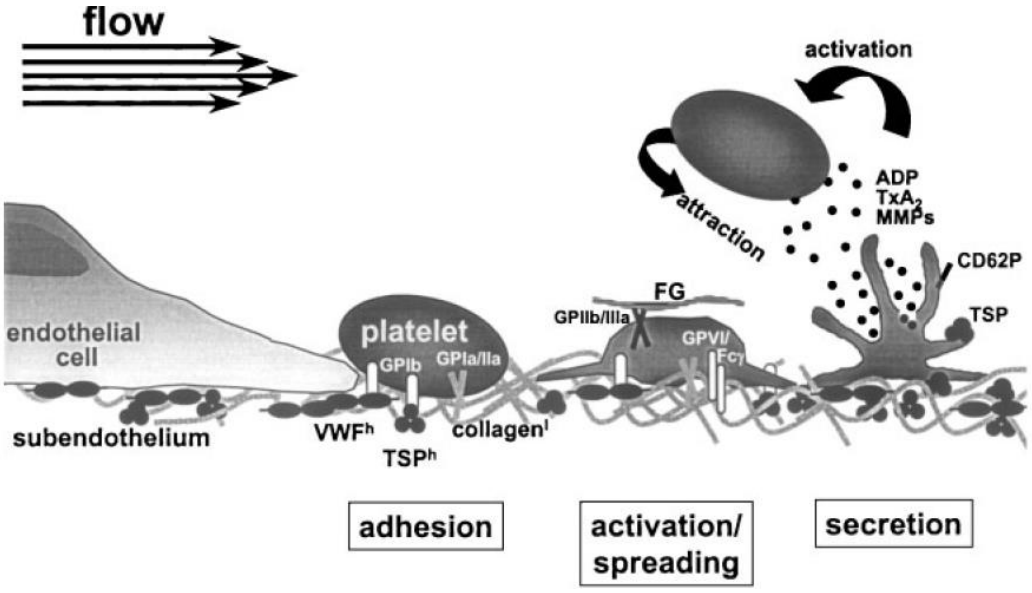

Figure 1.13. A simplified diagram shows platelet adhesion process (Jurk and Kehrel, 2005). Abbreviations; VWF von Willebrand factor, TSP1 Thrombospondin-1, FG Fibrinogen, ADP Adenosine diphosphate, CD62p P-selectin, MMPs Membrane microparticles, TXA2 Thromboxane A2.

1.1.2.1.1.1 Platelet Granules and Contents

It has become obvious that platelets contain three different types of granules; α-granule, dense granule and lysosomes. Each granule contains many of substances, which increase the inflammatory and immune response, procoagulant activity and wound-healing modulators etc., which are released in case of platelet activation. It has been stated that there are 284 proteins stored in the α-granule (Maynard et al., 2007). About 40-100 α-granules are found per platelet (White, 2007). Protein contents are either released into fluid phase as soluble effector proteins such as chemokines CCL5, CXCL8 (IL-8), thrombin and TSP1, or expressed onto platelet surfaces like adhesion receptors such as P-selectin (CD62p) (King and Reed, 2002, Gawaz et al., 2005, King et al., 2009). The dense granules specialise in cell-activation processes through secreting molecules like serotonin, histamine, and adenine nucleotides such as ATP and ADP, and divalent cation (Ca^{2+},Mg^{2+}) that might act as pro-inflammatory molecules (O'Sullivan and Michelson, 2006, King et al., 2009). However, lysosome granules comprise lysosome components similar to the other lysosome containing cells, for example, lysosomal membrane proteins (LAMPs), hydrolases and cathepsins, and also elastase, responsible for C5 cleavage of the complement system (Table 1.1) (King et al., 2009, Cheng, 2012). The mass substances contents in α-granule are more abundant than dense granules (Table 1.1) (Michelson, 2007).

α-granule	
Adhesion molecules	P-selectin (CD62P), von Willebrand Factor (vWF), thrombospondin, fibrinogen, Glycoproteins GPIb/IX/V, GPIIb/IIIa (αIIbβ3), GPIV, integrin αvβ3, fibronectin
Chemokines	Platelet basic protein (platelet factor 4 (PF4, CXCL4) and β-thromboglobulin), MIP-1α (CCL3), RANTES (CCL5), MCP-3 (CCL7), growth-regulated oncogene-α (CXCL1), ENA-78 (CXCL5), IL-8 (CXCL8)
Coagulation pathway	Factor V, VII, multimerin, factor VIII, XI, XIII
Fibrinolytic pathway	α_2-Macroglobulin, plasminogen, plasminogen activator inhibitor-1 (PAI-1), Protein C.
Growth and Angiogenesis	Basic fibroblast growth factor, epidermal growth factor, hepatocyte growth factor, insulin-like growth factor 1, transforming growth factor β (TGF-β), vascular endothelial growth factor-A (VEGF-A), vascular endothelial growth factor-C (VEGF-C), platelet-derived growth factor (PDGF)
Immunologic molecules	β1H Globulin, factor D, C1 inhibitor, IgG
Other proteins	Albumin, α1-antitrypsin (α1-AT), Gas6, histidine-rich glycoprotein, high molecular weight kininogen, osteonectin protease nexin-II (amyloid beta-protein precursor)
Dense granules	
Ions	Ca, Mg, P, pyrophosphate
Nucleotides	ATP, GTP, ADP, GDP
Membrane proteins	CD63 (granulophysin), LAMP-2
Transmitters	Serotonin, Histamine
Lysosomes	
β-glucuronidase, β-hexosaminidase, β-galactosidase, Elastase, *N*-acetylglucosaminidase Collagenase, Cathepsins, Endoglucosidase, Heparanase	

Table 1.1. The contents of platelet granules; α-, dense granules and lysosomes (Jurk and Kehrel, 2005, Michelson, 2007, Cheng, 2012).

1.1.2.1.1.2 Platelet Receptors

The response of platelets to the activator is mediated through platelet surface, and receptors called integrins are the predominant receptors on platelet surface (Faull et al., 1994). These integrins are crucial for platelet activation process (Ruggeri and

Mendolicchio, 2007, Michelson, 2007, Varga-Szabo et al., 2008). There are several receptors on platelet surface but few will be selected in this section (Table 1.2) (Figure 1.14). There are two essential roles for platelet receptors either acting to mediate platelet-platelet interaction or to mediate platelet-ECM interaction.

Platelet adhesion		**Platelet activation**	
Receptor	**Ligand**	**Receptor**	**Agonist**
GPIb-IX-V GPIbα (CD42b) GPIbβ (CD42c) GPIX (CD42a) GPV (CD42d)	vWF, TSP1	GPVI	TSP1, Collagen, laminin
α2β1 (GPIα-IIα) (CD49b/CD29)	Collagen, TSP1	P_2Y_1, P_2Y_{12}	ADP
αIIbβ3 (GPIIb/IIIa) (CD41/CD61)	Fibrinogen, TSP1, vWF, Fibronectin Vitronectin	PAR1, PAR4	Thrombin
α5β1 (CD49c/CD29)	Fibronectin, TSP1	TP	ThromboxanA_2(TXA_2)
αvβ3 (CD51/CD61)	Vitronectin, TSP1	α2-AR	adrenaline
α6β1 (CD49f/CD29)	Laminin, TSP1	GPIIIb, GPIV (CD36)	TSP1, Collagen,
Integrin associated protein (IAP/CD47)	TSP1		

Table 1.2. Some of the platelet receptors associated in the process of adhesion and activation. Note that some receptors are bi-function receptors involved in both platelet adhesion and activation like GPVI is considered for TSP1 and collagen acting in adhesion and activation.
ADP: adenosine diphosphate, AR: adrenergic receptor, GP: glycoprotein, PAR: protease activated receptor, TP: thromboxane receptor, TXA2: thromboxane A2, vWf: von Willebrand factor (Eriksson, 2008, Cheng, 2012).

The integrins are composed of two essential subunits; alpha (α) and beta (β). The α and β subunits associate together in order to form the ligand-binding head and also form legs. The specification of low and high binding affinity of integrin arises from the legs configuration (Mould and Humphries, 2004).

There are binding sites within the integrins which facilitate the binding with their ligands. RGD binding sites are identified as (arginine-glycine-aspartic acid), recognised as recognition sites for their ligands that possess RGD motif. It has been found that several

members of integrin family recognise RGD motif in their ligands. Therefore, mimicking RGD motifs can block the interaction sites on the integrin, leading to altering the physiological process (Takagi, 2004, Cini et al., 2009). It has been noted that even though RGD recognition binding site in the integrins favour RGD motifs in their ligands, integrins can also offer secondary binding sites by selectivity through α-subunit of the integrin while β-subunit is specified for RGD motifs interaction (Takagi, 2004).

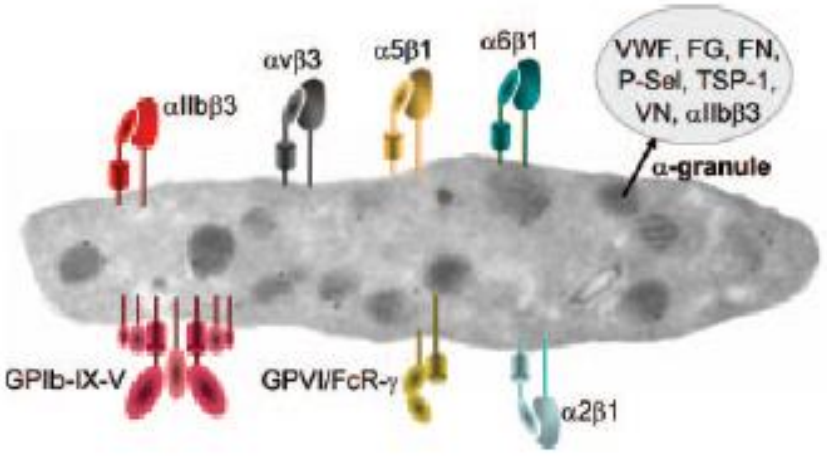

Figure 1.14. The most dominant adhesion receptors expressed on resting platelet surfaces. The adhesion receptors on intact circulating platelets are inactive molecules which require conformational changes mainly after the tethering step where the GPIb-IX-V adheres onto vWF and TSP1, sending inside and outside signals. The α-granule contains the adhesion proteins; Fibrinogen (Fg), Fibronectin (Fn), P-selectin (CD62p), Thrombospondin-1 (TSP1) and von Willebrand factor (vWF) (Denis and Wagner, 2007).

1.1.2.1.2. Platelet Tethering and Adhesion

In the first crucial step, haemostasis is initiated when circulated platelets bind and adhere onto the exposed extracellular matrix proteins (ECM) from the injured vascular system through specific receptors expressed on resting platelet surfaces. The adhesion process depends on a dynamic condition called shear rate, and stress on vessel injury (Kroll et al., 1996). In ruptured veins, the rate of shear and stress of blood flow are increased more than normal blood flow. The shear rate is the platelet speed in the layer next to the vessel wall. It is also identified by the time required for interaction between the exposed ECM and platelet integrins (Kroll et al., 1996, Weller, 2008).

Initially, the circulated or the exposed von Willebrand factor (vWF), high multimeric molecular weight compound, from sub-endothelium layer interacts directly with platelet

adhesion receptor GPIb-IX-V complex under high shear rate, or interacts indirectly with collagen, a major matrix protein, exposed also in sub-endothelial cells forming a bridge for platelet adhesion by acting as a cofactor with the adhesion receptor. This part of the adhesion process is called the tethering step (Figure 1.13).

This initial process allows the other exposed matrix components to interact with tethered platelets, especially ECMs which are unable to interact under high shear stress (Ruggeri, 2009, Auton et al., 2010). Only dimers and higher multimers of vWF have the ability to activate and crosslink the interaction with GPIb-IX-V complex (Jurk and Kehrel, 2005).

vWF is produced by megakaryocytes and endothelial cells. It is found in extracellular matrix layer expressed by sub-endothelium and also secreted by α-granules of platelets and Weibel-Palade bodies of endothelial cells (Schmugge et al., 2003, Ramasamy, 2004).

The tethering step caused by vWF is also engaged by TSP1 which plays a major role in crosslinking between sub-endothelial matrix and platelet glycoprotein matrix receptors. TSP1 is known as an alternative substrate for vWF in the adhesion process to bind to GPIb under high shear conditions (Figure 1.13) to up-regulation the activation of a number of complex receptors on surface of tethered platelets such as GPIIb/IIIa (αIIbβ3) by 50% (Auton et al., 2010, Ruggeri, 2009). Another adhesion receptor is proposed to be involved in tethering step so that the binding of vWF to collagen receptor (GPVI) may trigger the interaction of platelet receptor complex (GPIb-IX-V) (Clemetson and Clemetson, 1995).

Following the tethering step, platelets adhere onto collagen through GPIa/IIa (α2β1) which in turn leads to platelet activation (Kehrel, 1995). The deficiencies of platelet receptor GPIa/IIa (α2β1) in patients lead to uncontrolled bleeding disorders (Kehrel et al., 1988). Other platelet receptors like CD36 (GPIIIb, GPIV) and GPVI play a crucial role in platelet adhesion and signalling activation by collagen (Kehrel et al., 1998, Polgar et al., 1997, Tandon et al., 1989). GPVI is one of the immunoglobulin superfamily members which confer signalling and adhesion response to collagen in association with Fc receptor γ-chain (Chen et al., 2002). There are also adhesion receptors involved in the interaction with ECM proteins, such as integrins αvβ3 (for vitronectin), α5β1 (for fibronectin), and α6β1 (for laminin) (Michelson, 2007).

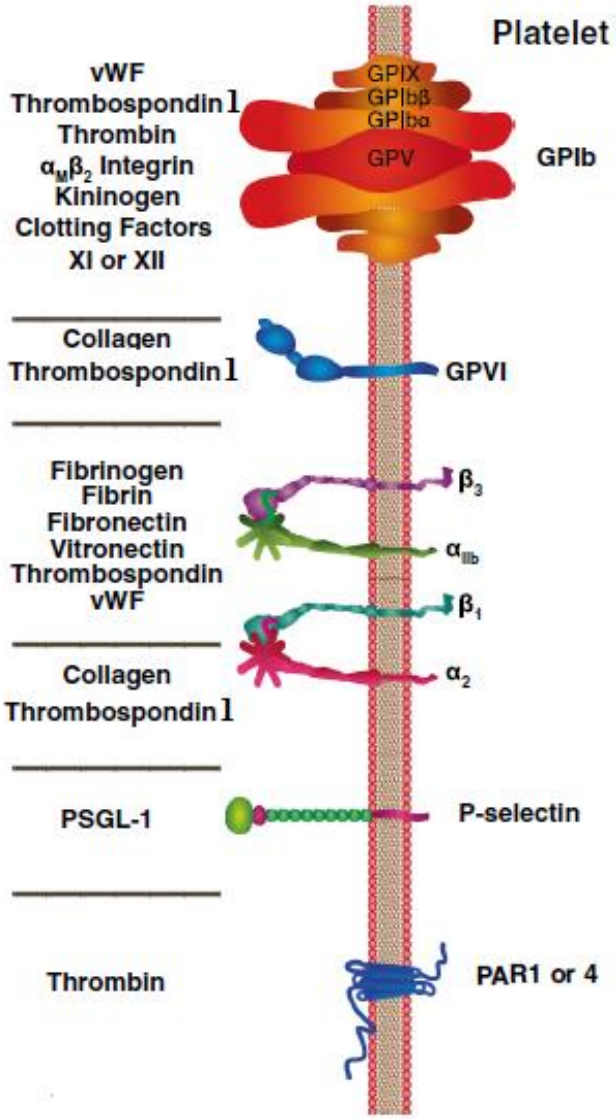

Figure 1.15. The interaction between agonists and variety of platelet receptors leads either to platelet adhesion or activation. It shows TSP1 interacting with GPIb/V/IX complex, GPVI, αIIβ3, and α2β1. Adapted from (Menter et al., 2014).

1.1.2.1.3 Platelet Secretion

Platelets secrete several components from either α-granules or dense granules, in order to support the interaction with vascular cells and to stimulate other peripheral intact platelets to vessel injury site by forming platelet aggregation. ADP is one of the secreted components of platelets from dense granules. As a result of releasing ADP, dense granules release all the contents but cannot be replaced (McNicol and Israels, 1999). The stimulation of ADP on platelet surface receptors mainly occurs toward two receptors known as P_2Y_1 (Jin et al., 1998) and P_2Y_{12} (Hollopeter et al., 2001). The role of P_2Y_1 is to mediate calcium release and platelet shape change and subsequently lead to aggregation transformation (Fabre et al., 1999), whereas, P_2Y_{12} receptor is suggested to force platelets for further stimulation and then may lead to continued aggregation (Dorsam and Kunapuli, 2004). There is also a crucial platelet agonist, released from activated platelets, which plays an important role in platelet shape change, adhesion and secretion process. This potent agonist is thrombin, which is a ligand for receptors called protease-activated receptors (PAR) PAR-3 and PAR-4 on platelet membranes. It has been shown that by targeting thrombin, thrombus formation was decayed in thrombosis models (Ni et al., 2001, Coughlin, 2005, Dubois et al., 2006, Mangin et al., 2006). There are also some secreted components such as thromboxane-A_2 (TXA_2) (Thomas et al., 1998, Puri, 1998), epinephrine (Pozgajova et al., 2006), ATP (Coleman et al., 2004), and leptin (Bennett, 2005) that potentiate the activation on the surface of platelets (Denis and Wagner, 2007). The TXA_2 acts via thromboxane receptor on other platelets for attraction and activation (Habib et al., 1999). The receptors for these agonists are mostly considered to be heterotrimeric coupled G-proteins. Furthermore, it has been shown that the anaphylatoxin C5a of the complement system potentiates platelet signalling stimulation, promoting further platelet aggregation via G-coupled receptors (Woulfe, 2005). The G-coupled receptors are divided into three families with different function: Gq-, G_i-, and G12/13 families. To activate these receptors, P_2Y_1 triggers the activation of Gq-receptors, leading to an increase in Ca^{2+} release from dense granule, platelet shape change and aggregation (Fabre et al., 1999), whereas, the attachment of ADP to P_2Y_{12} triggers the activation of G_i receptors, resulting in reduced levels of cyclic adenosine monophosphate (cAMP) (Oury et al., 2006).

Serotonin (5-hydroxytryptamine, 5-HT) is found in platelets and secreted to enhance platelet aggregation via retention of their pro-coagulant proteins, such as TSP1 and fi-

brinogen on platelets' surface (Dale et al., 2002). During platelet activation, Ca^{2+} influx occurs through tabular system by Ca^{2+} pumps which are vital activators for platelets activation and aggregation (Nesbitt et al., 2003).

The stress hormone adrenaline is also released from activated platelets (Paul et al., 1999). The latter stimulates platelets via α2-adrenogenic receptors (α2-ARs) (Grant and Scrutton, 1980).

There are also other secreted components like P-selectin (CD62P) and CD40L located on the membrane of α-granules. Once the activation is initiated, the secreted glycoproteins enhance platelet binding to monocytes and neutrophils (Singbartl et al., 2001).

These inflammatory immune-cells have the ability, by P-selectin dependent mechanism, to engage and roll on activated/adhered platelets by binding through the P-selectin glycoprotein ligand (PSGL-1) of P-selectin (Furie et al., 2001, Zarbock et al., 2009).

The ligand receptors of P-selectin differ from cell to cell, for example, by binding to neutrophils-leukocytes-endothelial-cell-adhesion-molecule-1 (LECAM-1) (Picker et al., 1991), endothelial-cell-leukocyte-adhesion-molecule-1 (ELAM-1) (Polley et al., 1991), and other ligands like sialy oligosaccharide (Foxall et al., 1992).

CD62p is a membrane glycoprotein called GMP-140 expressed in platelets in the event of activation (Johnston et al., 1989, Stenberg et al., 1985). The P-selectin is composed of five regions as follows: lectin region, EGF domain, nine tandem-repeats of complement binding protein-like, transmembrane domain, and cytoplasmic tail (Menter et al., 2014).

1.1.2.1.3.1 Platelet Procoagulant Microparticles (PMPs)

Platelet microparticles (PMPs) are sub-micron-vesicles which are sized between 0.05-1.0μm (Horstman and Ahn, 1999, Gelderman and Simak, 2008). PMPs inherit all receptors of the platelet membrane. PMPs are produced from the effect of Thrombin, collagen and complement component C5-9 mediated-platelet activation (Sims et al., 1988, Wiedmer et al., 1989, Abrams et al., 1990). There are also some agonists like TSP1, ADP, and epinephrine that are also able to trigger PMP release (Heinz Joist et al., 1974, Bode and Miller, 1986, Sims et al., 1989, Horstman and Ahn, 1999). It has been found that PMPs expose GPIb, GPIIb/IIIα, P-selectin (CD62p) and TSP1 (George et al., 1986, Gawaz et al., 1996b). Therefore, it has been demonstrated that PMPs provide matrix grid for platelet adhesion, in particular through GPIIb/IIIα onto the sub-endothelial layer

(Merten et al., 1999) and also possibly through TSP1. Most of the exposed receptors and proteins on PMPs surfaces can be used as biomarkers for identification (Horstman and Ahn, 1999).

Moreover, phosphatidylserine (PS) is a phospholipid surface of platelet membrane that is exposed during activation on the outer membrane of platelets, and also inherited on PMPs (Zwicker et al., 2012). Therefore, PMPs are called procoagulant microparticles due to their potent activity of triggering the activation of coagulation pathway by exposing PS, tissue factor (TF), and the receptor for coagulation factor Va which induces prothrombinase reaction by which vitamin K dependent coagulation factors is particularly involved in triggering the coagulation pathway (Horstman and Ahn, 1999).

It has been reported that PMPs seriously induced thrombin generation leading to coagulation pathway activation due to the exhibition of the negatively charged phospholipids (i.e., phosphatidylserine) (PS) on the outer membrane (Wolf, 1967, Horstman and Ahn, 1999, Berckmans et al., 2001, Zwicker et al., 2012). Clinically, the increasing levels of PMPs have been noticed in patients with serious diseases like disseminated intravascular coagulation (Holme et al., 1994), myocardial infarction (Gawaz et al., 1996a) and cardiovascular disease (VanWijk et al., 2003). Therefore, measuring PMP levels in plasma by specific biomarkers may help for screening disease prognosis in future, and so for treatment and protection.

1.1.2.1.4. Platelet Aggregation

After firm adherence onto the exposed surfaces, all platelet components are released in order to recruit other circulated platelets into the targeted area (Figure 1.13). However, it has been shown *in vitro* and *in vivo* that the GIbα sub-unit of GPIb/V/IX complex is responsible for platelet recruitment, on the intact circulated platelets (Wu et al., 2000, Kulkarni et al., 2000, Bergmeier et al., 2006). Therefore, the GPIbα is essential for the initiation process of adhesion onto the exposed surfaces and at the same time functions to recruit other intact platelets for aggregation (Denis and Wagner, 2007).

Another important receptor for platelet aggregation, integrin GPIIb/IIIa (αIIbβ3-integrin) is one of the major platelet receptors considered to be a central platelet activation receptor via the interaction with Fg. It is found in both platelet membrane and α-granules (Bennett, 2005).

The deficiency of αIIbβ3-integrin leads to dysfunction of the platelet plug formation which results a bleeding syndrome called Glanzmann thrombasthenia in humans (Nurden, 2005). In addition, αIIbβ3-integrin has been knocked out by deleting the β3 sub-unit in mice which reproduced the syndrome. Several symptoms were reported e.g. having sustained bleeding time, haemorrhage in GIT, abnormal platelet accumulation, and clot retraction (Hodivala-Dilke et al., 1999). Therefore, targeting thrombus formation has been observed by interfering with αIIbβ3-integrin in mice using antagonists which then highlighted the importance of the integrin in the aggregation process (Ni et al., 2000, Smyth et al., 2001, Gruner et al., 2003, Mangin et al., 2006). There are various ligands that bind to the complex, such as Fg, vWF, TSP1 and Fn. However, the inactive states of these ligands prevent the ability to bind to the GPIIb/IIIa complex.

However, it is believed that TSP1 is not as important as vWF in thrombosis process due to pointing out the low concentration of TSP1 in plasma. Therefore, TSP1 cannot take the lead in thrombosis in the absence of vWF and Fg (Denis and Wagner, 2007). Despite the fact of the low plasma concentration of TSP1, the concentration of TSP1 was measured in vessel injury sites after activating platelets and this increased the levels in plasma up to 40μg/ml within minutes (Legrand et al., 1997, Lamy et al., 2007). Furthermore, about 59mg/L of TSP1 was also measured from the α-granules contents of 10^9 platelet/L (Mosher et al., 1985). Therefore, the variation of TSP1 concentrations before and after activation would indicate the importance of TSP1 in the process of thrombosis either by interacting with αIIbβ3-integrin or GPIbα which might lead to a stable thrombotic plug formation in vascular injury site.

The activation of platelets by vWF leads to signalling through the GPIb/V/IX complex and links platelets to sub-endothelial cells. The interaction between vWF and GPIb/V/IX complex is very important to activate GPIIb/IIIa complex via platelet α-granules secretions, which subsequently activate the complex (Jurk and Kehrel, 2005, Ruggeri, 1993). As a result of GPIIb/IIIa activation, soluble fibrinogen is allowed to bind to adhered platelets through its ligand receptor and also may lead to granule secretion (Figure 1.13) (Jurk and Kehrel, 2005, Luscher and Weber, 1993).

Besides platelet ligands, low-density lipoproteins (LDL) are considered as ligand modulators in mediating platelet activation via interaction with the GPIIb/IIIa complex. Raised LDL plasma levels are found in patients who have discontinued their treatment with statins (used as drug to decrease the levels of cholesterol). This may lead to platelet hyperactivity (Jurk and Kehrel, 2005). In addition to LDL which may enhance the

formation of atherosclerotic plaques, P-selectin/PSGL1 or CD40L/CD40 play a role in recruiting T-lymphocytes to contribute to thrombus formation (Falati et al., 2003) and also the CD40L was shown to associate with GPIIb/IIIa complex leading to the stability of thrombus formation (Andre et al., 2002). Within blood circulation, the accumulated platelets and monocytes are presumed to have activities in atherosclerotic plaques formation after surgery (Esposito et al., 2003, Huo et al., 2003). The interaction between platelets and leukocytes forming platelet-leukocyte aggregates occurs between Mac-1 on leukocytes and GPIb on platelets that would lead to solve the reason for a rapid clearance phenomenon of transferred chilled platelets (Hoffmeister et al., 2003).

1.1.2.2 Coagulation Pathways

The components of coagulation pathways mostly react by close contact to activated platelets. At sites of vessel injury, activated platelets express the negatively charged lipoprotein called phosphatidylserine (PS). Thereby, the activation of coagulation cascade is triggered which is also triggered by TF and various mediators that exposed on PMP. These can contribute to haemostasis/thrombosis by generating thrombin (Stassen et al., 2004).

There are two pathways contributing together in thrombosis/haemostasis process. Extrinsic and intrinsic pathways are associated in converting soluble Fg into insoluble mesh fibrin by transforming pro-enzymatic components into active enzymes, leading to the formation of pro-thrombin (II) into thrombin (IIa) down the coagulation cascade. The interaction between coagulation components requires calcium ions and occurs on the phospholipid exposed surfaces such as PS on activated platelets. Factor X, the centre of the cascade, is the convergence point of the coagulation cascade from each pathway that contributes in converting FX into FXa. The extrinsic pathway depends on TF for activation while the intrinsic pathway depends on the contact factors called kallikrein and pre-kallikrein (Oikonomopoulou et al., 2012).

The activation of the intrinsic pathway is initiated by close contact between XII and the contact factors, whereas the activation of the extrinsic pathway occurs rapidly in the presence of TF, which is a lipoprotein expressed from damaged cells including activated platelets, activated monocytes, and endothelial cells. In the extrinsic pathway, factor VII is converted into VIIa by TF (Figure 1.16) (Markiewski et al., 2007). The zymogens FIX and X are converted into active serine proteases which contribute with VIIa in con-

verting II into IIa in the presence of calcium ions. The mesh of fibrin is deposited on the platelet surface receptor GPIIb/IIIa after converting Fg by IIa, which also stimulate the active XIIIa to initiate fibrin polymerisation into clot formation (Hoffbrand et al., 2001, Markiewski et al., 2007, Schouten et al., 2008).

Both of the pathways and their components are regulated in the damaged vascular site. There are natural plasma circulated regulatory proteins which control the main key targets of coagulation pathways, like controlling thrombin conversion, and fibrin clot lysis by anti-thrombin inhibitor (ATII) and by converting plasminogen into plasmin. Tissue factor pathway inhibitor (tFPI), present in circulating blood and also platelets, and controls Xa, VIIa, and TF. In addition of controlling II, ATII also controls the conversion of XIa, IXa, and Xa. Heparin cofactor II, moreover, binds to IIa to inhibit the activity (Figure 1.16). In addition, protein C and S are inhibitors for FV and FVIII while thrombomodulin binds to active IIa (Markiewski et al., 2007, Schouten et al., 2008).

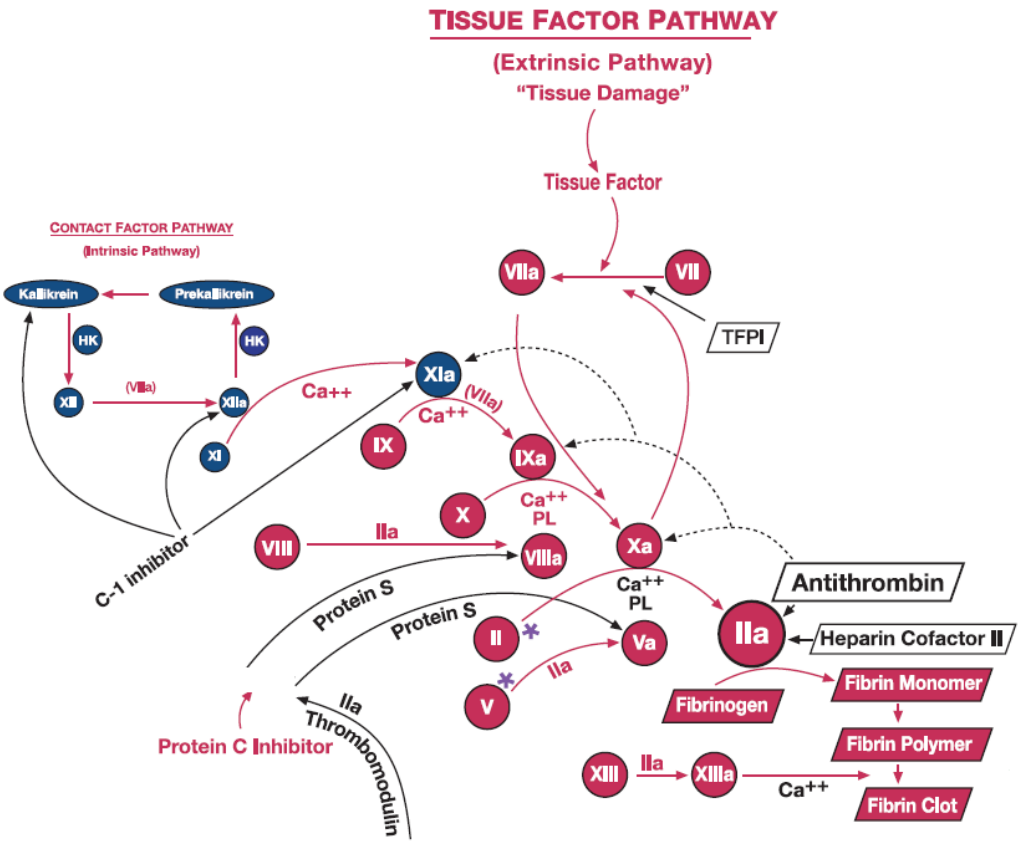

Figure 1.16 A simplified diagram of the coagulation pathways, drawn with red arrows, including the regulatory components drawn with black arrows. Adapted from (Gorbet and Sefton, 2004).

1.2. The Convergence and the Cross-talk Between Haemostasis and the Complement System

The two systems are vital for providing first-line defence against microorganisms and protection from blood loss at the site of vessel injury. There are several studies which have observed the binding of complement components; C1q, C3, C4, and C5-C9 (MAC), on activated platelets, leading to further platelet activation (Hamad et al., 2010). The binding of C3 on activated platelets was through P-selectin (CD62p) (Del Conde et al., 2005). As a result of complement cascade activation, either by pathogenic invaders or activated platelets in vascular tissue, the chemotactic C5a acts on C5a receptors of endothelial cells, monocytes, and leukocytes which in turn respond by expressing TF (Muhlfelder et al., 1979, Ikeda et al., 1997), which may increase the opportunity of initiating the activation of coagulation pathway. Indirectly, the anaphylatoxins C5a and C3a induce the regulation of inflammatory cytokines, which in turn influence the production of tumour necrosis factor (TNF-α) that finally stimulates monocytes to express TF (Markiewski et al., 2006). Moreover, there are a few studies about the positive regulator properdin (of AP) in binding to intact and resting platelets. Therefore, one of the aims of this study is to investigate the binding activity of native properdin forms including active oligomerised forms, which may trigger platelet activation. In addition, the serine protease MASP-2 of LP has been observed recently to activate II into active IIa (Krarup et al., 2007). Besides, MASP-1 converts II into IIa, and also plays a part in converting fibrinogen into fibrin by cleaving the β-chain and subsequently leading to stable fibrin clot by converting FXIII into FXIIIa (Figure 1.17) (Hajela et al., 2002, Krarup et al., 2008, Hess et al., 2012, La Bonte et al., 2012).

In contrast, it has recently been shown that IIa can cleave C5 into C5a and C5b in C3 deficient mice, so no C5 convertase was formed. Moreover, pure thrombin was incubated with human C5 and the cleavage products were observed as mentioned previously (Huber-Lang et al., 2006). Moreover, C3 and C5 have been recently found to be substrates cleaved by IIa, FXIa, FXa, and IXa and also plasmin (Amara et al., 2010). Besides, elastase is a potent enzyme, which is released from activated platelets (James et al., 1985), and also from leukocytes, that cleaves C5 into C5a and C5b (Ward and Hill, 1970, Wetsel and Kolb, 1983, Vogt, 2000).

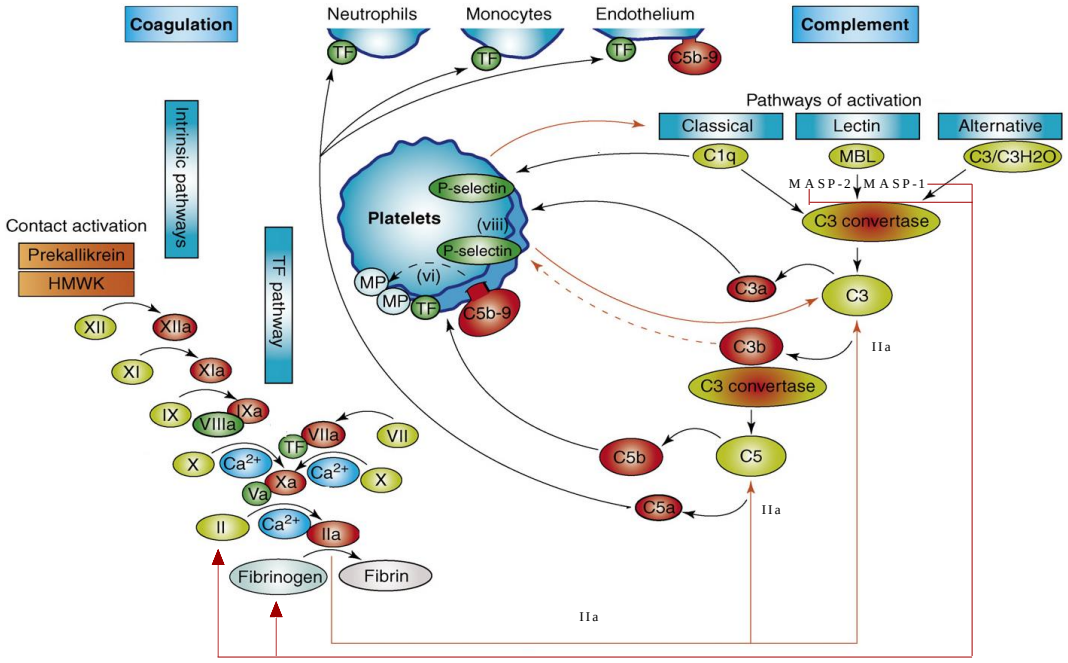

Figure 1.17 A simplified diagram of the crosstalk between the coagulation pathway including platelets, and the complement systems, adapted from (Markiewski et al., 2007).

1.3. The Interference between Haemostasis and the Complement System

In view of the convergence of activation in ab/normal conditions between the haemostasis and complement systems, there is also interference between their regulator proteins to control further activation in both systems.

The fluid-phase regulators of complement system, fH (Vaziri-Sani et al., 2005) and vitronectin (S-protein) (Asch and Podack, 1990), were found to bind to activated platelets via the GPIIb/IIIa complex to prevent complement activity. The membrane-bound complement regulators were observed to be expressed on activated platelets such as CR2, CR3, CR4, DAF, and C1q receptors which prevent C3/C5 convertase formation and the disruption of C1 complex formation of the CP (for more details, see pages 21-24).

On the other hand, soluble C1-inhibitor (C1-INH) of the complement system was found to bind to ECM proteins like collagen-IV and laminin (Patston and Schapira, 1997) exposed in injured vessels, which suggested that the interaction occurred in order to eliminate the danger of the complement activation (Davis et al., 2008). It has also been shown to bind P-selectin (CD62p) expressed on activated platelets (Cai & Davis, 2003).

In contrast, C1-INH interacts in the contact pathway of coagulation system through binding to Kallikrein, FXIIa, and FXIa to prevent the downstream activation (Gigli et al., 1970, McConnell, 1972, van der Graaf et al., 1983, Harpel et al., 1985, Davis et al., 2008).

1.4. Thrombospondin Overview

Thrombospondin (TSP) is a family of extracellular glycoproteins forming multi-domains. There are five thrombospondin molecules, distinct in function and structure i.e. TSP1, TSP2, TSP3, TSP4, and TSP5. The latter is also called cartilage oligomeric matrix protein (COMP) (Roberts, 2008).

In 1971, thromobospondin-1 (TSP1) was first discovered on activated platelets (Baenziger et al., 1971). The TSP1 is mainly produced by megakaryocytes in bone marrow where platelets (Thrombocytes) are produced by cellular fragmentation. The name of thrombospondin was given as it was first isolated from platelets (Lawler et al., 1978, Roberts, 2008). There are, however, different cells that produce TSP1 including endothelial cells (McPherson et al., 1981, Mosher et al., 1982), smooth muscle cells, glial cells, fibroblasts and macrophages (Frazier, 1987).

At vessel injury sites where tissue factor (TF) is exposed by both endothelial cells and platelets, the activation of intrinsic coagulation pathway is initially triggered, as previously described. As a result of the activation, the formation of fibrin clot occurs in order to form haemostatic plug as a physical barrier preventing blood loss (Figure 1.11).

However, platelets co-operate with the coagulation cascade promoting aggregation. The aggregation process, as stated previously, is initiated through platelet receptors. These platelet receptors bind to potent agonists such as von Willebrand factor (vWF), TSP1, and collagen. All these have in common the facilitation of the adhesion process on the exposed sub-endothelial cells via their respective receptors. Subsequently, the adhering platelets send out signals to facilitate or initiate further activation leading to aggregation, by platelets surface receptor GPIIb/IIIa complex where been involved (Jurk and Kehrel, 2005). Moreover, TSP1 interacts with the platelet receptor i.e. GPIIIb (GPIV) (alias CD36) and it has been reported that the CD36 was also responsible for platelet activation by TSP1, which is called a scavenger receptor (Asch et al., 1993, Chen et al., 2000). TSP1 also binds to fibrinogen/fibrin and provides contact strength with aggregated platelets (Mosher, 1990). TSP1 binds to most platelet β1-integrin receptors like α2β1, α3β1, α4β1, α5β1, α6β1, and α9β1, and also binds β3 integrins like αvβ3, αIIbβ3 and to the integrin-associated protein (IAP/CD47) including binding to heparin sulphate proteoglycan (Lawler et al., 1988, Adams and Lawler, 1993, Yabkowitz et al., 1993, DeFreitas et al., 1995, Gao et al., 1996b, Bonnefoy et al., 2008).

1.4.1. Thrombospondin Structure

The family of extracellular thrombospondins can be divided into two subgroups depending on their structure, A and B. Subgroup-A includes TSP1 and TSP2. Both members of subgroup A are composed of homo-trimeric glycoproteins with similarity in structure but the patterns are discriminated in term of regulation and expression (Simantov et al., 2005), whereas it has been reported that the expression of TSP1 was identified as a homo- and hetero-trimeric structure using Swiss 3T3-fibroblasts (O'Rourke et al., 1992). The subgroup-A is distinguished from subgroup-B in having properdin or thrombospondin (TSR)-like (Type-I) repeats (Figure 1.18).

As reviewed by Tucker, the supergene family of TSR repeats represented in TSP1/2 shows a high degree of homology with other proteins composed of TSR domains (Type I) (Tucker, 2004). The molecules found to have TSR repeats similar to TSP1 include complement proteins such as C8, C9 and properdin (Lawler and Hynes, 1986, Esemuede et al., 2004). As mentioned before, the release of TSP1 from α-granules can be induced by thrombin, by which explains that TSP-1 was first called thrombin-sensitive protein (Baenziger et al., 1971, Baenziger et al., 1972).

The other TSPs assigned to subgroup-B have a distinct molecular architecture which typically forms pentameric structures (Adams and Lawler, 2004, Roberts, 2008). The members of the subgroup-B are all characterised by the deficiency of procollagen homology domain (PCH), which is considered a linking region, and also lack of the thrombospondin-like-repeats (TSR) (Type-I) which is found, however, in TSP1 and TSP2 with three repeats. Whereas, extra epidermal growth factor (EGF)-like repeats (Type-II) emerges as a distinguishable structure between subgroup-B members, while only three repeats are present in TSP1 and TSP2. TSP5 (COMP) is clearly distinct from TSP3 and TSP4 of subgroup-B and diverging from their (NH_2)-terminal domains (NTD) (Sodersten et al., 2006). The carboxyl terminal region presents in all the members of both sub-groups.

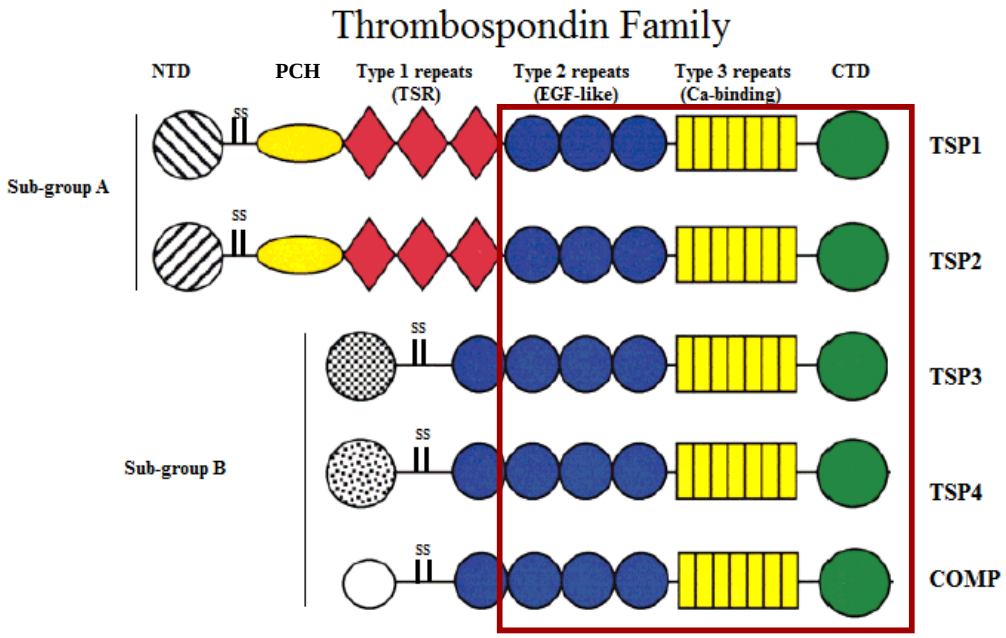

Figure 1.18. Schematic representation shows the relation of TSP-1 with other TSPs by comparing their domain structure. N-terminal domain (NTD). C-terminal domain (CTD). Adapted from (Adams and Tucker, 2000).

The 450kDa TSP1 is a homo-trimeric glycoprotein composed of three subunits. Each subunit is sized 150-180kDa. The three identical polypeptide chains are linked by disulfide bonds close to the N-terminal sequence (Figure 1.19) (Lawler et al., 1978, Lawler and Simons, 1983). Each subunit of TSP1 is characterised by six domains. The NH_2-terminal globular region contains a unique heparin-binding domain. The oligomerisation occurs via the coiled-coil domain which mediates the interactions between three subunits known as translational oligomerisation, and is subsequently stabilised by inter-chain disulfide linkage forming a helical structure (Adams and Lawler, 2004).

The other domains are procollagen-like (homology) domain (PCH), three thrombospondin-like repeats (Type-I) which are also called thrombospondin structural homology repeats (TSRs), three EGF-like repeats (Type-II), seven calcium-binding repeats (Type-III) as formed in calmodulin, and lastly COOH-terminal globular domain (Lawler et al., 1978, Lawler and Hynes, 1986, Esemuede et al., 2004).

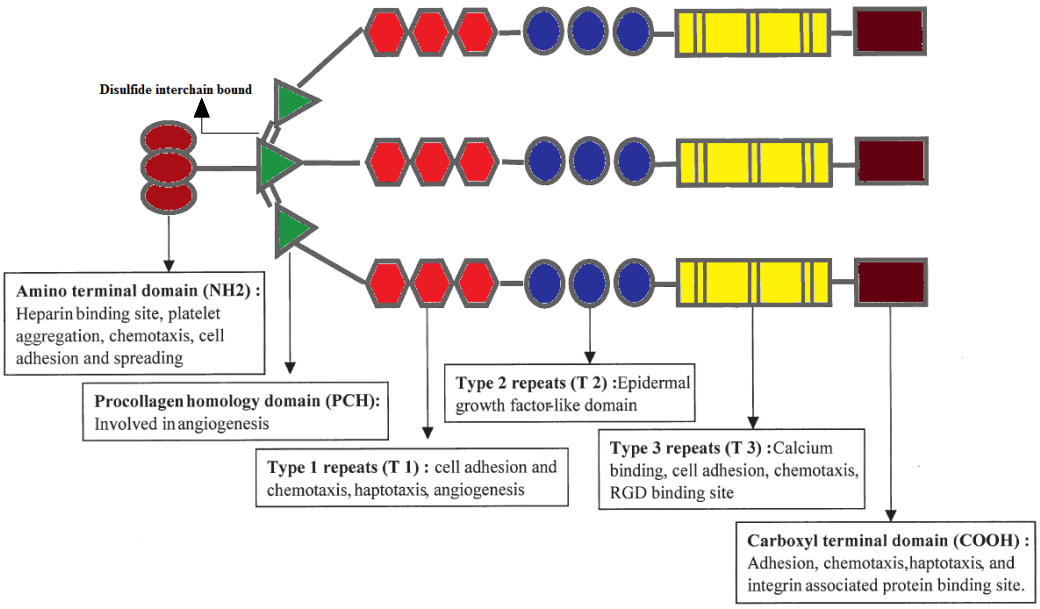

Figure 1.19. 3D diagram represents the homo-trimeric structure and functions of TSP1 for each domain. Adapted from (Esemuede et al., 2004).

1.4.2. The Thrombospondin Gene family

The genes encoding TSP1 and TSP2 are localised in human chromosome 15q15 and 6q27, respectively (Jaffe et al., 1990, LaBell et al., 1992). The human genes encoding TSP3, TSP4 and TSP5 are positioned in the human chromosomes; 1q21 (Adolph et al., 1995), 5q23 (Newton et al., 1999), and 19p13.1 (Newton et al., 1994), respectively. The similarity between TSP1 and TSP2 genes can be determined through their intron/exon boundary positions for the region encoding Type-I repeats (TSRs) as well as the procollagen homology domain (PCH). The length difference between the introns of TSP1 and TSP2 is significantly discriminated. The gene of TSP1 sized about <20kb is smaller than the TSP2 gene sized about >30kb (Hennessy et al., 1989, LaBell and Byers, 1993).

1.4.3. Functions of Thrombospondin-1

TSP1 is a multifunction glycoprotein which plays an essential role following injury, exposing the ECMs in sub-endothelial layer. TSP1 is able to interact with heparin sulfate proteoglycans, heparin, collagen, laminin, fibronectin (Fn), etc., and also with plasma proteins such as fibrinogen (Fg), histidine-rich glycoproteins, vWF and plasminogen (Frazier, 1987). In addition, TSP1 is considered to be responsible for cell-cell interactions including the interaction between cells and proteins, e.g. growth factors, cytokines, and coagulation proteins in vascular injury site (Lahav, 1993, Bornstein and Sage, 1994, Bornstein, 1995, Chen et al., 2000). Thus, TSP1 can be called a matrix-cellular protein. It is also involved in cell proliferation, migration and apoptosis regulation either in physiological or pathological conditions, for instance, in inflammation, angiogenesis, wound healing and haemostasis (Chen et al., 2000). Despite the fact that TSP1 is involved in the extracellular matrix, it has been found as a fluid-phase plasma protein in normal conditions with varied concentrations 40-300ng/ml (Switalska et al., 1985, Booth and Berndt, 1987). TSP1 is considered as a secreted protein and form 20-30% of the secreted proteins, and also is stored in α-granules of platelets by forming 3% of the total stored proteins (Esemuede et al., 2004), hence the concentration of TSP1 in platelet activation condition is about 30-100µg/10^9 platelets (Bonnefoy et al., 2008). That concentration of the secreted TSP1 is also measured after platelet activation and the plasma levels of TSP1 increased up to 40µg/ml (Legrand et al., 1997, Lamy et al., 2007). That would indicate the importance of TSP1 in particular events like haemostasis/thrombosis, where platelets raise the plasma levels of TSP1.

However, the binding partners for TSP1 have been located within TSP1 domains. The N-terminal domain is responsible for binding to glycosaminoglycan, calreticulin (endoplasmic reticulum resident protein 60 (ERp60)) and Fg, heparin and CD36 (Legrand et al., 1992, Murphy-Ullrich et al., 1993, Calzada et al., 2004, Goicoechea et al., 2000, Tan et al., 2006). Meanwhile, the partners for TSR Type-I are glycosaminoglycan, CD36, collagen and Fn (Guo et al., 1992, Asch et al., 1992, Takagi et al., 1993, Panetti et al., 1999). Other binding partners are not located precisely in TSP1 but are located between EGF domains and C-terminal domains. These partners are β1, β3 integrins, collagen, vWF, laminin and Fn (Pimanda et al., 2004, Galvin et al., 1987).

1.4.3.1. The Role in Platelet Activation, Adhesion and Platelet Aggregation

The interaction between TSP1 and platelets has been thoroughly investigated. As stated previously, TSP1 is released from α-granules by thrombin-mediated platelet activation. TSP1 attaches to platelet surface through the interaction with IAP/CD47 via the RGD sequence of TSP1 (page 34), and other receptors on platelet surfaces like αvβ3 (Lawler and Hynes, 1989) and αIIbβ3 (Karczewski et al., 1989) (Table 1.2). Moreover, as stated previously (page 48) the TSP1 non-covalently binds to β1 and β3 chains of some integrins on platelet surfaces, leading to either initiation of the activation, or further activation. The β1 integrins are α2β1, α3β1, α4β1, α5β1, α6β1, and α9β1, which are all considered as ligand receptors on platelet surfaces for TSP1 (Lawler et al., 1988, Adams and Lawler, 1993, Yabkowitz et al., 1993, DeFreitas et al., 1995, Gao et al., 1996b, Bonnefoy et al., 2008). Therefore, the binding to IAPs/CD47 by TSP1 has been found triggering platelet activation in association with the following integrins: αvβ3 (Gao et al., 1996a), αIIbβ3 (Chung et al., 1997) and α2β1 (Wang and Frazier, 1998).

However, TSP1 is Ca^{2+} dependent agonist for mediating platelet activation. As a result of binding TSP1 to platelet surface, platelet receptors increased from 1-2000 molecules on resting platelets to 14-16000 on stimulated platelets (LEGRAND et al., 1988). In other words, large amounts of TSP1 bind to activated platelets surfaces during the activation process after influx Ca^{2+} (Esemuede et al., 2004).

Thus, TSP1 initiates platelet adhesion and aggregation and then leads to clot formation (Bacon-Baguley et al., 1990, Tuszynski and Kowalska, 1991) through allowing Fg to bind to GPIIb/IIIa complex. It has been assumed by Silverstein et al. that the initiation of platelet aggregation is mainly triggered and supported by binding TSP1 to Fg-bound αIIbβ3 integrin (Silverstein et al., 1986). In vascular injury site, Fg attaches to the platelet surface via GPIIb/IIIa. In order to stabilise this interaction in thrombus formation, TSP1 increases the interaction efficacy between Fg-bound GPIIb/IIIa by creating a bridge between the complex (Bonnefoy et al., 2001).

The NH_2-terminal globular domain of TSP1 is characterised as having a high activator affinity toward platelets, because a reduction of platelet aggregation occurred on average 35-50% after neutralising TSP1 using antibodies, however, the ability of TSP1 on platelet aggregation is still debated (Kasirer-Friede et al., 2001).

All TSP1 terminal domains, NH_2 and COOH, have been investigated in the involvement for platelet aggregation (Legrand et al., 1994, Rabhi-Sabile et al., 1996), whereas, the C-terminal independently activates platelets via binding with CD47 (Trumel et al., 2003, Voit et al., 2003, Neuhaus et al., 2004). Moreover, it has been showed that by inducing washed platelets with cell-binding domain (CBD) peptide of C-terminal TSP1, platelet aggregation was observed with rapid rising levels of tyrosine phosphorylation (Chung et al., 1999).

The association between IAPs/CD47 and the following activation of αIIbβ3 integrin is a result of RGD motif of TSP1 interaction with platelet receptor. This specific interaction induces Fg to immobilise and aggregate with αIIbβ3 integrin (Chung et al., 1997). It was clear that TSP1 promoted the association between CD47 and αIIbβ3 by inducing conformational changes to the extracellular domain of αIIbβ3 (Fujimoto et al., 2003). Furthermore, TSP1-mediated platelet activation was able to enhance collagen induced platelet aggregation via α2β1. That activation by TSP1 was dependent for CD47 after using platelets from CD47-deficient mice (Chung et al., 1997, Chung et al., 1999). That would indicate the involvement of CD47 in the initiation process of platelet activation by TSP1.

GPIIIb (CD36, GPIV) has been identified as an integral platelet plasma membrane receptor for TSP1 after targeting the sequence motif (CSVTCG) of TSP1 using monoclonal antibody against the motif (LEGRAND et al., 1988, Asch et al., 1992, Beiso et al., 1990).

Even though, after using platelets from CD36-deficient mice, platelets were activated by TSP1 and that was an indication of the ability of TSP1 may interact with other platelet receptors (Kehrel et al., 1991). Moreover, an investigation by Legrand et al., used monospecific antibodies targeting the TSP1 heparin-binding domain (HBD) to block the binding interaction with Fg and CD36. The antibodies significantly altered the expression levels by decreasing thrombin-mediated-platelet aggregation and the secretion response (Legrand et al., 1992). That late response of platelet aggregation would indicate other receptors to be involved in TSP-mediated platelet activation. Therefore, TSP-mediated platelet activation is either triggered through CD36 or CD47, or also through other receptors, as stated previously.

Moreover, TSP1 is considered as a secreted protein and also expressed in the subendothelial cells layer as ECM. However, the ECMs proteins are the fundamental bases for the structural frame in supporting tissue organisation (Tan and Lawler, 2009).

Therefore, once the ECM is exposed to bloodstream, intact platelets initially tether onto the expressed ECM components like TSP1 via the GPIb/V/IX complex and GPVI, α2β1 and most of the adhesion receptors of intact platelets, as stated previously.

Another role for TSP1 is a direct contact to ECM components like collagen types I, III, IV, vWF, Fn, vitronectin, and laminin etc., (Galvin et al., 1987), and subsequently leading to strengthening the binding grip between platelets and ECM for adhesion process.

Another role of TSP1 is that the interaction with the exposed vWF would protect vWF from proteolysis by ADAMTS13. It has been found that the TSP1 binds to A3 domain of vWF which is the binding domain for ADAMTS13. It has been suggested to cause a slower proteolysis process for vWF (Pimanda et al., 2004). It has been reported that the use of TSP1 knock-out mice resulted in reducing platelet activation for up to 20% comparing to 48% of platelet activation in WT mice in the presence of vWF that is generated under endothelium stimulation. Therefore, TSP1 has been indicated to be involved in the protection role of vWF from proteolysis by ADAMTS13 (Bonnefoy et al., 2006). ADAMTS13 (A-Disintegrin-And-Metalloprotease-with Thrombospondin-type-I-motifs-13) is a proteolytic enzyme in plasma. It cuts high molecular weight vWF into smaller fragments in plasma or ECMs to reduce platelet-tethering events (Hubbard et al., 2015, de Groot et al., 2015).

The various roles of TSP1 in platelet activation, and binding lipoproteins, cholesterols and triglycerides (atherogenic particles) indicate the participation of TSP1 in the pathophysiology of cardiovascular disease, atherosclerosis and coronary heart disease. For instance, high levels of LDL define the risk of atherosclerosis disease. Atherosclerotic disease develops through a combination of cell accumulation on the endothelium and vascular smooth muscle cells (VSMC) close to atherosclerotic lesions. An injury -for example- in the arterial wall, can cause an increase level of TSP1, and also atherogenic particles (cholesterol, LDL and Triglycerides) in plasma. The injured arterial wall, then, can trigger uncontrolled events of cellular migration and proliferation. These cells are VSMC and inflammatory cells like macrophages, which can be transformed into foam cells (fatty cells), that produce and release high levels of TSP1 and store cholesterol or LDL. That event would also stimulate platelets to adhere into the injury site leading to triggering uncontrolled thrombosis event (Ross, 1993, Wight et al., 1985, Moura et al., 2007). Moreover, the circulated LDLs accumulate and precipitate into endothelial or sub-endothelial cells and, then, increase the incident rate of accumulating circulated platelets and other cells (Goldstein and Brown, 1975). Recently, it has been observed *in*

vitro that LDL precipitates or binds to platelets through GPIIb/IIIa complex (Tetik et al., 2008). However, it has been found that TSP1 binds to very-LDL (vLDL) with high affinity which may link with atherosclerosis disease (Muraishi et al., 1993).

1.5. The Association of TSP1 with the Adhesion Process of Pathogenic Bacteria on Host Cells Including Platelets

In vascular injury sites, there is an opportunity for bacteria to colonise into inflamed sites due to the density of several regulatory proteins released either for complement or haemostasis system and which, therefore, can be used for protection against the immune system. For instance, the defence ability of *Neisseria meningitidis* against complement attack has been recently observed by the binding to C4bp and fH (Jarva et al., 2005, Lewis et al., 2010). In addition, for example, *Streptococcus pneumoniae* also protect its surface from complement activation via expressing several surface proteins which interfer with C3b deposition and/or binding to fH (Tu et al., 1999, Ren et al., 2003, Jarva et al., 2002). There are different serotypes of *S. pneumoniae* by which may differe in expressing low or much quantity of these pneumococcal surface proteins. That may illustrate how such serotypes of bacterial species would be defeated and become susceptibility for killing by the complement system (Shaper et al., 2004).

On the other hand, microbial colonisation and dissemination likely depends on the ability of pathogen-associated molecular patterns (PAMPs), like lipopolysaccharide (LPS) or glycosaminoglycan (GAG), to bind to pattern recognition receptors (PRRs) on activated platelets, like Toll-like receptors (TLRs), which could lead to further platelet activation (Clark et al., 2007), and also likely to result in colonisation for microbial pathogens through binding to a variety of ECMs proteins secreted by platelets.

Furthermore, among the bacterial protection mechanisms from complement activation where initiated in vascular injury, TSP1 is released from activated platelets in abundant amounts, and has been recently suspected of contributing toward the adhesion and dissemination process for several pathogenic bacteria such as *S.pneumoniae* and *Staphylococcus aureus* (Rennemeier et al., 2007, Niemann et al., 2009).

Chondroitin sulphate (CS) is one of several components released from activated platelets and retained on the surface (Ward and Packham, 1979). The previous studies

(Vischer et al., 1997) and the current results observed the binding capability of TSP1 to CS. CS is a derivative from GAG which contains chains of sulphated galactose sugar *N*-acetylgalactosamine (GalNAc), and also is similar to varieties of the conserved materials of bacterial cell wall represented as peptidoglycan layer, especially Gram-positive bacteria. Therefore, it has been found that TSP1 strongly binds to G+ve bacteria, but with less binding efficiency toward G-ve bacteria, and that what has been indicated is the association with bacterial pathogens through binding to peptidoglycans and host cells. Therefore, TSP1 may have effects on colonisation and dissemination (Rennemeier et al., 2007).

1.6. Hypothesis

The importance of the complement system and platelet interaction was introduced in the introduction regarding the conclusion of microbial clearance and the prevention of blood loss, respectively. The cross-talk and the interference between complement system and platelet interaction were also introduced from the view point of synergism.

The hypothesis is focused on two different components called Properdin and TSP1 related to two different systems; complement system and platelet interaction, respectively. TSP1 is composed of a number of highly conserved structural motifs, called Thrombospondin domains or thrombospondin repeats Type-I (TSRs). Like thrombospondin, a unique complement regulatory protein, properdin, is nearly entirely composed of TSR domains. The similarity in TSRs between TSP1 and Properdin was identified with 47% identity (Goundis and Reid, 1988). The interaction of TSP1 with platelets causing platelet activation and adhesion has been established for many years. On the other hand, Properdin has been proved by many researchers to increase the substantial protection of the complement system against microbial infection (Ali et al., 2014).

Therefore, the cross-talk and the interference between the complement system and platelet interaction might include TSP1 and Properdin from the view point of the structural homology of the TSRs between the components. Therefore, the hypothesis in this study will investigate if there is a novel link between haemostasis/thrombosis (Platelet interaction) and the complement system with respect of the two proteins. This link connects two essential biological activities required to limit the damage following blood vessel injury, i.e., the prevention of blood loss and the protection from infection.

1.7. Aims of the thesis

Clear indications for the cross-talk and the interference between haemostasis/thrombosis and complement systems have recently been established from several different perspectives. My task was to explore to what extent TSP1 and Properdin are involved in this cross-talk and interfere, based on their highly similar conserved domain structure. Each one of these molecules has been previously studied in the context of their specific systems but little or no attempts were made to explore their roles in the cross-talk and the interference between their defined systems (i.e. the possible role of TSP1 on complement activation and the possible role of properdin on haemostasis/thrombosis).

This work was conducted to explore new possible functions of TSP1 and Properdin in the cross-talk or interference between the systems because of the striking degrees of identity in the composition of their TSR (Type-I) domains. Therefore, this work was designed to achieve the following goals;

i. To investigate the potential role of native as well as of highly oligomerised recombinant Properdin to bind to resting and activated washed human platelets;
ii. To define and assess the role of recombinant TSP1 and highly oligomerised Properdin on activating intact and washed peripheral blood platelets.
iii. To compare the effects of both Properdin and TSP1, on platelet adhesion;
iv. To monitor the release of a platelet activation markers called P-selectin (CD62p) and thrombin;
v. To screen for the release of platelet microparticles (PMPs) from peripheral blood platelets in generating thrombin in response to exposure to recombinant properdin and TSP1;
vi. To assess the binding ability of native and highly oligomerised Properdin to extracellular matrix proteins (ECMs) such as, Collagen types I, III, IV, and von Willebrand factor (vWF) and fibronectin (Fn) as the most expressed proteins in sites of vascular injury, and compare it with the binding to different atherogenic factors such as cholesterol, triglycerides and low-density-lipoprotein (LDL).

Another essential part of my study was to define to what extent TSP1 interferes or interacts with the complement system. To investigate its role as potential platelet

agonist, recombinant TSP1 was added to functional assays to measure Lectin pathway (LP) and Alternative pathway (AP) functional activity. I also monitored C3 and C4 deposition on activated platelets and on their released activation markers such as chondroitin sulphate (CS). I compared this with the LP or AP mediated deposition of complement activation products on Mannan in presence or absence of recombinant TSP1 or recombinant highly oligomerised Properdin.

To investigate a possible role of TSP1 on complement activation on the surface of capsulated microbial pathogens like *Streptococcus pneumoniae*, I coated ELISA plate with formalin treated bacteria.

These aims required production and purification of the full-length recombinant TSP1 and Properdin in order to define the functional activities of both proteins. The first part of my work therefore involved the establishment of TSP1 encoding expression plasmids, transfection of host cell lines, screening single transfectants for TSP1 protein expression and large scale protein production and protein purification for both TSP1 and Properdin.

Chapter 2
(Materials & Methods)

2. Materials and Methods

2.1 Materials

This section will provide all the information about the commercial chemicals and materials of media, buffers, kits, oligonucleotides, vectors, antibodies and proteins were used for the current study.

2.1.1 Chemicals and media

Chemical commercial name	Manufacture	Catalogue No.
1kb plus DNA ladder	Invitrogen	10787-018
Agarose, electrophoresis grade	Melford	MB1200
Ampicillin	Sigma	
Bromophenol blue	Sigma	B-7021
Calcium chloride	Sigma	C3306
Ethanol	Fisher	
Ethylenediaminetetraacetic acid (EDTA)	Sigma	CAS 60-00-4
Ethidium Bromide	Sigma-Aldrich	
Glycerol	Sigma	G5516
Glycine	Fisher Scientific	BP381-1
High fidelity polymerase	New England Biolabs	
Isopropanol	Fisher	
L-Agar Bas	LAB M Limited	
Magnesium chloride	Sigma	M8266
3-(N-Morpholino) propanesulfonic acid (Na.MOPS)	Sigma	M-9381
Potassium Acetate (K-acetate)	Sigma	P5708
potassium chloride (KCl)	Fisher	P/4240/53
T4 DNA ligase	Promega	M180A
Purified BSA 100X	New England BioLabs	B9001S
Tris Base	Fisher Scientific	BP152-1
Calcium chloride	Sigma-Aldrich	
HEPES	Sigma-Aldrich	H7006
Magnesium chloride	Fisher Scientific	M8266
Sodium Chloride	Fisher Scientific	BP358-212
Sodium hydrogen carbonate	Fisher Scientific	

Sodium Citrate	BDH Anala R	102424L
Tri-sodium Citrate	Fisher Scientific	S/3280/6
Tris-hydrochloride	Fisher Scientific	BP153-1
Sodium carbonate an hydrous	FISONS Analytical Reagent	S/2920/53
Human Albumin 20% Solution	ZENALB 20	
D-Glucose an hydrous	Fisher Scientific	G/0500/61
Toluidine Blue	Fluka Analytical	89640
Phosphate buffered saline tablet	Sigma-Aldrich	P4417
Prostaglandin E1	Sigma-Aldrich	
Fibrinogen from Human Plasma	Sigma-Aldrich	F3879
Adenosin 5`-diphosphate sodium salt	Sigma-Aldrich	A2754
Formalin Solution 10% Neutral Buffered	Sigma-Aldrich	HT50-1-1
Dimethyl Sulphoxide (DMSO)	Sigma-Aldrich	
Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA)	Fisher Scientific	E3889
Heparin sodium salt	Sigma-Aldrich	H4784
Calcium Inophore A23187	Sigma-Aldrich	A23187
Mannan	Sigma-Aldrich	M7504
Zymozan	Sigma-Aldrich	Z-4250
N-acetylated BSA	Invitrogen	
Tween 20	Fisher Scientific	P1370
Barbital	Sigma-Aldrich	
Ethanol	Fisher Scientific	
Sigma Fast p-Nitrophenyl Phosphate tablet	Sigma-Aldrich	N2770-50SET
Horse Blood Defibinated	Thermo Scientific	SR0050C
Bovine serum albumin	Sigma	A3294
Blood Agar Base	Oxoid	CM0055
T4 DNA Ligase	New England BioLabs	M0203S
Fetal Bovine Serum (Inactivated)	Sigma	F7524
Heparin Sepharose 6 Fast Flow	GE Healthcare	17-0998-01
Coomassie Plus™ Protein Assay reagent	Thermo scientific	1856210
HBSS (1X) Hanks' Balanced Salt Solution ($CaCl_2$, $MgCl_{2)}$	Gibco by life technologies	14025-050
BD Vacutainer 9NC Sodium Citrate (0.105M)	BD Vacutainer	367691
GeneJuice Transfection Reagent	Novagen	70967

Dulbecco's Modified Eagle's Medium	Sigma	D5796
CHO-S-SFM II (1X) (+) L-GlutFamine	Gibco by life technologies	12052-098
F-12 Nutrient Mixture (1X)+GlutaMAX	Gibco by life technologies	31765-027
Penicillin/Streptomycin	Sigma	P4333-100
1X-Trypsin-EDTA solution	Sigma	T3924-100
Hygromycin B	Invitrogen	10687-010
L-Glutamine 200mM	Sigma	G7513
Pefablock SC (AEBSF)	Roche	11429868001
GeneJuice Transfection Reagent	Novagen	70967
dNTPs	Invitrogen	U1511
Chondroitin sulphate	Sigma	C9819
N,N,N,N,- Tetramethyl-ethylenediamine (TEMED)	Sigma	T9281
2-Mercaptorthanol	Aldrich chemistry	M6250
Brilliant Blue R-250	Fisher scientific	BP101-25
Ammonium Persulphate	Sigma	A3678

Table 2.1. Chemicals substances and media with their origin manufacturer and catalogue number were used.

2.1.2 Buffers and solutions

Agarose DNA Electrophoresis buffer	
Tris-Acetate-EDTA Buffer	10mM Tris-HCL 1mM EDTA 10mg/ml Ethidium bromide pH8.4

Competent cell preparation buffers	
TfbI solution	30mM K-acetate 50mM $MnCl_2$ 100mM KCl 10mM $CaCl_2$ 15% glycerol pH 7.4
TfbII solution	10mM Na-MOPS 75mM $CaCl_2$ 10mM KCl 15% glycerol pH 7.4

Proteins Purification buffers	
Thrombospondin purification buffers	
Binding Buffer	20mM Tris pH 8.0
Washing Buffer	20mM Tris 150mM NaCl pH 8.0
Elution Buffer	20mM Tris 500mM NaCl pH 8.0

Properdin purification buffers	
Binding Buffer	Phosphate buffer saline (Tablets) (PBS) 100mM NaCl 5–10mM Imidazole pH 8.0
Washing Buffer	Phosphate buffer saline (Tablets) (PBS) 100mM NaCl 10mM Imidazole pH8.0
Elution Buffer	Phosphate buffer saline (Tablets) (PBS) 100mM NaCl 500mM Imidazole pH 8.0

SDS-PAGE and Western blot buffers	
Stacking Gel (5%)	3.4ml dH_2O 0.83ml of 30% Bis-acrylamide gel 0.63ml of 1M Tris-HCl (pH6.8) 0.05ml of 10% (w/v) SDS 0.05ml of 10% (w/v) ammonium per-sulphate 0.005ml TEMED
Resolving Gel (12%)	4.9ml dH_2O 6ml of 30% Bis-acrylamide gel 3.8ml of 1M Tris-HCl (pH8.8) 0.150ml of 10% (w/v) SDS 0.150ml of 10% (w/v) ammonium per-sulphate 0.006ml TEMED
Resolving Gel (8%)	7.9ml dH_2O 3ml of 30% Bis-acrylamide gel 3.8ml of 1M Tris-HCl (pH8.8) 0.150ml of 10% (w/v) SDS 0.150ml of 10% (w/v) ammonium per-sulphate 0.012ml TEMED
Resolving Gel (6%)	4.9ml dH_2O 6ml of 30% Bis-acrylamide gel 3.8ml of 1M Tris-HCl (pH8.8) 0.150ml of 10% (w/v) SDS 0.150ml of 10% (w/v) ammonium per-sulphate 0.006ml TEMED

SDS Loading Dye	100mM Tris-HCl 4% SDS 5% β Mercaptoethanol 10% Glycerol 0.2% (w/v) Bromophenol blue pH 6.8
SDS-Transfer Buffer for Trans Turbo System	45ml of (17.5g Glycine 0.375g SDS 6g Trizma Base In 500ml d.H2O) Mixed with 5 ml methanol to become 10% (v/v) pH 8.3
SDS-Running Buffer	20mM Tris base 150mM glycine 0.038% SDS pH 8.3

ELISA buffers	
Tris-buffer saline (TBS)	10mM Tris-HCL 140mM NaCl 2.5mM $CaCl_2$ 2.5mM $MgCl_2$ pH 7.4
TBS-Blocking buffer	10mM Tris-HCL 140mM NaCl 2.5mM $CaCl_2$ 1% (v/v) HSA pH 7.4
Washing buffer	10mM Tris-HCL 140mM NaCl 2.5mM $CaCl_2$ 0.005% (v/v) Tween 20 pH 7.4
Coating buffer	15mM Na_2CO_3 35mM $NaHCO_3$ pH 9.6
Barbital buffer saline (BBS)	4mM barbital 145mM NaCl 1mM $MgCl_2$ 2mM $CaCl_2$ pH7.4

EGTA-Barbital buffer saline (BBS)	4mM barbital 145mM NaCl 10mM EGTA 1mM $MgCl_2$ pH7.4

Washing Platelets buffers	
HEPES buffer saline (HBS) (pH7.4 & pH6.0)	150mM NaCl 1mM $MgSO_4$ 5mM KCl 10mM HEPES pH 7.4 and pH 6.0

Anti-coagulant solution	
Acid Citrate Dextrose anticoagulant (ACD)	0.085M tri-sodium citrate 0.071M citric acid 0.11M glucose pH 6.0

Table 2.2. Buffers and solutions were used in different technique with their recipes.

2.1.3 Commercial Antibodies & Proteins

Purchased Antibodies & Proteins	Manufacture	Catalogue No.
Goat polyclonal IgG Anti-human TSP1 raised in mouse	Sigma	
Mouse polyclonal IgG Anti-human TSP1 raised in goat	Sigma	
Mouse monoclonal IgG Anti-human TSP1 raised in rabbit	Abcam	
Mouse monoclonal IgG Anti-human P-selectin CD62P raised in rabbit	Sigma	
Rabbit polyclonal Anti-human C3c IgG	Dako	
Mouse polyclonal Anti-human TSP1 IgG	Sigma-Aldrich	
Chicken monoclonal anti-human C4c	Immunsystem AB	
Mouse monoclonal anti-human MBL	ANTIBODYSHOP	
Mouse monoclonal anti-human fibrino-gen	Santa cruz Biotech.	
Mouse anti-poly-histidine-tag	Sigma	
Anti-mouse polyclonal IgG1 antibody	Sigma	
Anti-rabbit polyclonal IgG1 antibody	Sigma	
anti-goat polyclonal IgG1 antibody	Sigma	
Anti-chicken polyclonal antibody	Sigma	
Human CD41 αIIb	Abcam	
Human Fibrinogen	Sigma-Aldrich	
Human Plasma Thrombin	Sigma	
Human Collagen type I, III and IV	Sigma	
Human Fibronectin	Sigma	
Human von Willebrand Factor (vWF)	HTI	
Human CD36 (100-200 aa) (100 peptide sequence)	Abcam	
Human CD36-IgG-Fc	R&D	
Human CD47-IgG-Fc	R&D	
CD42b (GPIbα) (GPIb/V/IX)	Abcam	
CD42d (GPV) (GPIb/V/IX)	Abcam	
Human Low-density Lipoprotein (LDL)	Sigma-Aldrich	

Human Triglycerides	Sigma-Aldrich	
Human Cholesterol	Sigma-Aldrich	C8667
Calcium ionophore A23187	Sigma-Aldrich	

Table 2.3. The antibodies and proteins were used provided with their origin manufacturer and catalogue number.

2.1.4 Commercial Kits

Purchased Kits	Manufacture	Catalogue No.
QIAquick gel extraction Kit	Promega	Cat# 28704
Wizard plus SV minipreps DNA purification system	Promega	Cat# A1460
Nucleobond®Xtra Midi Kit	Macherey-Nagel	Cat# 740410.50
ZYMUPHEN MP-Activity Kit	HYPHEN BioMed	Cat# 521096
Thrombin (Human) ELISA Kit	Abnova	Cat# KA0511

Table 2.4. The commercial kits were used provided with their origin manufacturer and catalogue number.

2.1.5 Oligonucleotides

All primers were purchased from Europhin MWG Biotechnology at 100 pmol/µl. And those primers were prepared at concentration 5 pmol/µl.

Primer	Primer sequence
hTSP-1-Forward-KpnI	5'-GGT ACC ATG GGG CTG GCC TGG GG-3'
hTSP-1-Reverse & Stop-HindIII	5'-AAG CTT TTA GGG ATC TCT ACA TTC GTA TTT CAG GT-3'

Table 2.5. The designed oligonucleotide primers provided with their sequences for PCR used to amplify the cDNA of TSP1. They were manufactured by Europhin MWG Biotechnology.

2.1.6 Vectors

Purchased Vectors	Manufacture	Catalogue No.
pGEM-T Easy vector	Promega	Cat# A137A
pCEP4	Invitrogen	Cat# V044-50

Table 2.6. The purchased vectors used for cloning process of TSP1.

2.1.7 Instruments

Name of Instrument	Manufacture
Thermocycler machine; Biometra T1^{Plus} Thermocycler	Thermo scientific
Trans-Blot® Turbo™ Transfer System	Bio-Rad Cat#170-4155
iMark™ Microplate Reader	Bio-Rad Cat#168-1135
Inverted Microscope	Cat#
UV transmission light	Cat#
Mini-PROTEAN® Tetra Cell	Bio-Rad Cat#165-8025
Nanodrop 1000 Spectrophotometer system	Thermo Scientific Cat# 165-8025

Table 2.7. The instruments were used during the PhD study.

2.2 Methods

2.2.1 DNA Molecular Technique

2.2.1.1 Generation the DNA construct of human TSP1 by Polymerase Chain Reaction (PCR)

A normal PCR was used to amplify human genome of TSP1 from human cDNA using two designated primers; hTSP-1-Forward-Kpn1 and hTSP-1-Reverse&Stop-HindIII primers (Eurofins MWG Operon). The forward primer binds to a specific sequence on the antisense strand of the cDNA, whereas, the other sense of a specific strand is amplified via the reverse primer. The master mix consisted of a High fidelity polymerase (Finnzymes, Cat# F-530S) and a fixed molar concentration of $MgCl_2$ in high fidelity polymerase phusion buffer 5x (Finnzymes) and also dNTPs mix (Promega, Cat# U1511) are required for DNA template synthesis. The reaction mixture was incubated in several steps each has different temperature for total 35 cycles, to gain the amplification of the DNA target sequence, using the automated thermal cycler machine. 95°C was the first temperature to denature the DNA template followed by 64°C which was the annealing process to allow primers to anneal to their DNA target sequence. 75°C was the third temperature as an optimal temperature for generating or synthesis the new DNA strands using DNA polymerase. After that, the reaction products of PCR were run on 1% agarose gel using electrophoresis to analyses the size of the generated DNA of the PCR products which were visualised using Ultraviolet Trans-illuminator (Bio-Rad). That was due to mixing the amplified DNA with Bromophenol blue (loading dye 6X) and Ethidium bromide (Et Br) (Sigma-Aldrich). The last one intercalates itself between the double strand DNA in order to attach with nucleic acids to be visualised and detected under UV-light in fluorescent emission. Agarose gels were pictured using Kodak EDAS 290 camera.

By using a standard PCR reaction, each PCR reaction mixture consisted the following

Reaction mixture contents	Volume per reaction
cDNA [1:10] 10ng	1 µl
High fidelity phusion buffer (5x)	5 µl
dNTP mix. (10mM)	0.5 µl
hTSP-1-Forward-KpnI	2.5 µl
hTSP-1-Reverse & Stop-HindIII	2.5 µl
High fidelity-DNA phusion polymerase (5U/µl)	0. 25 µl
Nanopure distilled water	13.25 µl

Table 2.8. The recipes of PCR reaction for amplifying DNA of TSP1.

The primers were used to amplify the cDNA target sequence of human TSP1 in the restriction enzyme's sites of Kpn1 and HindIII.

Primer	Primer sequence
hTSP-1-Forward-KpnI	5'-GGT ACC ATG GGG CTG GCC TGG GG-3'
hTSP-1-Reverse & Stop-HindIII	5'-AAG CTT TTA GGG ATC TCT ACA TTC GTA TTT CAG GT-3'

The cycling programme was:

Cycle	Temperature	Time	Manner
Initial denaturation	98°C	1:30 minute	
Denaturation	98°C	15 second	 **15 Cycles**
Annealing	70°C (-0.8C/cycle)	30 second	
Extending	72°C	40 second	
Denaturation	98°C	15 second	 **25 Cycles**
Annealing	58°C	30 second	
Extending	72°C	40 second	
Final extending	72°C	5 minutes	
Cooling down	4°C	∞	

Table 2.9. The program process was used to amplify the DNA of TSP1 using thermocycler machine.

2.2.1.2 DNA purification from TAE-agarose gel (band prep)

After cutting the interesting DNA band under UV-light by clean and sharp scalpel, all DNA fragments were purified from agarose gel by using QIAquick Gel Extraction Kit (Promega). The sliced Gel were placed in 1.5µl eppendorf tube and then weighted and dissolved in three volumes of QG buffer (1 volume of gel into 3 volumes of QG) (provided by the kit) at 55°C for 5-10 minutes with shaking. The dissolved gel was transferred into the QIAquick column and then the column was centrifuged for 1 minute at 6000xg. The silica column is prepared to be sensitive for binding DNA. The column then was washed by adding 500µl QG buffer and centrifuged for 1 minute. Next, 750µl PE buffer (Ethanol) was added for washing purpose and then centrifuged for 1 minute. The QIAquick column was centrifuged again to release the residual ethanol amount of PE buffer at maximum speed for 1minute. The DNA was then eluted by adding 50µl elution buffer EB into clean 1.5µl eppendorf tube.

2.2.1.3 Chemical competent *E.coli* Top10F strain

In order to generate the competent *E.coli* Top10F to has the ability to up take plasmid DNA, the protocol of (Hanahan, 1983) was followed by treating the bacterium with ice cold providing shock condition in the presence of Ca ions followed by heating by which presumably rendering the adherence of the plasmid DNA into the cells. This method briefly depends on shocking in ice and heating at 42°C or 37°C for 45 seconds or 5minutes respectively, by which allowing the DNA plasmid to be up taken by bacterial cells, and then shock in ice. However, the actual mechanism of entering the plasmid DNA into the competent cells is still unknown.

2.2.1.4 Chemical preparation of competent *E.coli* Top10F strain

By a full loop of sterile platinum wire immersed in *E.coli* strain frozen stock to streak it onto LB agar plate in order to get one single colony. At 37°C, the streaked plate was incubated for 16-18 hours. Next, one single colony was inoculated into 5ml of non-selective medium (LB broth medium) containing 20mM $MgSO_4$ to obtain an increase in cell density, and then cells grew at 37°C for overnight incubation. After incubation, 100ml LB broth medium was incubated in shaker incubator at speed 250-300rpm with

1ml overnight culture at 37°C for 2-3hours until the absorbance of the optical density (OD) reach 0.7–0.8 Abs at 550nm. Then, by centrifugation, cells were harvested at 2000xg for 10 minutes. After that, 30ml of ice cold TfbI buffer was used to re-suspend the cells pellet following incubation on ice for 30minutes. Next, cells were centrifuged again at 2000xg for 10minutes at 4°C and then, 4ml of TfbII buffer was used to re-suspend the cells pellet in order to distribute/aliquot of 100µl in each 0.5ml eppendorf tube to store them at -80°C or for immediate transformation.

2.2.1.5 Heat & Cold shocking Transformation of competent *E.coli* Top10F strain

2µl of ligation product was transformed into 50µl competent *E.coli* (Top10F strain) by mixing gently the contents, and then incubation in ice for 20 minutes and then, the mixture of cells was heat shocked at 37°C for 5minutes or 42°C for 45seconds. Repeatedly, the mixture was returned again into the ice for 2 minutes to complete the transformation. The mixture was placed in falcon tube provided with 450µl of non-selective medium (LB broth), which allows to produce/synthesis the plasmid DNA encoded with antibiotic resistance gene, and then incubated at 37°C for 1hour with gently shaking. Meanwhile, 30µl X-gal and IPTG were spread on the LB agar containing 100µg/ml ampicillin. After incubation of the transformed competent cells, all amounts of transformed cells were poured and spread onto the LB agar plates followed by incubation for overnight at 37°C. After overnight incubation, blue colonies were banned of isolation while white single colonies were selected and cultured again into 5ml LB broth medium containing 100µg/ml ampicillin and then incubated for overnight at 37°C with gently shaking for plasmid DNA purification process.

2.2.2 Cloning of Molecular DNA

2.2.2.1 A-Tailing of PCR products

There are such enzymes can provide Adenine (A) to the 3'terminus of PCR DNA products in order to make blunt ends. As a result of A-tailing, the T-overhang in pGEM-T Easy vector (Promega) can be used as a compatible with the PCR product for ligation. Taq-polymerase (Thermo Scientific) is one of those enzymes was used to provide DNA products with blunt ends by which the T-overhang can be ligated with using T4 DNA

ligase. The Taq-polymerase has a dependency of 5'-3' exonuclease activity. The reaction mixture of A-tailing was incubated with the PCR product at 72°C for 15minutes. The total mixture was 10µl containing as the following materials; 1µl of DNA Taq-polymerase (Thermo Scientific), 1µl Taq-polymerase reaction buffer 10x (containing $MgCl_2$), 1µl 0.2mM dATP (Invitrogen), and 7µl gel-purified PCR DNA product. After 15minutes incubation, the A-tailed PCR product was then stored at -20°C to be used later for the next step which is the ligation process for cloning into pGEM-T Easy vector.

2.2.2.2 Ligation and cloning PCR products with pGEM-T Easy Vector

pGEM-T Easy vector (Promega) was provided with a single thymidine (T) at 3'terminus in both ends to keep the vector opened in linear position. The T-overhang prevents vector from re-circularisation, and also provides compatible ends with the A-tailed PCR products (Mezei and Storts, 1994 and Robles and Doers, 1994) (Figure 2.1). 50ng/ µl pGEM-T Easy vector was ligated with (ng) the A-tailed DNA of PCR products in the presence of T4 DNA Ligase buffer and T4 DNA ligase. The volume of DNA needed for the ligation was calculated using the equation given by (Sambrook et al., 1989):

ng insert of DNA=

$$\frac{\text{(ng vector DNA) x (kb size of the insert DNA)}}{\text{(Kb size of the vector)}} \times \frac{\text{insert}}{\text{vector}} \text{ molar ratio}$$

The ligation reaction mixture was completed in final volume of 10µl Nano pure water. The reaction mixture containing as the following materials; 1µl pGEM-T Easy vector (50ng/µl) and then mixed with amount of insert to assess 3:1 molar ratio (insert:vector) (x)µl the PCR product (x ng), 1µl T4 DNA ligase (5units) (Promega) and 5µl T4 DNA ligase buffer (2x) (Promega). After that, the reaction mixture was incubated for overnight at 4C°. After that, the ligation products were transformed into the competent cells; *E.coli* (Top10F strain), in order to provide a Mini scale purification of plasmid DNA using (Miniprep protocol) as later will be mentioned.

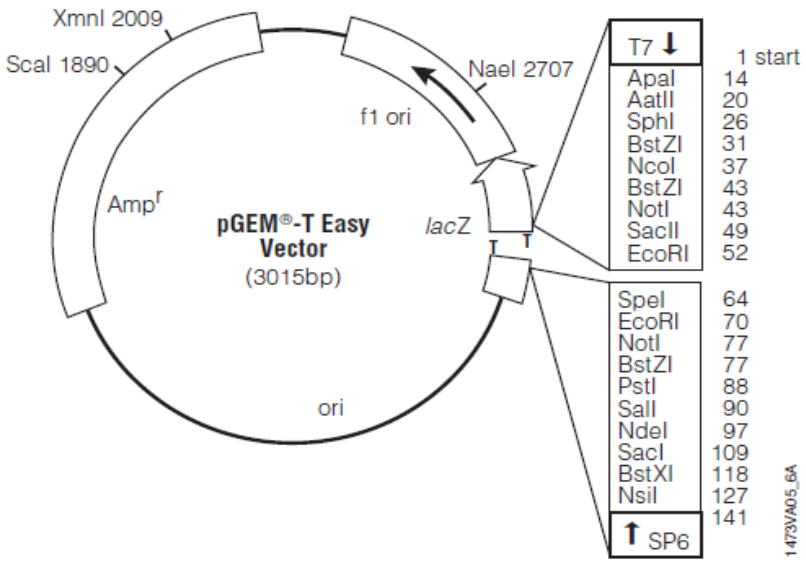

Figure 2.1. A diagrame shows T-overhang and the restriction sites of restriction enzymes. Adapted from the technical manual protocol of pGEM-T and pGEM Easy Vector Systems. *Promega*.

2.2.2.3 Restriction digestion of the DNA construct from the pGEM-T-Easy vector

The purified and isolated DNA fragment and DNA plasmid were digested as follow:

Reaction mixture contents	Volume per reaction
The purified DNA (1ug)	3µl
BSA 10µg/µl	2µl
Restriction enzyme buffer (2) 10X	2µl
Restriction enzyme (Kpn1) 10µg/µl	1µl
Restriction enzyme (Hind III) 10µg/µl	1µl
Deionized distilled water	11µl

Table 2.10. The recipes of restriction digestion reaction of the DNA construct of TSP1 in pGEM-T-Easy vector.

The reaction mixture was incubated in water path at 37°C for 1.5-2hours and then, was run on 1% gel in order to determine the size of the expected bands.

2.2.2.4 Sub-Cloning the DNA construct into the expression vector pCEP4/HygromycinB

The DNA construct of TSP1 in the pGEM-T easy vector (Promega) was digested by the same previous restriction enzymes and evaluated by agarose gel electrophoresis. Then, the purified DNA construct was ligated into pCEP4 vector (Invitrogen) using T4 DNA ligase procedure. It was essential to linearize the pCEP4 before the ligation process by using the same restriction enzymes were used for the previous vector. After that, the ligation products were transformed into the competent cells; *E.coli* (Top10F strain), in order to provide a large scale purification of plasmid DNA using (Maxiprep) as later will be mentioned.

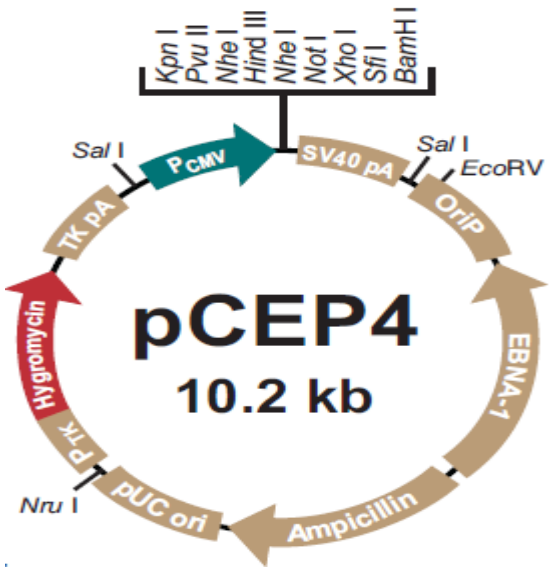

Figure 2.2. A diagrame shows pCEP4 vector circle map. Adapted from the technical manual protocol of pCEP4 Vector. *Invitrogen*.

The DNA consturct of human properdin has already engineered into pSecTag2/hygroB expression vector to have the following restriction sites; HindIII and XhoI. The vector contains START codon (ATG) of the N-terminal sequence and the C-terminal poly-histidine tag followed by STOP codon (TAA), and the plasmid was kindly provided by Dr. M.A Youssef (University of Leicester, UK) (Ali et al., 2014).

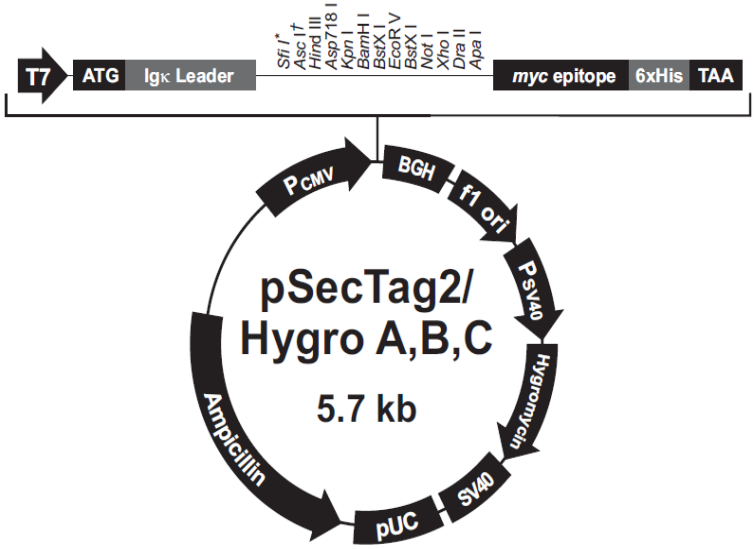

Figure 2.3. A diagrame shows pSecTag2/Hygro vector circle map. Adapted from the technical manual protocol of pSecTag2/Hygro Vector. *Invitrogen.*

2.2.2.5 Isolation and purification of plasmid DNA

2.2.2.5.1 Isolation and Purification using Mini scale (Miniprep) of plasmid DNA (pGEM-T Easy vector/ TSP1 DNA construct)

Plasmid DNA was isolated and purified by using Wizard plus SV Miniprep DNA Purification System (Promega). The 5ml of the overnight culture, containing the white transformed colony provided with 100µg/ml ampicillin, was centrifuged for 10minutes at 13000xg in order to re-suspend the pellet using 250µl cell re-suspension solution. Then, 250µl cell lysis solution was added in order to damage the competent cell wall to release/free the plasmid DNA. The mixture, in 1.5ml eppendorf tube, was inverted gently for 4 times and all tubes kept for 1-5minutes at room temperature (RT). After that, 10µl alkaline protease solution was added and mixed and then incubated at RT for 5minutes. Next, 350µl neutralisation solution was added and then mixed well by vortex. All cell debris was eliminated by centrifugation at maximum speed 13000xg for 10minutes. The clear supernatant was transferred into a spin column (provided from the kit) and washed with 750µl washing buffer (ethanol) followed by adding approximately 75µl Nuclease Free Water. Lastly, the plasmid DNA was eluted in clear tubes by centrifugation at maximum speed 13000xg for 1minute.

2.2.2.5.2 Isolation and Purification of Large scale of plasmid DNA (Maxiprep)

By using Nucleobound®Xtra Midi kit (Macherey-Nagel), large scale purification of plasmid DNA from the competent cells (TOP10F *E.coli*) was processed. 10µl the starter culture having the desired plasmid DNA; pCEP4/HygromycinB/hTSP1 and pSecTag2/hygroB/hProperdin, was inoculated into 100ml LB broth medium consisting of 100µg/ml ampicillin and then incubated for 12-16hours under rotation manner for 200-300 rpm at 37°C. After overnight incubation, cells were centrifuged at 6000 xg for 15minutes at 4°C. Next, cell sedimentation was re-suspended by adding 4ml S1+RNase buffer. Next, by adding 4ml cells lysis buffer S2, the lysed cells were mixed gently by inverting the tubes 6-8times. Then, the lysate mixture was incubated at RT for 2-3minutes. The basic principle of the lysis process briefly provides alkaline condition

with SDS existence by which denaturing chromosomes and plasmid DNA. Neutralising buffer (S3) was pre-cooled on crushed ice (4°C), as stated on the kit protocol, and then 4ml of the S3 was added to the suspension mixture and then mixed gently by inverting the tube 6-8times until the suspension containing white flocculate formation. In the previous step of neutralising condition contains Potassium acetate by which forming precipitation for chromosomal DNA and the other cellular debris while the plasmid DNA returns to its natural supercoiled structure by which remains in the supernatant. Next, the suspension was incubated on ice for 5minutes. Afterward, clarification step was done in order to clarify the bacterial lysate by centrifugation at 13000xg for at least 40minutes at 4°C. In the meantime, the Nucleobound column was equilibrated with 2.5ml buffer N2. The basic principle for equilibration is to allow the plasmid DNA to bind to the anion-exchange resin in the column. After column equilibration, the clear lysate was filtered using the Nucleobound filter paper. Then, the filtered clear lysate was transferred into the Nucleobound column by letting to empty or pass by gravity. Next, washing step was processed twice using 10ml of buffer N3 and then the plasmid DNA was eluted by 5ml of buffer N5 into Beckman tubes. Afterward, precipitation step was processed in order to precipitate the eluted plasmid DNA by adding 3.5ml isopropanol. The plasmid DNA was obtained by centrifugation at 15000xg for 3 minutes at 4°C. After discarding the supernatant, the plasmid DNA pellet was washed by 2ml 70% ethanol followed by briefly vortex the pellet and centrifuged at 15000 xg for 10minutes at RT. The ethanol was removed carefully using pipette tip and the plasmid DNA pellet was dried at RT for 10minutes. Finally, the plasmid DNA pellet was reconstituted or dissolved in a desire volume (500µl) buffer TE (Promega) or sterile deionized water for transfection usages.

2.2.3 Cell Culture Unit Techniques

In this section all dealing with cell culture must be in sterile condition using safety cabinets and also sterile tools e.g. pipets, etc., by spraying 70% Ethanol or Industrial Methylated Spirits (IMS) into the equipment. The cells were chosen for transfection, were used to determine the certain concentration of the selective antibiotic (HygromycinB) that the eukaryotic cell line could not survive at the certain condition. Firstly, the eukaryotic cell line need to be grown in 6 well plate for 2-3 days till become 50-60% confluent. Secondly, cells need to be washed at once by PBS while the previous serum media

require to be replaced with new serum media supplemented with different concentrations of HygromycinB beginning from 50µg/ml, 100µg/ml, 150µg/ml, 200µg/ml, 250µg/ml, and 300µg/ml and go further for high concentration if it is required. Ultimately, the treated cells require incubation under observation day by day. Through the observation, dead cells can be noticed with the higher concentration of HygromycinB while cells continue to grow on the low concentrations. Therefore, the certain concentration of the antibiotic by which a point kill and prevent non-transient cells from growing should be determined. The basic protocol of tissue culture technique was followed as described by (Phelan, 2007).

2.2.3.1 Human Embryonic Kidney (HEK 293) Cell Line Transfection

HEK293 cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented by the manufacture with L-glutamine and high glucose concentration (Sigma). The media was supplemented with 10% Heat Inactivated Fetal Bovine Serum FBS (GIBCO) and also Penicillin/Streptomycin (Sigma) was added as 1ml:100ml media to be an equivalent 0.1mg. The HEK293 cell line was incubated in the presence of 5% CO_2 conditions at 37°C until the growth become 40–70% confluence. After all, 100µl serum-free medium (for example, RPMI1640 (Sigma) or Opti-MEM (GIPCO)) was added into a sterile 1.5ml eppendorf tube. Then, 3µl GeneJuice Transfection Reagent (Novagen) was mixed by vortex with the free serum medium. The reaction mixture (GeneJuice reagent/serum free medium) was incubated for 5min at RT. Next, 1µg of the prepared plasmid DNA was added into the reaction mixture and mixed gently by pipetting. The reaction mixture (GeneJuice reagent/serum free medium/DNA) was incubated for 15min at RT. After that, the entire volume of the mixture was transferred to the grown HEK293 cell line as drop wise in complete growth DMEM. Then, the cells were incubated in 5% CO_2 at 37°C for 24-72hours. After that, the transient HEK293 cell line was trypsinised using 1ml EDTA-Trypsin (1X) (Sigma), and then the cell line was resuspended in DMEM serum media containing a selection of 180µg/ml HygromycinB (Invitrogen) by which at this concentration the non-transient cells were killed, however, the transient cells (up taking DNA plasmid) can grow under no effect of HygromycinB due to having HygromycinB resistance gene within the DNA plasmid.

Following, the cell suspension was transferred and distributed into 24-well plates, and incubated until cells were confluent. The expression of recombinant human TSP1 was screened by using Dot blot and confirmed by Western blot. Any well showing positive results in both Dot blot and Western blot techniques was split and diluted into 96-well plates in order to obtain a single cell colony that produces TSP1. Finally, after selecting the single cell colony of HEK293 cell, the cells were split into 6-well plates and then, after confluence, transferred to 25cm small flasks and later to 75cm medium flasks by which later cells transferred into 225cm^2 large flasks for protein production and also for freezing propose in liquid nitrogen.

2.2.3.2 Chinas Hamster Ovary K1 (CHO-K1) Cell Line Transfection

CHO-K1 cell line was cultivated in F-12 Nutrient Mixture (1X)+GlutaMAX media (GIBCO), which was supplemented with 10% Heat Inactivated FBS (GIBCO). Penicillin/Streptomycin (Sigma) was added as 1ml:100ml media to be an equivalent 0.1mg. The CHO-K1 cell line was incubated in the presence of 5% CO_2 conditions at 37°C until the growth become 40–70% confluence. The same procedure of HEK293 cell was followed for DNA plasmid transfection using GeneJuice Transfection Reagent (Novagen).

After that, the transient CHO-K1 cell line was trypsinised using 1ml 1X-Trypsin (Sigma), and then cells were re-suspended in F-12 Nutrient Mixture (1X)+GlutaMAX (GIBCO) serum media containing a selection of 300µg/ml HygromycinB (Invitrogen) by which the non-transient cells were killed. The expression of human recombinant Properdin was confirmed as previously described in HEK cell line transfection protocol.

2.2.3.3 Protein Production

2.2.3.3.1 Large scale production of TSP1 and Properdin

2.2.3.3.1.1 Large scale production of TSP1

A single clone of transfected HEK293 cell line with pCEP4/HygromycinB/TSP1 plasmid DNA was transferred into cell culture polystyrene angle neck large flask 225cm² (Corning) with vent cap. The flask is made with single polystyrene layer 225cm^2 in size to maintain such detachable cells like HEK293 cell line, which are weaker in attaching to polystyrene, in order to prevent disturbing such cells in washing process. The 4-5 flasks provide enough growing cell layer that is able to synthesis a mass production of TSP1 approximately 0.5mg/ml of the first batch. The trypsinised transfected cell line was transferred into 500ml DMEM media supplemented with 10% FBS, 5ml/500ml Penicillin/Streptomycin and 180µg/ml HygromycinB. The mixture of DMEM media containing trypsinised cells was split into 5 polystyrene large flasks 225cm^2 where each flask takes about 100ml medium. The transfected HEK293 cell line incubated for 4-5 days till reach the optimum confluence (80-90%) by which to obtain mass production of TSP1. After that, warmed PBS was required for washing process for the cell layer by 3times with awareness of detachable cells; meanwhile, 500ml of DMEM serum free media was prepared without adding FBS and split into the 5 flasks. The transfected cell line was incubated again with the serum free medium at 37°C for 60-72hours with providing 5% CO_2. Lastly, the media was harvested and replaced by another new serum media. The supernatant was collected for TSP1 purification purposes.

2.2.3.3.1.2 Large scale production of Properdin

The same procedure was used for large scale production of human TSP1; it was also followed to obtain large scale production of human Properdin with varieties of materials supplementation. The media was used for Properdin production was CHO-S-SFM II (GIBCO) which is suitable to maintain CHO cells for recombinant proteins production while HygromycinB was supplemented in concentration 300µg/ml. In addition, the flasks required for protein purification for CHO cell line were triple flasks vent/close cap (Thermo scientific). The flasks composed of three parallel surfaces (Nunc) offer culture area of 500cm^2 representing 175cm^2 for each layer. The 4-5 flasks provide

enough growing cell layer that is able to synthesis a mass production of Properdin approximately 0.250mg/ml for the first batch.

2.2.4 Protein Purification

2.2.4.1 Purification of recombinant human TSP1 (TSP1) and highly oligomerised recombinant human Properdin (Properdin)

In order to purify a mass production of TSP1 from transfected clone of HEK293 cell, the serum free media was harvested, collected and also replaced with another new serum-free media prepared for a second batch. This process was repeated almost three times. The harvested media was centrifuged at high speed 4500rpm for 10-15 min to sediment and remove the cells remnants. The supernatant was collected in 1L bottle and mixed with 100ml 10X Tris-HCl (1X = 20mM Tris-HCl, pH 8.0), and then the pH adjusted to 8.0 followed by filling the bottle with distilled water up to 1L. 20mM Protease inhibitor of Pefablock SC (4-(2-Aminoethyl)-benzenesulfonyl fluoreide, hydrochloride white crystalline powder) (AEBSF) (Roche) was required to prevent the possibility of protein proteolysis. Meanwhile, 2-1ml Heparin Sepharose 6 Fast Flow (GE Healthcare) was added into a chromatography column (Bio-Rad) then washed several times for sepharose equilibration using binding buffer until the content of preservative ethanol in the heparin sepharose washed away. Non-mutated recombinant human TSP1 was purified on top of the prepared column by letting the media to flow through the heparin sepharose column upon gravity in cold room at 4°C.

The principle of purification depends on the specification of the structure of human TSP1 which consists of heparin binding domain (HBD) within the N-terminal domain which allows TSP1 to bind to heparin. In addition, the TSR domain was found to bind heparin. Thus, TSP1 will be captured by heparin binding affinity using chromatography column. The other expressed proteins from the cell line were free of binding to Heparin Sepharose 6 Fast Flow.

After passing the media through the column, the column was washed using washing buffer three times for 10ml each. Next, TSP1 was eluted using the elution buffer containing 500mM NaCl. The eluted TSP1 was collected in 1.5ml eppendorf tubes and required 1ml for each fraction; 4–5 fractions. The purified TSP1 was analysed by SDS-

polyacrylamide gel electrophoresis (SDS-PAGE) and native PAGE, and also followed by Western blot.

The previous process was technically as the same as Properdin purification process; however, the binding affinity of Properdin was used toward a Nickel-Sepharose chromatography column and due to the engineering mutation was processed in the C-terminal of Properdin. The mutation was considered to have 6 His-tag residues for purification purposes. Therefore, the protein can be trapped using His GraviTrap column (GE Healthcare). The buffers were used for Properdin purification are PBS contain different concentrations of imidazole (Table 2.2).

2.2.4.2 Protein Dialysis

In order to neutralise the NaCl within the purified TSP1 or eliminate imidazole within the purified Properdin, a membrane of snakeskin 10kDa cut off (Thermo) was used. The dialysis depends on the specification of membrane permeability which allows the high concentration of salts or imidazole to exchange to the low concentration medium. Thus, protein elution fractions were poured into the membrane and then the membrane was closed well. Furthermore, the membrane was placed in a beaker containing PBS pH 7.4 and, then, left for stirring on a stirring instrument for overnight in cold room at 4°C. After that, the whole amount of the dialysed sample was concentrated using a concentrator tube 10 or 30kDa cut off (Millipore).

2.2.4.3 Purification of recombinant human TSP1 using Gel filtration HPLC

After protein purification, dialysis and protein concentration, the purified protein was passed through a filtration column in order to eliminate of any existence of contaminant proteins by High Performance Liquid Chromatography (HPLC) using dextran gel known Hiload^{TM} 16/60/Superdex^{TM} 200/prep-grade column with geometric column volume (Vc) 120ml (Kindly provided by Dr. Russel Wallis, University of Leicester).

The principle of gel filtration column depends on the porous beads that are able to separates molecules upon their sizes as large molecules pass faster than smaller molecules via the beads through the column (Stellwagen, 1990, Laurent and Laurent, 1964). Therefore, the large molecules such as TSP1 540kDa, emerge first from the column through the external volume while the contaminant proteins access the internal volume through the beads and emerge later. The HPLC is computerised to measure eluted pro-

teins via sensor. Measuring protein occurs for each fraction using Milli-absorbance Unit (mAU) that is converted into histogram leading to illustrate protein concentration.

The superdex^{TM} 200 column was equilibrated by filtered Tris-HCL buffer. Meanwhile, after protein dialysis process, 5ml of the total volume of the purified TSP1 was loaded into the column using 5ml syringe (BD Corning). Then, the elution step was computerised by monitoring continuously the flow rat of pumping out 1ml/min of eluted TSP1 known as fraction via the column. The eluted volume was monitored through using absorbance at wavelength 280nm. The amount of eluted TSP1 appears on the monitoring screen as a histogram peaks which explain passing proteins through the column with different peak elevation. Passing protein through the column depends on the type of column used and the molecular weight of the purified protein. After all, the interested fractions depends on their histograms were collected and electrophoresed using SDS-PAGE as well as western blot to investigate and identify which fraction that refer to TSP1 with purity of any contaminants.

2.2.4.4 Tag Proteolysis

Human recombinant Properdin was engineered to have 6xHis-tag for purification purposes. That tag was cleaved using Tobacco Etch Virus Protease (TEV) (Purchased from Biochemistry department, University of Leicester). 1Unit of TEV protease is able to cleave 100μg tagged protein. Therefore, 500μg/1ml recombinant Properdin was incubated with 10U TEV protease in phosphate buffer for overnight at 4°C. After incubation, the reaction was transferred to the heparin sepharose chromatography column in order to purify properdin depending on the conserved TSR domain by which can bind to heparin, then, the column was extensively washed several times using PBS to eliminate the remaining of TEV protease. Finally, Properdin was eluted using PBS containing 140mM NaCl . Fractions of eluted protein were concentrated in order to test the function of the protein.

2.2.4.5 Calculation of Protein Concentration

To measure protein concentration, it was estimated automatically using the Nanodrop 1000 system (Thermo scientific) which depends on UV-V spectrum which can calculate protein concentration upon protein absorbance at 280nm (A280). The system can estimate the concentration using micro-volumes. However, Bradford assay using Coomass-

ie Plus™ Protein Assay reagent (Thermo scientific) was used to quantify the total protein concentration in comparison to standard protein. 300µl of Coomassie plus reagent was placed in 96-well flat plate. Known concentration of standard BSA was serially diluted starting from 2mg/ml. Then, an equal volume, for example 20µl, from each dilution was added into separate wells in duplicate. The same amount for the purified protein was added while the last well was considered as blank. All the wells contents were mixed gently by pipetting and then incubated for 10min at RT. The color, was formed, was absorbed at wavelength 595nm using Bio-Rad iMark microplate reader. To analyse the data, the average result of each standard dilution was calculated and fitted into a standard curve. Y was representing the weave length OD 595nm while the X was the protein dilutions. Lastly, the concentration of the purified protein was calculated by placing the final average absorbance value amongst other standard concentrations of BSA.

2.2.5 Protein Techniques

2.2.5.1 Sodium Dodecyl Sulphate (SDS) Poly-Acrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE can be used to check the purity of protein fractions after chromatography technique. In order to assess the characterization of TSP1, the recombinant protein was run on SDS-PAGE and native PAGE under reducing and non-reducing conditions, respectively. In order to break down disulphide bonds (S-S), which bind the polypeptide chains together, within the protein structure, β-Mercaptoethanol (Reducing agent) (Sigma) was added into the protein 5X loading dye containing SDS (100mM Tris-HCl, 4% SDS, 5% β-Mercaptoethanol, 10% Glycerol, 0.2% (w/v) Bromophenol blue, pH 6.8). Moreover, to initiate the denaturation process, boiling the loading mixture was processed at 95°C for 5min. The protein structure will become linear (un-folding condition) because of the heating process. SDS works as an anionic detergent and also keeps the protein linear. Thus, the denatured polypeptides become negatively charged; hence, protein migrated by electrophoresis according to the protein molecular size. Therefore, in order to estimate the protein size, protein markers of known molecular weight were used.

30µl each fraction was mixed with 7µl of protein loading dye with and without SDS. Then, the SDS tubes were heated for 5 min at 95°C. All tubes; SDS or without SDS, were loaded into 8% SDS-PAGE and 6% native PAGE, respectively. The usage of 6% native PAGE was to estimate the size of native structure of TSP1 in non-reducing condition. Both gels were run in running buffer 1X with and without SDS at 150V for about 60 min. The protein, first of all, migrates into the stacking gel, and because of having high percentage of pores, that admits to categorise different proteins on different molecular sizes at the same time. Next, the protein is deposited like a layer onto resolving gel. The latter can separate proteins according to their molecular weight. Lastly, the size of protein was estimated according to protein molecular weight compared with molecular weight standard.

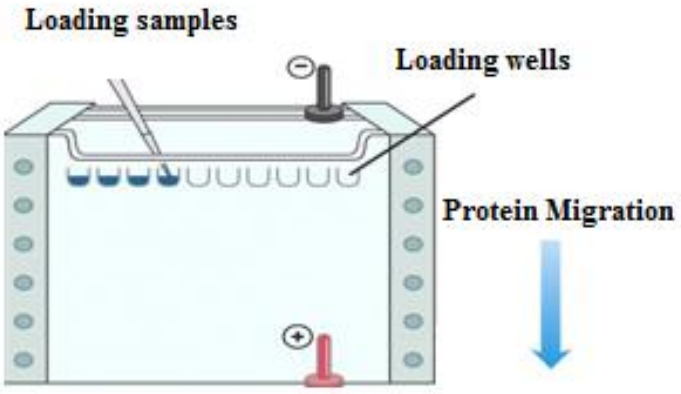

Figure 2.4. Schematic illustration shows the principle of Protein Electrophoresis.

2.2.5.2 Coomassie Blue Stain

Using Coomassie brilliant blue R-250 to visualise proteins was first implemented in 1963 by Fazekas de St. Groth and colleagues (Fazekas de St Groth et al., 1963). After two years, Coomassie brilliant blue R-250 was, then, used by Meyer and Lambert for staining proteins in polyacrylamide gel (Meyer TS, 1965).

In order to visualise protein after electrophoresis either using SDS or native PAGE, the gel was carefully taken off from the supporting glass plates and then placed in filtered Coomassie brilliant blue R-250 (Fisher Scientific). The gel with stain was slowly shaken in a circular manner for 30 min. After that, the gel was placed in Coomassie De-stain solution. In order to obtain proper stain decolourisation, the gel was gently shaken in a circular manner. That process was repeated several times until the background of the gel become clear. Therefore, the protein can be easily seen and compared with the protein marker (Ladder) (Invitrogen) in order to estimate the molecular weight of the interesting protein. The polyacrylamide gels were visualised under using white Trans-illuminator. All gels were scanned using scanner connected to computer in order to visualise better imaging.

2.2.6 Immune Blotting Techniques

2.2.6.1 Dot Blot

For short time and fast check-up to many clones of transient HEK293 and CHO-K1 cell lines for TSP1 and Properdin expression, respectively, Dot blot was used to analyse and determine the positive clones that express the proteins. In this technique, 2-5µl of purified proteins, served as positive controls, and 50-70µl of culture supernatant from different clones were spotted onto nitrocellulose membrane. At RT, the membrane was left to dry and then blocked using 5% skimmed milk powder (Oxoid) in PBS for 1 hour under shaking condition. The skimmed milk blocks the residual protein binding sites in the membranes. Following the blocking step, the primary antibodies, a monoclonal anti-human TSP1 (Abcam) to detect the expressed TSP1 was prepared using 1:1000 in blocking buffer while polyclonal anti-histidin Horse Radish Peroxidase (HRP) conjugate (Sigma) was prepared using 1:2000. The antibodies were applied into the membranes and incubated for 1hour at RT. After that, the membranes was washed three times in washing buffer (PBS containing 0.05% Tween 20) for 5min each. During the washing step, the secondary antibody, goat anti-mouse Horse Radish Peroxidase (HRP) conjugate was prepared using 1:5000 in blocking buffer for TSP1 while the membrane of Properdin was ready for detection. Next, the secondary antibody was applied into the membrane and incubated for 1hour at RT. Then, washing was processed three times for 5min each. Lastly, the membranes were incubated with Horse Radish Peroxidase substrate (Luminata Crescendo) (Millipore) for one minute and then wrapped in cling film and placed in an autoradiography cassette to be exposed to autoradiography film (X-ray film) (Fuji Film) for 1–30 min depending on the signal intensity.

2.2.6.2 Western Blot

The Western blot technique was used in order to recognise and identify the protein of interest using monoclonal antibody. Elution fractions of the purified proteins were migrated first via SDS or native PAGE and then blotted into nitrocellulose membrane. After migration on SDS-PAGE or native PAGE, the polyacrylamide gel orientated the anode side by side facing the nitrocellulose membrane towards the anode while the nitrocellulose membrane orientates the cathode.

The gel was taken carefully from the casting glass and then the stacking gel was removed. The remaining gel is called the resolving gel, which needs to be blotted on the

nitrocellulose membrane. The items required for Western blot are PAGE, nitrocellulose membrane, six sheets of Whatman filter paper (3mm) and transfer buffer. First of all, the Western blot transfer buffer was prepared then 45ml of the transfer buffer was mixed with 5ml absolute methanol. Secondly, three sheets of Whatman filter paper were immersed in the transfer buffer and placed onto the trans-blot cassette. Thirdly, nitrocellulose membrane was placed on the top of Whatman filter papers towards the cathode. Fourthly, the resolving gels were immersed first in the transfer buffer and then placed on the nitrocellulose membrane (face to face). Fifthly, the other three sheets of Whatman paper were immersed in the transfer buffer and then placed on the top of the gel, and then air bubbles were removed gently (Figure 2.5). Protein transfer was technically carried out using Trans-Blot® Turbo™ Transfer System (Bio-Rad) at 25V and 2.5mA (Amp) for 7-10min.

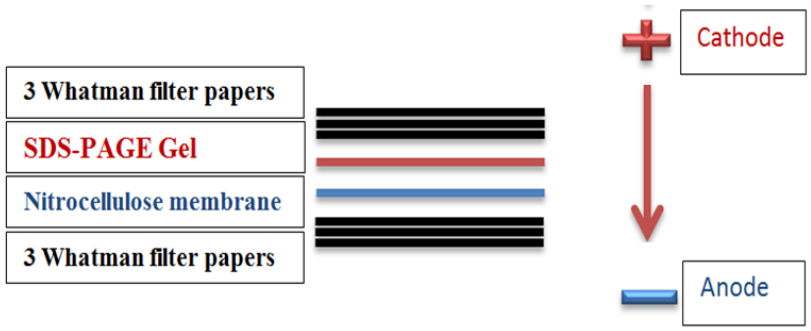

Figure 2.5. Schematic diagram shows the principle of Western blot using Trans-Blot® Turbo™ Transfer System (Bio-Rad) and the composed materials.

After protein transferring process, the nitrocellulose membrane was blocked using blocking buffer by following the same process of the Dot blot technique. Finally, the expressed protein can be detected via immunological interaction using specific monoclonal or polyclonal antibodies, and then, the size of the expressed protein can be identified.

2.2.7 *In Vitro* Studies

2.2.7.1 Human Blood Platelets Preparation

Human platelets were isolated and extracted from blood of human healthy volunteers using a method of preparing and washing platelets described by many authors (Mustard et al., 1972, Cazenave et al., 1983, Cazenave et al., 2004, Polanowska-Grabowska and Gear, 2004). It is vital that the volunteers should not take anticoagulant tablets like Aspirin for at least four days. Taking blood from human volunteers has been ethically approved (Ethical approve No. asa31-a1d5). 5ml of blood was drawn from healthy volunteers using a vacutainer tube containing sodium-citrate anticoagulant (BD). The sodium citrate is basically diminish the ions of calcium and magnesium with no changing of blood pH (Tucker et al., 2012).

The blood sample was then immediately mixed with $1/6^{th}$ volume ratio (v/v) of ACD anticoagulant (Acid Citrate Dextrose) (Kulkarni et al., 2004). The puropose of adding the ACD was to reduce the pH of blood as well as chelate ions of calcium to prevent clot formation (Tucker et al., 2012). It is crucial to use one or both of the above anticoagulants instead of using EDTA or heparin as performing to damage or modify the function of platelets, respectively (Cazenave et al., 2004).

The blood samples were centrifuged in order to obtain Platelet Rich Plasma (PRP) at low speed (900rpm) (143*g*) at RT for 20min with no deceleration force as that will disturb the red blood cell (RBC) layer. PRP was then transferred into clean 15ml tubes and mixed gently by inversion after adding Prostaglandin E1 (PGE1) (Sigma) with a final concentration of 200ng/ml. The PGE1 was used to inhibit the platelet activation for 30 min during washing process (Mustard et al., 1972, Cazenave et al., 1983, Cazenave et al., 2004). PRP was spun down in order to obtain Platelet Poor Plasma (PPP) for 15min at the speed of 1750rpm (600*g*) at RT. Next, the supernatant was discarded and the platelet pellet was re-suspended carefully with 1ml HEPES buffer saline (HBS) pH6. Basically, HEPES buffer is the suitable buffer for platelet preparation while other pH buffers such as Tris-HCL prevent platelet reaction and also stimulate others (Cazenave et al., 2004).

After re-suspension, HBS was added up to 10ml into the re-suspension platelets. PGE1 was added and mixed gently by inversion. The platelets were then centrifuged for 15min at speed 1750rpm (600*g*) at RT. Again, the supernatant was discarded and the platelet pellet was re-suspended into a small volume of 1–2ml HBS pH7.4. A platelet

count was performed using the AcT Diff cell counter system or the manual method with haemocytometer slides. The platelet count was adjusted to 200–150x10^3/µL; in HBS containing 2mM $CaCl_2$. The washed platelets were quickly used for the experiments due to the fact that the platelets had >30minutes since PGE_1 was added from the last step.

2.2.7.2 Collection Human Serum

Serum was extracted from blood of human healthy volunteers. Taking blood from human volunteers has been ethically approved (Ethical No. asa31-a1d5). 5-10ml of blood was drawn from healthy volunteers in plain tube with no addition of anticoagulants. After taking blood, tubes were then immediately placed on crashed ice in ice box until blood completely clotted for 1-1.5 hour. The clotted blood then was centrifuged at 3500rpm for 10-15 mintues. Serum was aliquoated in eppendorf tubes 300µl each and then frozen at -80°C (Kulkarni et al., 2004).

2.2.7.3 Preparation of extraction Platelet secretions and Microparticles (PMPs)

Platelet secretions including α-Thrombin and Microparticles (MPs) were isolated from washed platelet donated from healthy volunteers as previously described. First of all, intact washed platelets were activated using different agonists in order to monitor the highest level of the secreted α-Thrombin from α-granule. The following agonists; 10µg/ml TSP1, 10µg/ml highly oligomerised Properdin, 30µM adenosine diphosphate (Sigma) (positive control), 10µg/ml human collagen Type-I (Sigma) (positive control) and finally PBS (negative control), were used for monitoring platelets activation. All the prepared agonists were incubated in 1.5ml eppendorf tubes containing intact washed platelet (200-300x10^3 platelet/µl) for 10min under static condition at 37°C. After the incubation, all tubes were centrifuged at high speed 7,000–10,000 rpm for 3-4min until observing platelet pellets with clear supernatant. Ultimately, the 90% supernatants were taken into another 1.5ml clean eppendorf tubes with care not to disturb the pellets. All the supernatants were ready for screening the levels of α-thrombin using Thrombin (Human) ELISA Kit (Abnova). All samples were then marked by date and the name of agonist was used and then frozen at -80°C.

However, platelet Microparticles (PMPs) were also extracted from intact washed platelet supplemented with HEPES buffer containing Ca^{+2} as previously described. All the agonists, were previously mentioned, were used as well as 10µg/ml Calcium-Ionophore A23187 (Sigma) served as non-physiological positive control to analyse the experiment of detecting PMPs or thrombin generation assay. After activation, platelets were centrifuged twice at speed 1500g-2500g for 15min to sediment platelet while PMPs remained in the supernatant (Nomura and Fukuhara, 2004). For each time 90% of supernatants were taken into 1.5ml clean eppendorf tubes with no disturbance to the pellets. After the second centrifugation, the isolated supernatant was repeated again for the last centrifugation step at high speed 20,000g for 30min in order to precipitate the Microparticles (Mause and Weber, 2010, Merten et al., 1999). Lastly, all supernatants were then aspirated and then the pellets were re-suspended in Tris-hydrochloride buffer containing Ca^{+2},Mg^{+2}. All tubes were then marked by date and the name of agonist was used and then frozen at -80°C. Samples were then ready for platelet Microparticles investigation using ZYMUPHEN MP-Activity KIT (HYPHEN BioMed).

2.2.7.4 Bacterial strains

2.2.7.4.1 Preparation and fixation of bacterial strains

All bacterial stains *Streptococcus pneumonia* D39, *Staphylococcus aureus* DSM20233 *and Neisseria meningitides* group A-Z2491, B-MC58 were received and already fixed from with acknowledge to the following members of Lab 231 at University of Leicester; Dr. Byad Mawload, Mr. Ali Almansor and Mr. Saleh Alshamrani, respectively. D39 of *S.pneumonia* strain was incubated at 37°C with 5% CO_2 for overnight in brain heart infusion broth (BHI) medium. *S. aureus* was incubated in rotation incubator at 37°C for overnight in LB broth media. *N.meningitides* was incubated at 37°C with CO_2 for overnight in BHI broth media. All the bacterial strains were centrifuged for once at 3500 rpm. The supernatant media was decanted and then followed by adding 0.5% formalin in PBS for 1.30 hour at RT. Next, the bacterial strains were washed 3 times in PBS followed by centrifugation at speed 3500rpm for 10min. Each fixed bacterial pellet was inoculated by loop on blood agar plates for *S.pneumoniae* and *S.aureus*, and chocolate agar plates for *N.meningitidis*. All plates were placed on incubator for overnight providing 37°C temperature condition in the presence of 5% CO2. The absence of the growth would confirm bacteria were fixed during the formalin-fixation.

In the final washing step, all bacterial pellets were re-suspended in coating buffer for measuring the absorbance of the OD at weave length OD_{550} reach 0.6 Abs (absorbance) for all the strains.

After adjusting the OD, 100µl of bacterial suspension in coating buffer was placed in 96-well microtitration plate (Maxisorb, Nunc) for *in-vitro* investigations using binding ELISA (Lynch et al., 2004).

2.2.7.4.2 Preparation of *Streptococcus pneumonia* for counting Colony Formation Unit (CFU)

10µl from prepared *S.pneumoniae* was inoculated into 10ml of brain heart infusion broth medium supplemented with 20% heated fetal bovine serum (Inactivated) (Sigma) followed by an incubation for 6-7 hours at 37ºC in static condition. After several hours of incubation, the growth of Optical Density (OD) of the pneumococci was assessed at weave length OD_{550} until reach 1.6Abs (absorbance) which is an equal to $1x10^8$ CFU/ml of bacterial count, according to (Sutton, 2011).

The optical density was used in order to approximate the colony formation unit (CFU) in the suspension.

After that, aliquots of 500µl of the bacterial growth were placed in -80°C. Later on, counting the appropriate number of pneumococci CFU was prepared by serial dilution of 20µl of the bacterial suspension in 180µl PBS. Finally, 60µl each dilution was inoculated into blood agar followed by incubation for overnight at 37°C providing 5% CO_2. Colonies were counted for the possible dilution to be read out.

2.2.7.5 Enzyme Linked Immuno-Sorbent Assay (ELISA)

ELISA technique was used to quantify the binding capacity and also the measurement of platelet expression materials including P-selectin, α-Thrombin and PMPs caused by either TSP1 or highly oligomerised Properdin compared with different agonists. In addition, ELISA was used in order to determine the role of TSP1 in complement system by screening the levels of either C3 or C4 on different target surfaces including on thrombin-activated platelets. Moreover, the technique was used to distinguish the binding affinity of TSP1 to variety of different bacterial surfaces, which categorised in Gram-positive and Gram-negative bacteria, including the pathogenic substances; Mannan and Zymozan as polysaccharide materials extracted from *Saccharomyces cervisiae*. The

basic of ELISA types technique was followed as described by the following authors; (Clark et al., 1986, Crowther, 1995, Jeon et al., 2014).

2.2.7.5.1 Direct Binding ELISA

2.2.7.5.1.1 Binding assay to Extracellular Matrix proteins (ECM)

Extracellular matrix proteins were coated in micro-titer ELISA 96-well plates (Max-isorb, Nunc) in single concentration using 10µg/ml as follow: Human collagen Type-I, III, IV (Sigma), Fibronectin (Fn) (Sigma) and von Willebrand factor (vWF) (HTI), and also human Fibrinogen (Fg) (Sigma). The coating step was separately used for each protein using coating buffer for overnight incubation at 4°C. After overnight coating, the free residual protein binding sites in the plates were blocked with 300µl of 1% (v/v) Human Serum Albumin (HSA) in Tris-buffer saline (TBS) for 2-hours at RT. After incubation, plates were washed three times with 250µl of washing buffer.

Meanwhile, 10µg/ml of the following human Properdin native forms; dimer, trimer, tetramer and oligomerised Properdin (Kindly provided by Dr. Viviana P. Ferreira from University of Toledo (Ferreira et al., 2010)) and also 10µg/ml recombinant highly active oligomerised Properdin and recombinant human TSP1, were serially diluted using Tris-HCL buffer containing 2.5mM $CaCl_2$,2.5mM $MgCl_2$. All of which were applied into the coated materials in the microtitration plate in duplicates while the last coated wells in the plates were only received TBS for subtraction negative controls purposes. After 1-hour incubation at 37°C, wells were washed three times using washing buffer. Then, 100µl of diluted mouse polyclonal anti-human TSP1 (Sigma) or rabbit polyclonal anti-human Properdin (kindly provided by Dr. M.A, Youssif, University of Leicester, England) 1:5000 in washing buffer was added as primary antibodies. Next, plates were incubated for 1hour at 37°C followed by the three times of washing step. After that, in order to detect TSP1 and rhProperdin binding with human extracellular matrix proteins and human Fg, 100µl of diluted goat polyclonal anti-mouse alkaline phosphatase conjugated (Sigma) (1:5000) or goat polyclonal anti-rabbit alkaline phosphatase conjugate (Sigma) (1:10,000) in washing buffer as added as secondary antibodies, respectively. Next, plates were incubated for 1hour at 37°C followed by a last washing step three times. Following the washing step for three times, the tablets of alkaline phosphatase substrate (Fast p-Nitrophenyl Phosphate tablet sets, Sigma) were prepared in 20ml distal water or PBS. Then, 100µl of the prepared substrate was added into each well in order to determine the presence of the conjugated ALK-phosphatase by which developing the

chromogenic substrate to yellow color is an evidence for binding. Lastly, plates were incubated at RT away from direct light for 20min until the reaction between substrate and alkaline phosphatase reach the optimum desired color which then absorbed by measuring the color intensity at wavelength 405nm using Bio-Rad iMark microplate reader. All ELISA assays were repeated three times using fresh buffers for each time.

2.2.7.5.1.2 Binding assay to Lipids; LDL, cholesterol and triglycerides

As previous assay, all the target of human lipids; low density lipoprotein (LDL), cholesterol and triglycerides (Sigma), were coated into 96-well microtitration plate (Maxisorb, Nunc). Each well contained 1µg/well (10µg/ml) of lipids in 100µl of the coating buffer. LDL was coated using coating buffer while other lipids; cholesterol and triglycerides were coated by the use of absolute ethanol to dissolve lipids to coat them in plate for overnight incubation at 4°C. As described previously for blocking and washing process, 10µg/ml of TSP1, highly oligomerised Properdin and human fibrinogen (Fg) (Sigma) were serially diluted using TBS buffer. 100µl of each diluted protein were subjected into corresponding wells while the remaining last wells were received only TBS buffer used for subtracting negative control. ELISA process was followed as described previously of preparing primary antibodies for TSP1 and properdin. Mouse mAb anti-human-Fg (Santa cruz Biotechnology) was prepared in 1:1000. Secondary Antibody goat polyclonal anti-rabbit ALK-phosphatase conjugate (Sigma) was prepared in 1:10,000 for Fg. ELISA was completed as described before. All the ELISA assays were repeated three times using fresh buffers for each time.

2.2.7.5.1.3 Binding assay to Human Platelet receptors

As previous assay, 100µl of diluted human platelet receptors were coated into 96-well microtitration plate (Maxisorb, Nunc) as follow; 5µg/ml (500ng/well) αIIb (CD41) (Abcam), 3µg/ml (300ng/well) CD42b (GPIbα), 3µg/ml (300ng/well) CD42d (GPV) (Abcam) forming GPIb/V/IX, and 5µg/ml (500ng/well) CD36-IgG-Fc (CD36) (GPIIIb) (R&D), 5µg/ml (500ng/well) CD47-IgG-Fc (integrin associated peptide (IAP)) (R&D), 3µg/ml (300ng/well) 100-200aa of CD36 (Abcam). In addition, 5µg/ml (500ng/well) of

IgG-Fc peptide (R&D) was coated into 96-well microtitration (Maxisorb, Nunc) as a negative control for chimera products (CD36-IgG-Fc and CD47-IgG-Fc, R&D) binding assay. Plates were incubated for overnight at 4°C. As described previously, plates were blocked by blocking buffer and washed using washing buffer. ELISA process of preparing TSP1 and properdin and their antibodies was followed as described previously. All the ELISA assays were repeated three times using fresh buffers for each time.

2.2.7.5.1.4 Binding assay to Human Resting and Activated Platelets

In this section, human platelets were used in ELISA to assess the binding capability of the recombinant proteins; highly active oligomerised Properdin and TSP1, and also to native properdin forms; dimer, trimer and tetramer.

Platelets were extracted and washed from human blood healthy volunteer served in mixture of ACD anticoagulant and sodium-citrate as previously described. The washed platelets (200–150x10^3 platelet/µL) were then split into two tubes; the first tube was contained resting platelets which were fixed using HBS containing fixative reagent 0.2% formaldehyde (Sigma) pH7.4 for 20min at RT to stop platelet activation before screening (Goodall and Appleby, 2004), and the second tube was contained the activated platelets which were activated using 1U/ml human thrombin for 10min incubation at 37°C and followed by fixation process. After fixation, platelets were washed once by HBS and, then, replaced by coating buffer for coating platelets in 96-well microtitration plate (Maxisorb, Nunc) for overnight incubation at 4°C.

To assess or distinguish between resting and activated platelets, P-selectin was targeted as an activation biomarker for platelets using monoclonal antibody. Therefore, the intact and activated platelets were both subjected for screening the liability of native Properdin forms for binding by determining the difference between intact and activated platelets through targeting the P-selectin. The protocol of targeting the P-selectin will be discussed later in the functional assays section.

After coating process, plates were blocked using blocking buffer. Meanwhile, 10µg/ml of the following native Properdin forms and the oligomerised Properdin (Kindly provided by Dr. Viviana P. Ferreira from University of Toledo (Ferreira et al., 2010)) and also 10µg/ml recombinant highly active oligomerised Properdin and recombinant human TSP1, were serially diluted using TBS buffer containing 2.5mM $CaCl_2$,2.5mM $MgCl_2$.

The further steps of ELISA were followed as described previously of preparing primary antibody for properdin. In addition, the activation level of platelets was assessed by the detection of the expression of P-selectin (CD62p) using mouse mAb anti-human P-selectin (Sigma) 1:1000. Then, the substrate was prepared and processed for development, and reading the OD as previously described.

2.2.7.5.1.5 Binding assay of Fibrinogen to human activated platelets mediated by TSP1 and properdin

After washing intact platelets, platelets (150-200x10^3/platelet) were equally split into 1.5ml eppendorf tubes by which used for platelet activation using different agonists which were added into each tube as follow; 10µg/ml TSP1, 10µg/ml highly oligomerised properdin, 10µg/ml human collagen Type-I (Sigma), 30µM adenosine diphosphate (ADP) (Sigma), human thrombin 1U/ml (Sigma) and 10µg/ml human fibronectin while only PBS added to part of intact platelets served as negative control. All tubes were incubated at 37°C for 10min in static condition. After that, platelets were fixed as previously described. Next, platelets were centrifuged at 2500g for 3-5min. Then, the platelet pellets were re-suspended in equivalent amounts of coating buffer in order to coat the same platelet number. 100µl of each re-suspended platelets was added into corresponding wells in 96-well microtitration plate (Maxisorb, Nunc) and incubated for coating for overnight at 4°C. After incubation, plates were blocked for 2-hours at RT. Meanwhile, 100µg/ml human fibrinogen (Sigma) was serially diluted in TBS containing Ca^{+2},Mg^{+2}. 100µl of the prepared fibrinogen was added into corresponding wells. Plates were incubated for 1-hour at 37°C. Meanwhile, primary mouse mAb anti-human-Fg antibody (Santa cruz Biotechnology) was diluted 1:1000 in washing buffer. After washing, 100µl of each prepared antibody was added to the corresponding wells and then incubated at 37°C for 1-hour. After that, ELISA process was followed as described previously.

2.2.7.5.1.6 Binding assay to Gram-positive, Gram-negative bacteria and Polysaccharide substances

100µl of coating buffer of each *S.pneumoniae* D39, *Staphylococcus aureus and Neisseria meningitides* group A, B (OD_{550}=0.6 abs), and also 10µg/ml of Mannan (Sigma), Zymozan (Sigma), *N*-acetyl-albumin (Acetylated-BSA) (Invitrogen) and Chondronitin sulfate (SC) (Sigma) were all coated into 96-well microtitration plate (Maxisorb, Nunc). Plates then were incubated for coating for overnight at 4°C. Following blocking step, 10µg/ml TSP1 serial dilution was processed and placed into coated plates as previously described. After that, ELISA process was followed as described previously. ELISA assays were repeated three times using fresh buffers each time.

2.2.7.5.2 Direct & Indirect binding or Competition ELISA

2.2.7.5.2.1 TSP1 compete Mannose-binding lectin (MBL) on poly-saccharide binding sites

10µg/ml Mannan was coated in 96-well microtitration plates (Maxisorb, Nunc) for overnight at 4°C using coating buffer. Following blocking step using 1% BSA, plates were washed three times by washing buffer. Meanwhile, one single concentration of 10µg/ml TSP1 was prepared in TBS buffer containing Ca^{+2},Mg^{+2} in duplicate and pre-incubated in 37°C for 1-hour in eppendorf tubes containing different dilutions of soluble Mannan starting from 10µg/ml. After that, one single concentration of 10µg/ml recombinant human MBL (Kindly provided by Dr. Russell Wallis, University of Leicester) was prepared in each tube of the pre-incubated TSP1 and then mixed by vortex. 100µl of each tube was added in duplicate into 96-well plate after washing plate. Another 10µg/ml MBL was prepared in TBS buffer containing Ca^{+2},Mg^{+2} to monitor the binding of MBL to BSA which served as negative control, whereas, duplicated wells were received only TBS buffer served for subtract negative control of antibodies non-specific binding toward coated materials. Plates were incubated for 1-hour in 37°C. Following washing step, the binding competition of TSP1 and MBL to mannan was observed by adding 100µl of the diluted mouse monoclonal anti-human MBL antibody 1:2000 (ANTIBODYSHOP) in washing buffer to each well. After processing 1-hour incubation and washing, secondary antibody goat anti-mouse alkaline phosphatase conjugate (Sigma) was prepared 1:5000 in washing buffer. ELISA steps were followed as described previously. Values of the competitive TSP1 were assessed comparing between the binding MBL to mannan and BSA. On the other hand, the same experiment was processed of TSP1 and MBL which were incubated together in different serial dilution of soluble Mannan instead of pre-incubation step of TSP1.

The previous experiment was confirmed by coating other residual carbohydrates; 10µg/ml Mannan and Chondroitin sulphate (CS) (Sigma) in 96-well microtitration plates for overnight at 4°C. Following ELISA steps of blocking and washing, 10µg/ml human MBL was prepared in TBS with or without 10µg/ml TSP1. ELISA process was followed as described previously including the use of primary antibody for human MBL.

2.2.7.5.2.2 TSP1 compete binding human Properdin on residual carbohydrate

10µg/ml Chondronitin sulfate (SC) (Sigma) was coated in 96-well microtitration plates (Maxisorb, Nunc) for overnight at 4°C using coating buffer. Following blocking step, serial dilution of 10µg/ml human highly oligomerised Properdin with or without 10µg/ml human TSP1 were prepared in TBS (Ca^{+2},Mg^{+2}). The binding of Properdin to BSA was used to serve as negative control compared to binding with CS. The prepared proteins concentrations were pre-incubated in the microtitration plates for 1-hour at 37°C. After washing step, the competition of TSP1 to Properdin was investigated by targeting properdin binding using 100µl of the diluted Rabbit polyclonal anti-human Properdin 1:10,000 (Kindly provided by Dr. M.A, Youssif, University of Leicester, England) in washing buffer to each well. ELISA process was followed as described previously. ELISA assays were repeated three times using fresh buffers for each time.

2.2.7.5.2.3 TSP1 compete human Properdin on binding to human Factor B

10µg/ml of Purified human factor B (fB) (Kindly provided by Dr. Robert B Sim, University of Oxford, England) was coated in 96-well microtitration plates for overnight at 4°C using coating buffer. Following blocking step, serial dilution of 10µg/ml human highly oligomerised Properdin with or without 10µg/ml human TSP1 were prepared in TBS (Ca^{+2},Mg^{+2}). The binding of Properdin to BSA was used to serve as negative control compared to binding with factor B. The competition ELISA steps were followed as previously described in TSP1 competition MBL and Properdin binding on residual carbohydrates. The target was focused on the ability of human Properdin to bind to fB in the presence of human TSP1 in comparison with binding to fB without TSP1. The targeting was by using 100µl diluted Rabbit polyclonal anti-human Properdin 1:10,000 (kindly provided by Dr. M.A, Youssif, University of Leicester, England) followed by the secondary antibody alkaline phosphatase conjugate.

Overturned, the previous experiment was confirmed by coating 10µg/ml TSP1 and Properdin, while 10µg/ml fB was serially diluted in TBS buffer. ELISA steps were followed as previously described above. The targeting was by using primary antibody rabbit polyclonal anti-human fB (H-95) (Santa Cruz Biotechnology) was prepared 1:1,000 in washing buffer followed by the secondary antibody ALK-phosphatase conjugate incubated at 37°C for 1-hour. The remaining ELISA steps were followed as previously described.

2.2.7.5.2.4 TSP1 bind human C3(H_2O) and prevent the binding on Properdin

10µg/ml of purified human TSP1 and Properdin were coated on 96-well microtitration plates for overnight at 4°C using coating buffer. Following blocking step, single concentration of 10µg/ml the treating purified human C3 molecule with 200mM Methylanmin Hydrochloride (kindly provided by Dr. Hany Kenawy, University of Leicester) with or without serial dilutions starting from 10µg/ml TSP1 were prepared in TBS (Ca^{+2},Mg^{+2}). To treat the cleavage process of C3 in 200mM Methylanmin Hydrochloride, the mixture was incubated in pH8.0 at 4°C for overnight or 37°C for 1-hour (Mead et al., 1999, Mitchell et al., 2008, Neves et al., 2012).
Methylanmin Hydrochloride (Sigma) was used in 200mM concentration to convert C3 intact molecule into C3(H_2O) through C3 α-chains (Gadjeva et al., 1998, Tack, 1983) of N-terminal peptide (Osterberg et al., 1989) which is identical to the cleaved C3b active molecule that expose active glutamyl-carboxylate group, which is able to covalently binds to hydroxyl or amino groups (Law et al., 1979, Tack et al., 1980, Hostetter et al., 1982, Janatova and Tack, 1981, Gadjeva et al., 1998).

After proceeding the serial dilutions of TSP1 with C3(H_2O), 100µl of each dilution was placed on Properdin coated wells while 100µl of the single concentration C3(H_2O) was placed on both TSP1 and Properdin coated wells as well as BSA for negative control.
The competition ELISA steps were followed as previously described in TSP1 competition MBL and Properdin binding on residual carbohydrates. The target was focused on the ability of soluble human TSP1 to bind soluble C3(H_2O) and then would prevent the binding of C3(H_2O) to immobilised Properdin. Therefore, targeting C3(H_2O) was the key to find-out the binding affinity of C3(H_2O) to both immobilised Properdin and TSP1. 100µl of diluted rabbit polyclonal anti-human C3c antibody 1:5000 (Dako) was added to each well. ELISA steps were followed until finally adding the substrate as previously described.

2.2.7.5.3 Functional Assays

2.2.7.5.3.1 Platelet Functional assays

2.2.7.5.3.1.1 Quantification the Expression levels of CD62P (P-selectin) on human activated platelets

P-selectin is an important adhesive molecule secreted from α-granule. It is essential for platelet tethering process to allow platelet to adhere on either the extracellular matrix layer or to let other circulated platelets to accumulate. This functional assay is evaluating the secreted P-selectin which bound to the membrane of activated platelets using potent agonists in comparison with TSP1 and Properdin.

After the isolating and washing process of platelets and adjusting platelets numbers, the washed intact platelets (200–150x10^3/μL) were incubated with 10μg/ml of either TSP1 or Properdin at 37°C for 10min. Tow positive controls; 10μg/ml human collagen type-I, and 30μM ADP, were used separately, whereas, two negative controls were used; intact PBS added to platelet, and 10μg/ml HSA added to platelets. After 10min incubation, platelets were then fixed in HBS buffer containing fixative reagent (0.2% Formaldehyde) (Sigma) for 20min at RT. As previously described the washing of fixed platelets and coating step, platelets were then incubated in in 96-well micro-titration plates for overnight at 4°C. After overnight incubation, plates were blocked and then washed. The remaining steps of ELISA were followed as previously described. Primary antibody mouse mAb anti-human P-selectin (CD62P) human platelets (Santa Cruz) was prepared in 1:1000, and also followed by adding the secondary antibody polyclonal anti-mouse ALK-phosphatase conjugate (Sigma).

2.2.7.5.3.1.2 α-Thrombin detection assay

The activated Thrombin (IIa) is an essential molecule for processing coagulation cascade in haemostasis/thrombosis. IIa acts on fibrinogen (Fg) by converting soluble Fg into fibrin and also interfere to the interaction of the coagulation factors as a catalyser (Badimon et al., 1988).

This assay was designed for cell culture in order to detect the alpha thrombin (IIa) in human cell culture supernatants. This assay was used to detect the levels of α-thrombin being released from washed activated platelet mediated by different agonists including TSP1 and the highly oligomerised properdin. Therefore, after activating platelets for 10min at 37°C, platelets were spun down as described before. Then, the supernatants contents, where α-thrombin supposed to be involved, were investigated using Thrombin assay by following the protocol according to the manufacturer's instructions kit (Thrombin (Human) ELISA KIT) (Abnova). The kit based on sandwich ELISA through pre-coating plate with specific monoclonal antibody for human Thrombin. As well as, once thrombin binds to the pre-coated antibody, the thrombin will be captured by a biotinylated polyclonal antibody. The later antibody is recognized by a streptavidin-peroxidase conjugate. Finally, by washing away all unbound materials, the chromogenic peroxidase enzyme substrate allows to recognise the color intensity of the developing. Once the color reaches the optimum development desires comparing with thrombin standard (provided by the kit) then the color can be stopped by adding the stopping solution. The plate were read at wavelength of OD 405nm. The functional assay was repeated three times using fresh buffers for each time.

2.2.7.5.3.1.3 Thrombin generation by MPs Assay

Platelet Microparticles (PMPs), which are released from activated platelets, were used in the current study to measure the procoagulant activity of PMPs using ZYMUPHEN MP-Activity KIT (HYPHEN BioMed). As described previously, the effect of PMPs release of activated platelets was quantified to monitor the effects of TSP1 and properdin on thrombin generation. Following the protocol recommended in the manufacturer's instructions of this kit, this assay is based on enzymatic reaction between factors Xa and Va which mediate the conversion of pro-thrombin into thrombin in the presence of Ca^{+2} and Mg^{+2}. The coagulation factors Xa-Va bind to phospholipids of phosphatidylserine (PS) that exposed on PMPs surfaces. PS binds to annexin-V pre-coated with streptavidin (Figure 2.6). The prothrombotic activity of the PMPs (generating thrombin) is monitored through colour reactions after adding a thrombin-specific chromogenic substrate (provided by the kit). The development colour intensity requires to stop this chromogenic reaction using 2% citric acid to stop the enzymatic substrate conversion. The plates were read at wavelength OD 405nm. The protocol was followed in detail as described in manufacturer's protocol provided with the kit.

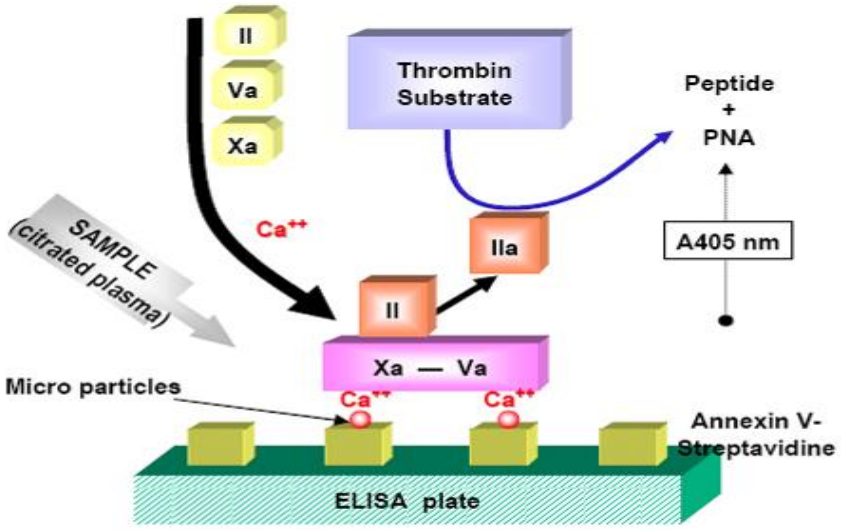

Figure 2.6. Schematic diagram shows the principle of Microparticles Procoagulant activity in Citrated Plasma and the composed materials, according to the leaflet of (Vissac et al.).

2.2.7.5.3.1.4 Platelets Adhesion Assay

96-well microtitration plates were pre-coated with 10µg/ml of each of the following extracellular matrix proteins; TSP1, collagen type-I and fibronectin (Fn), and also including highly oligomerised properdin, and 1% of HSA (human serum albumin solution, ZENALB 20) and BSA (bovine serum albumin, Sigma) which are served as negative controls. After overnight incubation at 4°C, plates were blocked with 1% BSA or HSA for 2-hours at RT. Meanwhile, washed intact platelets were prepared in HEPES buffer, and counting number was adjusted 150-200x10^3platelets/µl as described previously. After the incubation, plates were rinsed three times using phosphate buffer saline (PBS). 100µl of the prepared platelets was added in quintuplicate for each coated material and then incubated at 37°C for 30min in static condition. After incubation, the residual amounts of non-adhered platelet were thrown and then plates were washed once using PBS. The adhered platelets were fixed using 0.5% glutaraldehyde at RT for 10-20min. Meanwhile, staining solution was prepared in PBS using filtered 0.02% toluidine blue (Sigma) (Isenberg et al., 2008b). After fixation, the fixative solution was thrown and replaced by 50-100µl filtered staining solution. Next, plates were incubated for 5-10min at RT and then the adhered platelets were visualised under microscope using 20x magnification power lens. By using fixed camera within the microscope, the adhered platelets were photographed at least 5-times for every adhesive molecule including BSA and HSA. Lastly, the adhered platelets were counted using ImageJ program. Mean±SEM were calculated using GraphPad Prism-6.

2.2.7.5.3.1.5 Staining Platelets Aggregation under Light Microscope

After the washing process of isolating platelets, platelets were individually incubated in 1.5ml eppendorf tube containing known single concentrations of the following agonists in HBS buffer; 10µg/ml human Collagen and 30x10^{-5}M ADP, including 10µg/ml of both TSP1 and Properdin. However, washed platelets were also incubated with 10µg/ml HSA as a negative control. The incubation period of time was 2-min at 37°C. After incubation, an equal amount of activated platelets (approx. 100µl) from each tube was

placed on Polysine microscopic slide (VWR) as smears and then left for 10-15min for platelets adherence at RT. As described by (Isenberg et al., 2008a), the activity of platelets was stopped using 0.5% glutaraldehyde prepared in HBS buffer by immersing the microscopic slides into the solution for 10-min. Then, the slides were left at RT to dry. Next, the slides were stained using filtered 0.02% Toluidine blue stain (Sigma) for 3–5min in HBS buffer. Lastly, platelets aggregation was visualised under the light microscope using 20x0.6 magnification power lens. Platelets clumps triggered by TSP1 and Properdin were compared with the positive agonist controls and the negative control. The microscopic slides were mounted using mounts, and the smears were covered with coverslips and then kept at RT.

2.2.7.5.3.2 Complement system Functional Assays

2.2.7.5.3.2.1 C3b deposition assay

Human Complement system was activated on pre-coated human washed thrombin-mediated platelet activation with known platelets number as described previously. Also, plates coated with 10µg/ml mannan and CS. The activity of complement was assessed by investigating the levels of C3b deposition on surfaces. The C3b deposition was screened to investigate the functional activity of TSP1 on coated materials. All complement residual activators were coated on 96-well microtitration plate for overnight at 4°C, and followed by blocking using BSA for 2-hours at RT. Meanwhile, human serum was serially diluted in Barbital buffer saline (BBS), containing Ca^{+2},Mg^{+2}, beginning from 1:20 (5%) and also for some experiment 1:50 (2%) and 1:80 (1.25%). The dilution process was occurred on crushed ice. The diluted human serum was processed in duplication served as positive control. Another serial dilution of serum was prepared with exogenous of the following recombinant proteins; 10µg/ml TSP1 for investigating the impact on activating complement system, as well as in some experiments 10µg/ml highly oligomerised properdin was added in either BBS containing Ca^{+2},Mg^{+2} to activate all complement pathways or using BBS containing EGTA to block CP and LP pathways excluding AP pathway. The negative control was used for measuring C3b deposition on BSA. After blocking, plates were rinsed three times using washing buffer. 100µl of each serial dilution of serum was added in duplicate into matching wells; whereas, the last wells were only received BBS buffer for subtract negative control purpose, followed by

incubation for 1-hour at 37°C. Following washing process, 100µl of prepared primary antibody rabbit anti-human C3c IgG antibody (Dako) was added, which was diluted 1:5000 in washing buffer. Then, plates were placed in the incubator for 1-hour at 37°C. The secondary antibody polyclonal anti-rabbit ALK-phosphatase conjugate (Sigma) was prepared in 1:10,000. ELISA process was followed as described previously. The functional assay was repeated three times using fresh buffers for each time.

2.2.7.5.3.2.2 C4 deposition assay

In order to investigate the functional activity of TSP1 on complement system, the measurement of C4b deposition on mannan was used. This assay was likely processed as the same as C3b deposition procedure assay as described above. The buffer that used for serum dilution was TBS (Ca^{+2},Mg^{+2}). Human serum was serially diluted starting from 1:20 (5%) in duplicate served as positive control, whereas, 10µg/ml TSP1 was added into 5% serum. The primary antibody Chicken monoclonal anti-human C4c (Immunsystem AB) was prepared in 1:2000. The secondary antibody goat anti-chicken ALK-phosphatase conjugate (Sigma) was prepared 1:5000. ELISA process was followed as described previously in C3 deposition assay. ELISA assays were repeated three times using fresh buffers for each time.

2.2.7.5.3.2.3 Serum Bactericidal (Killing) assay (SBA)

Serum bactericidal assay (SBA) was assessed by evaluating the reduction levels of living bacteria (*S.pneumoniae* D39) in incubation time during 2-hours in human serum (McQuillen et al., 1994, Estabrook et al., 1997) with or without adding TSP1. 990µl of HBSS or BBS buffer containing Ca^{+2},Mg^{+2} was used for re-suspending 10µl of the frozen stocks of *S.pneumoniae* D39 ($1.87x10^{8}$ CFU/µl) followed by vortex to adjust the desired bacterial concentration needed for the experiment. The dilution became 1:100 by assuming bacterial counts approximately $1.8x10^{6}$ CFU/µl.

The bacterial suspension was transferred into several 1.5ml eppendorf tubes where should be received the following materials; 20% human active serum (positive control), heated-inactive serum (negative control) or only buffer (negative control). The controls are very important to know the actual surviving bacterial numbers during selective time points of incubation (every 30min for 90min incubation for example). The negative con-

trol of heated-inactive serum was assessed by increasing the serum temperature up to 60°C for more than 30min.

Different concentrations, 10µg/ml and 20µg/ml, of TSP1 were added individually to the prepared bacterial serum suspension tubes. The final volume of all tubes contents was 200µl. All tubes contents were incubated for 120min at 37°C under rotation at 120 rpm. Eventually, every sample was inoculated into Blood Agar Base (BAB), supplemented with 20% Horse blood (Thermo-Scientific) (500ml of agar+25ml Horse blood), for each time point incubation beginning from 0, 30, 60, 120min. The inoculation was assessed by serially dilution of known volume was taken from the bacterial reaction (20µl) into known volume of HBSS (180µl) for serial dilution purposes. Therefore, the dilution should be as following; (1)1:10, (2)1:100, (3)1:1000 and (4)1:10,000. By calculations, the expected numbers of counted colonies for each test tube in the dilution number (4) for the zero time point is approximately 60colony/60µl (180colony/well).

Lastly, 60µl of each dilution was inoculated as spot onto blood agar plate, and allowed to dry for approximately 5-10min in the safety cabinet laminar hood. All plates were inverted for incubation at 37°C providing 5% CO_2 for overnight.

2.2.8 Statistical Analysis

All data were evaluated statistically using GraphPad Prism version6.0 and presented as mean±SEM. The data were presented in curves either line graph or bar chart. The significance differences of the functional assays were statically analysed by Two Way ANOVA using Bonferroni's for multiple comparison compared to the –ve control, while some data were analysed per row and compared to the –ve control using Multiple t test using Sidak-Bonferroni test. The significance difference of P-value was considered to start from $p \leq 0.05$ or less.

It was taken into account that the platelets of every human healthy volunteer are differ from other volunteers regarding the sensitivity in response to agonists. Therefore, the experiments of binding both TSP1 and highly oligomerised properdin toward washed intact and activated platelets, and the experiments of quantifying the expression levels of either CD62P, α-thrombin or PMPs were repeated at least twice from different human healthy volunteers.

Chapter 3 (Results)

(Generation of recombinant human Thrombospon-din1 (TSP1) and human Properdin)

Generation of recombinant human Thrombospondin1 (TSP1) and human Properdin

The goals were mentioned in the late of chapter-1 (Introduction) about requiring both human Thrombospondin1 (TSP1) and Properdin which need to be purified as recombinant proteins. Therefore, it is investable to express and purify both proteins. To achieve that, it is essential to establish the cDNA constructs of both proteins, all of which in this chapter are going to be achieved and discussed.

3. Results

3.1 Engineering, Cloning and expression of human TSP1 and Properdin

3.1.1. Engineering and cloning DNA constructs of human TSP1 and Properdin

3.1.1.1. Engineering DNA construct of human TSP1

In order to assess the function ability of human thrombospondin-1 (TSP1) in platelet, protein-protein interaction, and in complement system, it requires mass production of full length structure of TSP1 without mutations or even providing such tags for purification purposes. TSP1 is encoded via open reading frame of 3,513 base pairs which encodes 1,170 amino acid residues (figure 3.7, 3.8). In order to estableish the expression of DNA consturct of TSP1 in pCEP4 vector, the templet of cDNA of human TSP1, was kindly provided by Dr. M.A Youssef (University of Leicester, UK), was used for amplification using specific oligonucleotides by utilising the PCR tool through thermocycler machine in order to generate hTSP1. The engineered DNA construct of TSP1 was to have the following restriction sites; HindIII and KpnI. The cDNA of TSP1 was generated under 35 repeated cycles require heating and cooling down waves depends on the optimum temperature for fusion polymerase and annealing the primers were used for PCR. The amplified DNA was visualised on 1% agarose gel (Melford) using electrophoresis. Observing and visualising the DNA of PCR products under UV light was compared with the DNA ladder (1kb) (Invitrogen). The engineered human DNA of TSP1 was visualised between 4000 bp and 3000 bp with size around 3,500 bp (Figure 3.1).

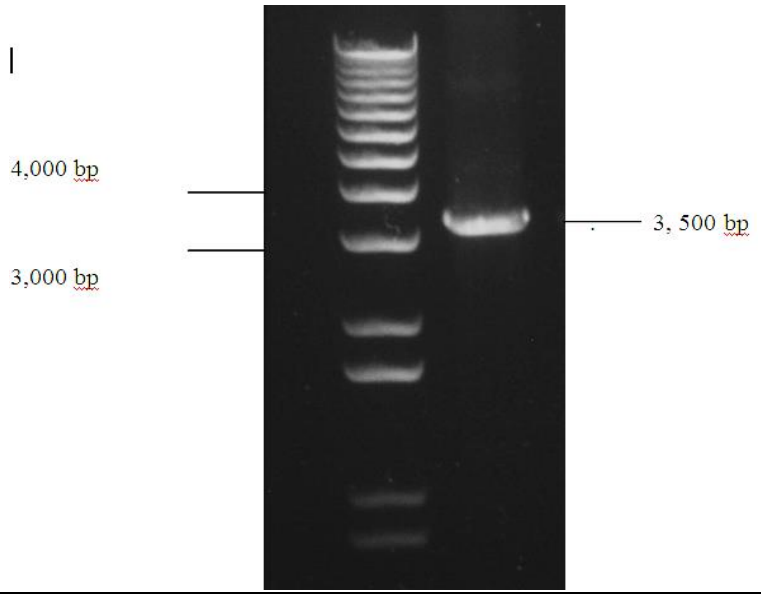

Figure 3.1. Generating the full length of DNA construct for TSP1 by Polymerase chain reaction (PCR). The coding sequence for TSP1 was generated with size 3500 bp by using high fidelity polymerase. 1 Kbp ladder was run along with the samples on 1% agarose TAE-gel.

3.1.1.2. Cloning the DNA constructs of TSP1 into pGEM-T Easy vector

The DNA PCR products of human TSP1 were cloned into pGEM-T Easy vector (Promega) which simplifies the cloning of the PCR products. T4 ligase enzyme (Promega) was used to ligate the full length of the generated DNA of TSP1, which treated to have A-tail, with the opened pGEM-T Easy vector, which has already T-overhang which is compatible toward the A-tailed PCR products. As a result of sub-cloning, the DNA plasmid was transformed into *E.coli* TOP10 strain in order to clone and generate the full length of the DNA construct of TSP1 including the endonuclease restriction sites; Kpn1 and HindIII. After cloning, plasmids have over length approximately 6500 bp containing the following; the DNA construct of TSP1 (around 3500 bp), and pGEM-T Easy vector (3018 bp). The DNA construct of TSP1 was digested from pGEM-T Easy vector using double digestion reaction by HindIII and Kpn1 restriction enzymes to cut the restriction sites of Kpn1 and HindIII where localised in the end of DNA segment of TSP1. It is clearly that the cloning in pGEM-T Easy vector was distinguished with two bands following each other (Figure 3.2). Thereby, the sub-cloned DNA of TSP1 was cloned again into the expression vector.

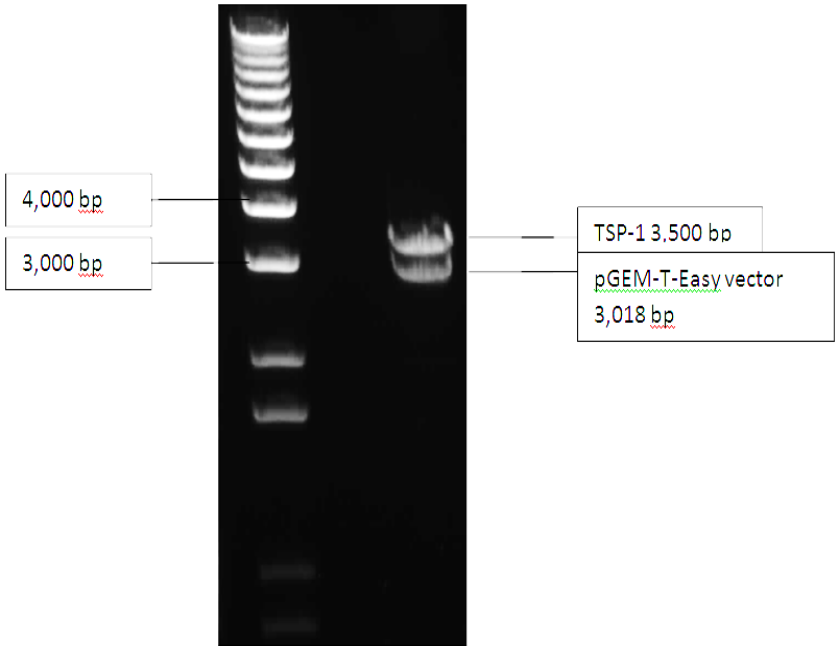

Figure 3.2. Generating the full length of DNA construct of TSP1 for cloning. By cloning the full length of TSP1 DNA construct into pGEM-T Easy vector (3.018kb) to provide DNA sticky ends on the restriction sites encoded into TSP1. The DNA construct was digested using two restriction enzymes; HindIII and Kpn1, resulting and observing two bands; a full length of DNA construct of TSP1 3.5 kb and pGEM vector 3.018 kb. 1 Kbp ladder was run along with the samples on 1% agarose TAE-gel.

3.1.1.3. Sub-cloning the digested TSP-1 DNA fragment into PCEP4 vector

The construct of human TSP1 was removed from pGEM-T Easy vector by double digestion using Kpn1 and HindIII restriction enzymes, and thereby ligated and sub-cloned into the expression vector pCEP4/hygroB. After cloning and transforming, the final construct in the expression vector was also double digested using the same restriction enzymes where were used previously. High copies of plasmids were obtained (Figure 3.3) for transfection process into eukaryotic cell line. Before proceeding a further transfection of the plasmids into the following eukaryotic cell lines; CHO-K1 cell, HEK293 cell, the clones of plasmid DNA were analysed for sequencing in the DNA sequence unit (PANCL) at University of Leicester, in order to confirm that the DNA constructs were inserted in the right frame, besides, ensuring no mutation have been generated during the PCR amplification process or from the effect of emission UV-light.

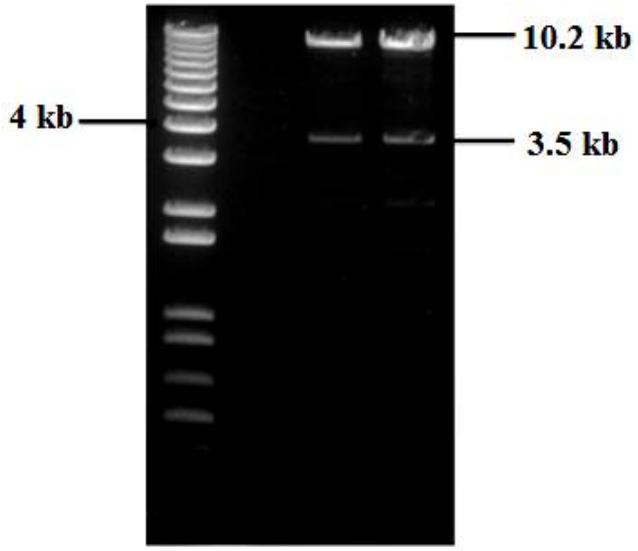

Figure 3.3. Generating the full length of DNA construct of TSP1 for transfection. By sub-cloning the full length of TSP1 DNA construct into pCEP4/HygromycinB vector 10.2 kb following the transformation into *E.coli* TOP10 strain was to provide large scale of DNA plasmid clone copies for transfection process. The DNA plasmid was digested using two restriction enzymes; HindIII and Kpn1, to make sure the plasmid has cloned well by visualising two bands; a full length of DNA construct of TSP1 3.5 kb and pCEP4/HygromycinB vector 10.2 kb. 1 Kbp ladder was run along with the samples on 1% agarose TAE-gel.

3.1.1.4. Engineering DNA construct of human Properdin

Human Properdin is encoded via open reading frame of 1,650 base pairs and encodes 550 amino acid residues. In order to estableish the expression of DNA consturct of Properdin in pSectag2/hygroB vector, the DNA plasmid of human Properdin/pSectag2/hygroB was kindly provided by Dr. M.A Youssef (University of Leicester, UK) (Ali et al., 2014), who had already engineered the DNA construct of human properdin to have the following restriction sites; HindIII and XhoI, and also sub-cloning the construct into pSectag2/hygroB expression vector which contains START codon (ATG) of the N-terminal sequence and the C-terminal poly-histidine tag followed by STOP codon (TAA). The tag was encoded to the DNA construct for protein purification and detection purposes by which nickel-chelating resin capturing histidine tags using chromatography columns, and detecting by using anti-histidine tag antibody (Sigma) for protein identification. The plasmid has over length approximately 6,850 bp containing the following; DNA construct of the full length of human Properdin with size around 1,650 bp and pSectag2/hygromycinB expression vector with size 5,200 bp. The

DNA plasmid was digested using double digestion reaction by HindIII and Xho1 restriction enzymes to cut the restriction sites of XhoI and HindIII where located in the expression vector. It is clearly that the sub-cloning in pSectag/hygroB vector was distinguished with two bands following each other (Figure 3.4). Thereby, the DNA plasmid containing the DNA construct of human properdin that ligated into pSectag/hygroB vector was ready for eukaryotic transfection usage.

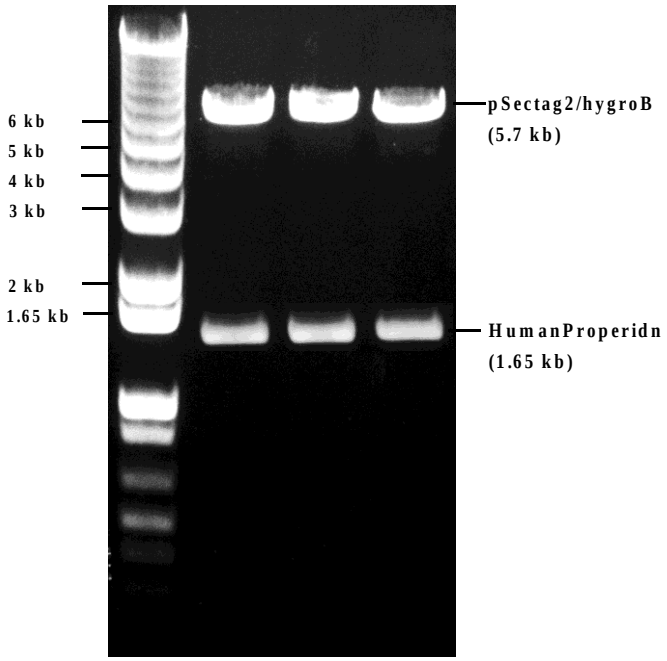

Figure 3.4. Generating the full length of DNA construct of human Properdin for transfection. By transforming the plasmid DNA, which was gifted by Dr. M.A Youssef, into *E.coli* TOP10 strain for obtaining large scale of plasmid clone copies, the plasmid DNA was double digested using HindIII and XhoI by which resulting two DNA fragments; full length of human Properdin DNA construct with size around 1.650 kb, and pSeqtag/HygromycinB vector with size 5.7 kb. 1 Kbp ladder was run along with the samples on 1% agarose TAE-gel.

3.1.2. The Expression of Human recombinants TSP1 and Properdin

Chinese Hamster Ovary cell line (CHO-K1) was transfected first with pCEP4/hygroB expression vector containing the engineered cDNA construct of human TSP1. Hygromycin-B (Invitrogen) as a selection antibiotic was added with 300μg/ml in order to maintain the cells that uptake the plasmid; otherwise the cells shall be vanished and killed. After two weeks of adding the selection antibiotic, all media of positive clones, which clearly shown some improvement in growing by looking to cell growth confluence, were taken into further process whereby subjecting the supernatant media into nitrocellulose membrane by which determining and screening the positive clones that expressed TSP1 using Dot blot technique via using monoclonal anti-human TSP1 (Abcam). A negative control was used as supernatant media of non-transient CHO-K1 cell. Unfortunately, human TSP1 was not successfully detected by using CHO-K1 cell line even with repetition and changing the transfection reagent for each time was not fulfilled with detection. That failed of detection was mainly refers to a reason which is related to some receptors on the CHO-K1 cell that strongly bind to the N-terminal domain of TSP1 (Clezardin et al., 1997).

Therefore, the transfection by using CHO-K1 cell line was shifted into HEK293 cell line (Human Embryonic Kidney cell). By following the same previous steps on CHO-K1 cell, finally human TSP1 was successfully expressed using HEK293 cell line with a selection 180μg/ml of Hygromycin-B (Figure 3.5).

Likewise, the same manner was followed for transfection the plasmid DNA containing the DNA constructs of human Properdin and pSeqtag/Hygromycin-B vector into CHO-K1 cell line. The transfection was successful of expressing human Properdin using polyclonal anti-histidine tag (Sigma) (Figure 3.6).

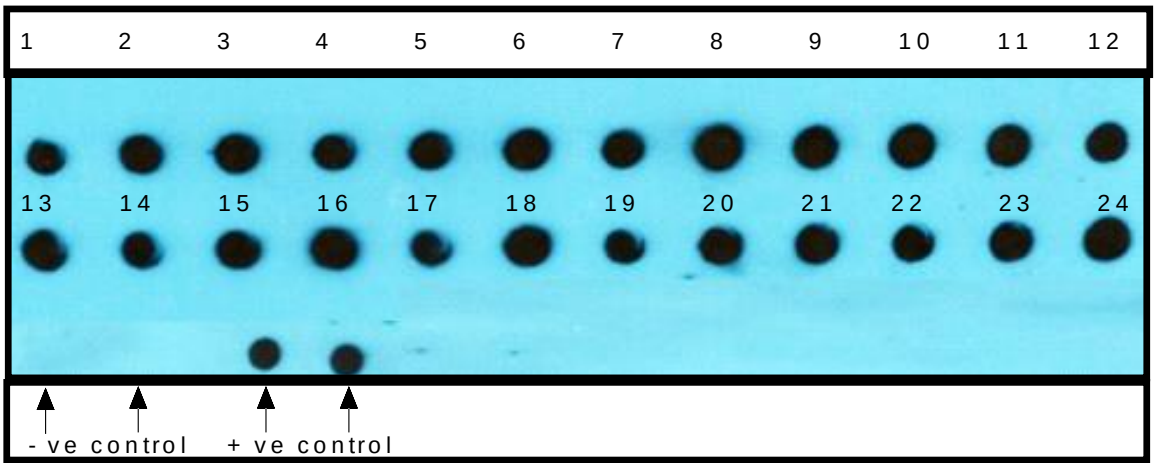

Figure 3.5. Dot blot screening test of numerous clones expressing human recombinant TSP1. TSP1 was detected using mouse monoclonal anti-human TSP1 (Abcam). The positive control represents the purified human TSP1 from activated platelet (Yee et al., 2003). The negative control represents a media supernatant of non-transient HEK293 cell line. All the selected clones were positive in expression. Thus, only 6 clones were randomly chosen for expressing and purifying mass production of TSP1. Positive clones were frozen in liquid nitrogen.

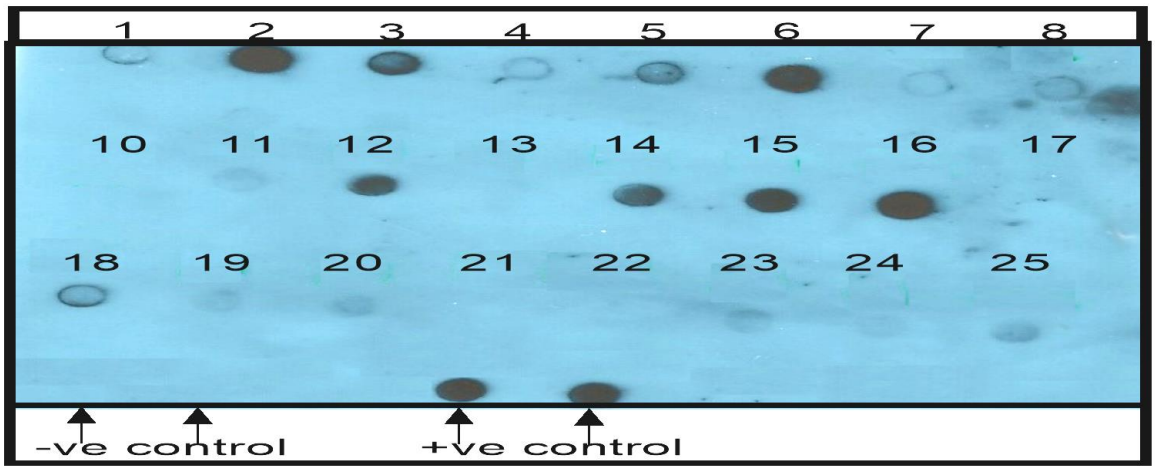

Figure 3.6. Dot blot screening test of numerous clones expressing human recombinant Properdin. Properdin was detected using polyclonal anti-histidine tag antibody (Sigma). The positive control represents purified recombinant mouse Properdin (tagged) (Kindly provided by Dr. M.A Youssef, University of Leicester, UK) (Ali et al., 2014). The negative control represents a media supernatant of non-transient CHO-K1 cell line. Few of the selected clones were positive to express human Properdin. Thus, the clones 2, 3, 6, 12, 15, 16 were chosen for expressing and purifying mass production of Properdin. The positive clones were frozen in liquid nitrogen.

3.1.3. Stable expression of human TSP1 and Properdin

As the ultimate and further goal of this project is to generate inhibitory monoclonal antibodies for both human TSP1 and Properdin, it obtains both purity and mass production

of TSP1 and Properdin. This aim has not achieved yet and shall be for future works. That because of the Medical Company in New Jersey, United States of America (USA) initially planned to provide the inhibitors since this project was in part funded by the company and whom has been involved in this promise, nevertheless, the inhibitory antibodies have not been provided yet. Therefore, 1mg/ml of both recombinant proteins were purified and dispatched to the company in the US, thereby; it is just to fulfil the agreement.

The TSP1 structure consists of five distinct domains. One of these domains is type-III (Calmodulin domain) which is responsible for maintaining the conformational changes in TSP1 by binding to calcium ions (Ca^{2+}) (Yee et al., 2003). Thus, the trace molarity of calcium may lead to less conformational changes which mean obtaining less binding activity to TSP1. Therefore, all the reagent buffers of experiments and protein purification should contain $CaCl^{2+}$, due to the lack of stability regarding calcium deficiency by which some regions in the TSP1 subsequently leading to be more fragile by proteolysis (Lawler and Hynes, 1986).

However, TSP1 was expressed as a recombinant protein from eukaryotic system of HEK293 without mutation or alterations in the structure of the protein, which refers to the fact that the DNA construct of TSP1 was engineered without amendments within the DNA sequence. The TSP1 was detected after the purification process using mouse polyclonal antibody against human TSP1 (Sigma), which binds to the N-terminal domain of TSP1, in reducing conditions by running the boiled TSP1 into SDS-PAGE using β-Mercaptoethanol reducing agent (Sigma), and therefore the TSP1 was electrophoresed and identified in size approximately 180kDa comparing with pre-stained molecular weight protein standard ladder (Invitrogen). Moreover, the native size of TSP1 was electrophoresed in non-reducing conditions into Native-PAGE and then detected in size around 540kDa using mouse monoclonal anti-human TSP1 (Abcam), which binds to type-III repeats in TSP1, comparing with HiMark Pre-Stained Molecular Weight Protein Standard (Invitrogen). The molecular variations of TSP1 in size between reducing and non-reducing (native) conditions mainly due to the existence of the disulphide bonds where located between the polypeptide chains to allow chains joining together in positions near to the N-terminal domain. Altogether, the TSP1 was expressed and secreted as a homo-trimer recombinant glycoprotein protein (Figure 3.11).

Properdin and TSP1 were sent to PNACL, Core Biotechnology Services, at University of Leicester for screening protein analysis by Mass Spectometry following the assess-

ment analysis of UniProtKB-SwissPort data bases usage for matching peptide cleaved sequences. The top scores of matching peptide sequence, were sent from PNACL, revealed the following ID Mapping proteins; P07996 and P27918, for human TSP1 and human Properdin, respectively, according to UniProtKB-SwissPort (Table 3.1 & Table 3.2). The process of using Mass Spectometry was required of running the proteins into SDS-PAGE followed by staining with Coomassie blue. Moreover, Mass Spectrometry required trypsin treatment to cleave proteins into small peptides with known cleavage sites; as trypsin cleave proteins at the carboxyl side between the amino acids arginine and lysine (www.promega.com. 2007).

3.2 Purification of recombinant proteins

The expression of the recombinant human TSP1 was not provided with mutation peptide for purification purposes, but expressing the protein as naturally expressed and with proceeding an easy purification tool using heparin affinity chromatography (Yee et al., 2003).

Nevertheless, the recombinant human Properdin was mutated by encoding poly-histidine residues tag, since sub-cloned into pSeqtag/HygromycinB vector, for purification purposes via using immobilised metal ($NiSO_4$) affinity chromatography (IMAC) (Bornhorst and Falke, 2000).

Therefore, the native structure of recombinant human TSP1 can be considered as a good immunogenic due to be without mutation, however, the histidine tag in the recombinant human Properdin can be removed using Tobacco Etch Virus (TEV) protease in order to be used for immunogenic for therapeutic approach purposes with a probability to stop or control thrombosis in further works in future.

3.2.1. Purification of human recombinant TSP1

Purification of TSP1 is based on the interaction affinity between heparin molecules, which attach to sepharose beads, and between heparin binding domain (HBD) within the N-terminal peptide sequence. Therefore, in order to break down the interaction affinity, higher concentrations of sodium chloride can break down the interaction between heparin molecules and the proteins containing HBD (Yee et al., 2003). Moreover, heparin is specified to bind to a wide range of proteins and polypeptides, a form of highly sulphated glucose-amino-glycan (Lei et al., 2008).

Heparin Sepharose 6 Fast Flow (GE Healthcare) was used to purify the recombinant human TSP1 as described in Chapter 2 in the protein purification section.

All the amount of media supernatant flowed through heparin sepharose columns, and then washed several times and protein fractions were collected after elution step. The fractions were analysed firstly using SDS-PAGE in reducing conditions (Figure 3.8) after the purification showing band with size 180kDa but with contaminants band served by the sepharose column. Therefore, all fractions were de-salted and concentrated using concentrator columns as previously stated in the chapter of methodology. Thereby, the concentrated TSP1 was filtrated through High Performance Liquid Chro-

matography (HPLC) as will be discussed later. After all, TSP1 was run again in non-reducing and reducing conditions, and then stained by Coomassie blue stain while Western blot was used to identified and determine the recombinant TSP1 in all conditions using mouse monoclonal anti-human TSP1 (Abcam) and goat polyclonal anti-human TSP1, respectively (Figure 3.10).

The purified TSP1 appeared on SDS-PAGE at size 180kDa in reducing conditions while in non-reducing conditions was at size 540kDa, providing a single band in all conditions.

3.2.2. Purification of human recombinant Properdin

The purification of recombinant human Properdin depends on affinity interaction between the amino acid residue of histidine tag and a transition metal Ni^{2+} ion. Thus, the higher molarity of imidazole can form interactions with the immobilized metal ion, leading to breaking down the affinity of the metal ions toward the peptide containing histidine tag residues (Bornhorst and Falke, 2000). His GraviTrap column (GE Healthcare) was used to purify the recombinant human Properdin.

The same following strategy of purifying and identifying TSP1 was used also for Properdin either by electrophoresis SDS-PAGE using Coomassie blue stain or Western blot using mouse monoclonal anti-human Properdin (SantaCruz Biotechnology). The purified Properdin appeared on SDS-PAGE with size 65kDa in reducing and non-reducing conditions with only single band (Figure 3.9 & 3.10) which revealed that the contaminants were washed away during purification process.

However, HPLC was not used for filtrating properdin for an obvious reason which will be discussed later. Instead of using HPLC, Properdin was washed several times in His GraviTrap column to eliminate chances of contaminant bands.

3.2.3. Filtration the recombinant human TSP1 using Gel filtration of High Performance Liquid Chromatography (HPLC)

In order to filtrate the recombinant human TSP1 from any contaminants, High Performance Liquid Chromatography (HPLC) was used to eliminate the contaminants proteins and also increase the purity of the recombinant by using dextran gel known Hiload^{TM} 16/60 /Superdex^{TM} 200/prep-grade column (Kindly provided by Dr. Russel Wallis, University of Leicester). As been described in the section of Methodology about the

principle of Gel filtration which separates proteins depends on their sizes; hence, the faster molecules pass through the column are the largest in molecular weight size and vice versa.

As can be seen through (Figure. 3.7), there is only one largest peak in the histogram firstly been eluted through the column due to high molecular weight of the trimeric glycoprotein of TSP1, which is remarkably observed with significant elevation peak. The first peak in the histogram was eluted between fractions 40 till 60 which were identified to contain TSP1 by SDS-PAGE and confirmed by western blot using goat polyclonal anti-human TSP1 antibody (Sigma). Following the first peak, there was a steady histogram showing no or slightly peak which is possibly to be contaminant proteins. Suggesting, using heparin sepharose chromatograph may be not specified for certain proteins but also contaminants can be eliminated during washing process by increasing salt concentrations up to a point that depends on the interaction stability and affinity of the interesting protein. Therefore, heparin sepharose is quit useful for such obtaining purity for protein purification.

All wanted fractions were run in SDS-PAGE in order to observe the purity of each band and also identified by western blot. Therefore, the pure fractions were concentrated to be ready for further experiments (Figure 3.10).

However, recombinant human Properdin was not filtrated through the column because of the purified properdin is probably a mixture of highly oligomer forms; dimer, trimer, tetramer and other higher forms including pentamers and hexamer, by which I need to investigate the mixture rather than investigating each form alone for binding and activating intact platelets. Therefore, by filtrating, the forms of properdin certainly will be separated. Instead, Properdin was washed several times by increasing the imidazole concentrations to eliminate any possible contaminates proteins in the column of His-GraviTrap.

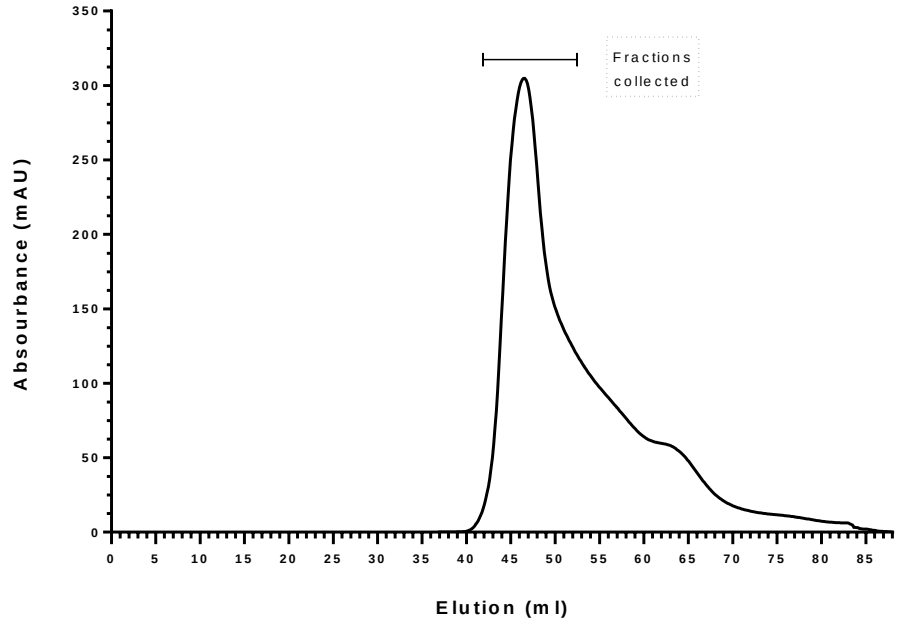

Figure 3.7. A histogram of HPLC shows one highest peak between fractions 40-60 for TSP1 filtration. The TSP1 was come out first from the superdex^{TM} 200 column, whereas, few peaks considered being contaminant proteins came out from the column later.

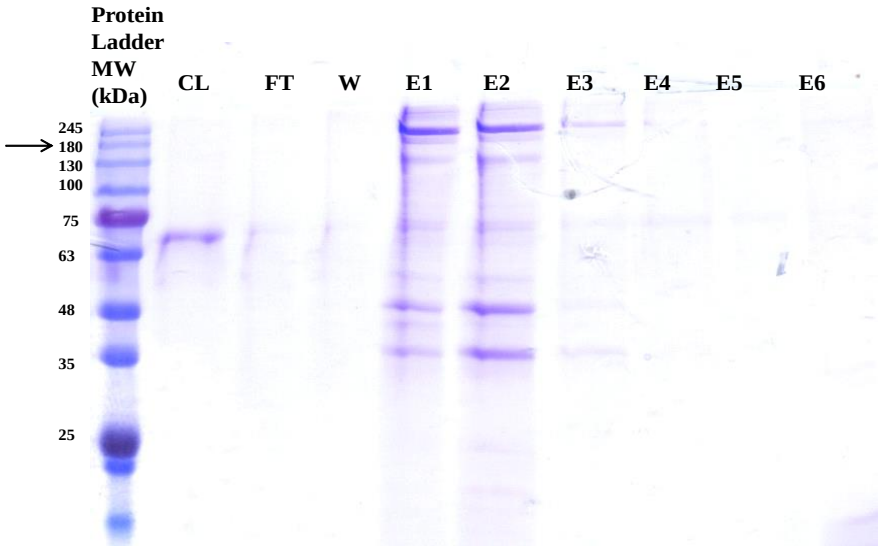

Figure 3.8. Purification of recombinant human TSP1 using Heparin Sepharose 6 Fast Flow (GE Healthcare). The purified TSP1 was run in 10% SDS-PAGE under reducing conditions using β-Mercaptoethanol and boiling the proteins for 5 minutes at 95°C. The gel was stained by Coomassie stain. TSP1 was observed on the band with size approximately 180kDa. Each well of loading sample on the gel was loaded by the following; CL: media column load, FT: media flow through, W: washing column, E1-6: elution fractions, MW: molecular weight marker.

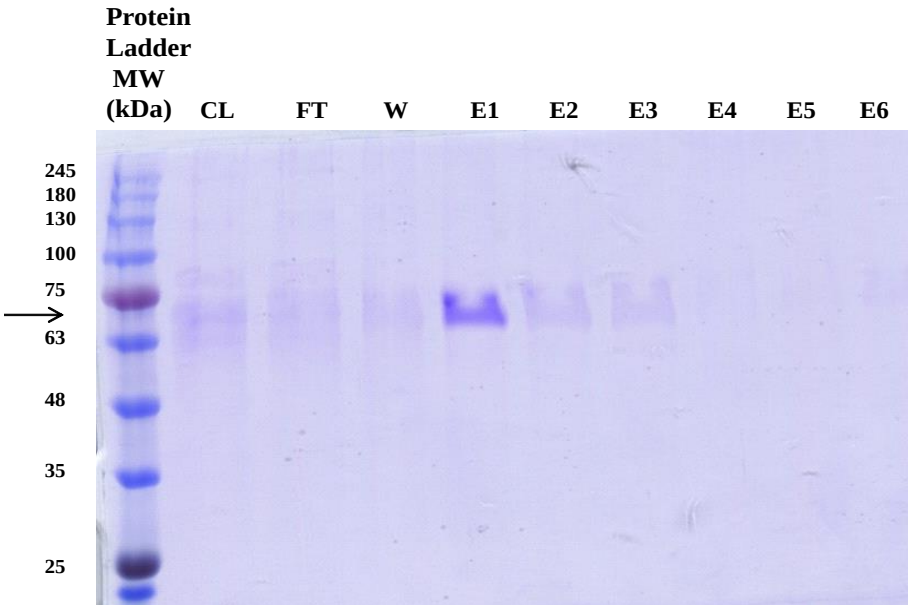

Figure 3.9. Purification of recombinant human Properdin using His GraviTrap column (GE Healthcare). The purified Properdin was run in 10% SDS-PAGE in reducing conditions using β-Mercaptoethanol and boiling the protein for 5 minutes at 95°C. The gel was stained by coomassie stain. Properdin was observed on the band with size approximately 65kDa. Each well of loading sample on the gel was loaded by the following; CL: media column load, FT: media flow through, W: washing column, E1-6: elution fractions, MW: molecular weight marker.

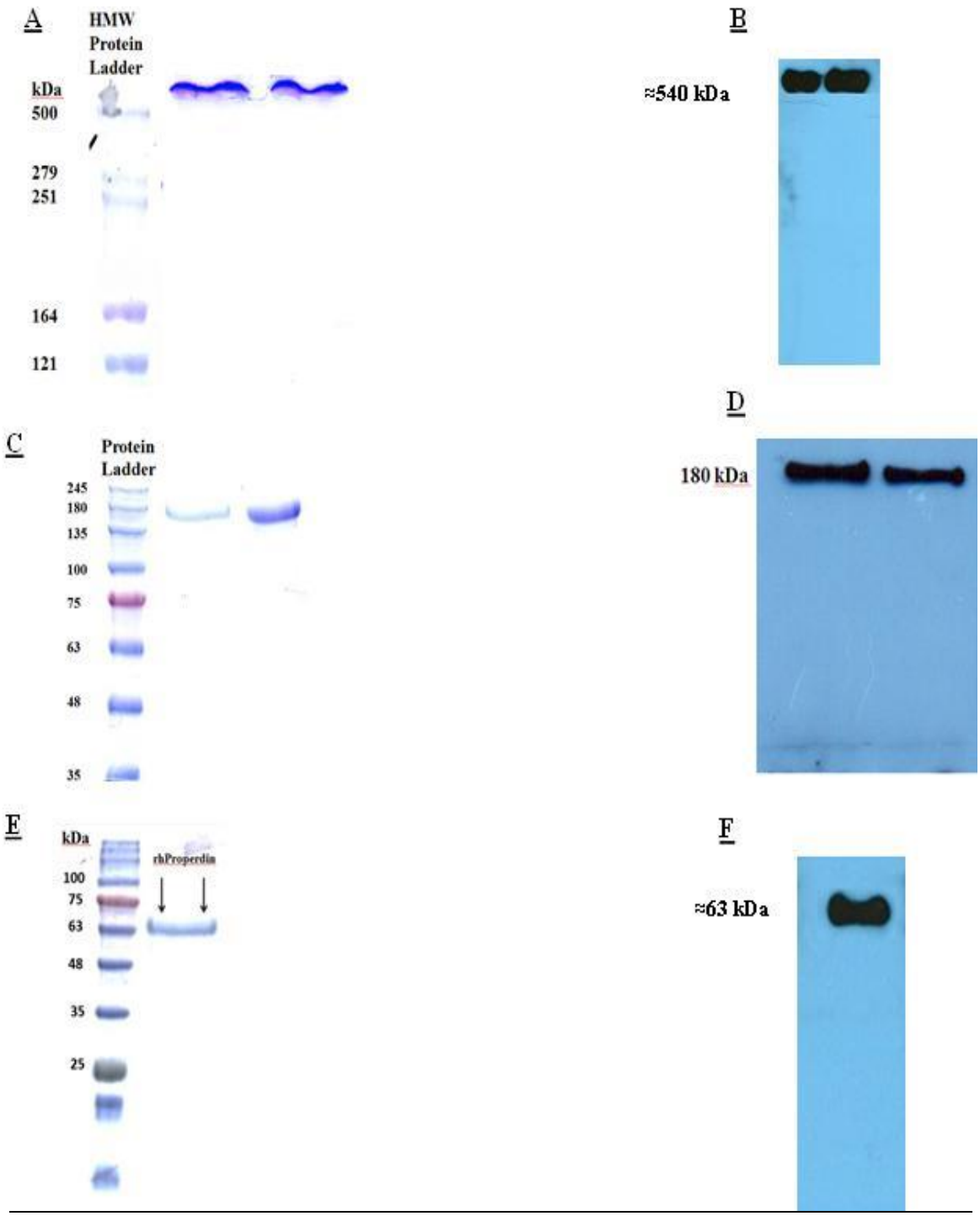

Figure 3.10. Purification of recombinant human TSP1 and Properdin, after using Gel filtration columns; Superdex^{TM} 200 column, and His GraviTrap column, respectively. (A) The purified and filtrated TSP1 was run in 6% Native-PAGE in non-reducing conditions and stained with Coomassie stain which showed one single band around 540kDa. (B) Western blot analysis was used to identify TSP1 using a mouse monoclonal anti-human TSP1 antibody (Abcam). (C) The purified and filtrated TSP1 was also run in 8% SDS-PAGE in reducing conditions using β-Mercaptoethanol and boiling process by which after staining observing one single band around 180kDa. (D) TSP1 was run under reducing conditions and identified by Western blot using goat polyclonal anti-human TSP1 (Sigma). (E) The purified Properdin was also run under reducing conditions in 12% SDS-PAGE. After staining one single band of approximately 63kDa is seen. (F) Properdin was identified by Western blot using mouse monoclonal anti-human properdin antibody (SantaCruz Biotechnology). Note; Properdin was not paased through HPLC column.

Protein	Matching peptide sequences		Observed Mass (Da)	Calculated Mass (Da)
	Peptide position	Peptide sequences		
TSP1	189-197	RDLASIARL	745.4310	744.4130
	1103-1110	KDFTAYRW	772.3630	771.3551
	101-110	RGTLLALERK	872.5380	871.5127
	1091-1099	RTLWHDPRH	924.4610	923.4613
	86-96	KGFLLLASLRQ	989.6020	988.6069
	50-61	KGPDPSSPAFRI	1030.4950	1029.4880
	164-174	RAQLYIDCEKM	1139.5700	1138.5328
	154-165	KSITLFVQEDRA	1207.6260	1206.6245
	288-300	RTIVTTLQDSIRK	1246.6980	1245.6929
	124-138	KAGTLDLSLTVQGKQ	1302.7540	1301.7191
	421-432	RTCHIQECDKRF	1346.6060	1345.5867
	968-981	KGTSQNDPNWVVRH	1372.6550	1371.6531
	216-229	RFVFGTTPEDILRN	1394.7280	1393.7242
	1077-1092	RNALWHTGNTPGQVRT	1550.7900	1549.7750
	810-823	RDNCQYVYNVDQRD	1573.6720	1572.6627
	109-125	RKDHSGQVFSVVSNGKA	1588.8420	1587.8005
	1041-1055	KQVTQSYWDTNPTRA	1595.7510	1594.7376
	201-217	KGGVNDNFQGVLQNVRF	1616.8200	1615.8067
	293-608	KEVPDACFNHNGEHRC	1681.7270	1680.7063
	662-676	KCNYLGHYSDPMYRC	1691.7020	1690.6868
	1018-1035	RDDDYAGFVFGYQSSSRF	1813.7810	1812.7591
	173-190	KMENAELDVPIQSVFTRD	1864.9200	1863.9037
	623-642	RFTGSQPFGQGVEHATANKQ	1875.9210	1874.8911
	137-155	KQHVVSVEEALLATGQWKS	1895.0520	1893.9949
	607-624	RCENTDPGYNCLPCPPRF	1949.8130	1948.7866
	460-480	RLCNSPSPQMNGKPCEGEARE	2147.9520	2146.9194
	20-42	RIPESGGDNSVFDIFELTGAARK	2195.0910	2194.0542
	264-286	KDLQAICGISCDELSSMVLELRG	2409.1720	2408.1386
	60-83	RIEDANLIPPVPDDKFQDLVDAVRA	2579.3740	2578.3279

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1    MGLAWGLGVL FLMHVCGTNR IPESGGDNSV FDIFELTGAA RKGSGRRLVK GPDPSSPAFR
61   IEDANLIPPV PDDKFQDLVD AVRAEKGFLL LASLRQMKKT RGTLLALERK DHSGQVFSVV
121  SNGKAGTLDL SLTVQGKQHV VSVEEALLAT GQWKSITLFV QEDRAQLYID CEKMENAELD
181  VPIQSVFTRD LASIARLRIA KGGVNDNFQG VLQNVRFVFG TTPEDILRNK GCSSSTSVLL
241  TLDNNVVNGS SPAIRTNYIG HKTKDLQAIC GISCDELSSM VLELRGLRTI VTTLQDSIRK
301  VTEENKELAN ELRRPPLCYH NGVQYRNNEE WTVDSCTECH CQNSVTICKK VSCPIMPCSN
361  ATVPDGECCP RCWPSDSADD GWSPWSEWTS CSTSCGNGIQ QRGRSCDSLN NRCEGSSVQT

421  RTCHIQECDK RFKQDGGWSH WSPWSSCSVT CGDGVITRIR LCNSPSPQMN GKPCEGEARE
481  TKACKKDACP INGGWGPWSP WDICSVTCGG GVQKRSRLCN NPTPQFGGKD CVGDVTENQI
541  CNKQDCPIDG CLSNPCFAGV KCTSYPDGSW KCGACPPGYS GNGIQCTDVD ECKEVPDACF
601  NHNGEHRCEN TDPGYNCLPC PPRFTGSQPF GQGVEHATAN KQVCKPRNPC TDGTHDCNKN
661  AKCNYLGHYS DPMYRCECKP GYAGNGIICG EDTDLDGWPN ENLVCVANAT YHCKKDNCPN
721  LPNSGQEDYD KDGIGDACDD DDDNDKIPDD RDNCPFHYNP AQYDYDRDDV GDRCDNCPYN
781  HNPDQADTDN NGEGDACAAD IDGDGILNER DNCQYVYNVD QRDTDMDGVG DQCDNCPLEH
841  NPDQLDSDSD RIGDTCDNNQ DIDEDGHQNN LDNCPYVPNA NQADHDKDGK GDACDHDDDN
901  DGIPDDKDNC RLVPNPDQKD SDGDGRGDAC KDDFDHDSVP DIDDICPENV DISETDFRRF
961  QMIPLDPKGT SQNDPNWVVR HQGKELVQTV NCDPGLAVGY DEFNAVDFSG TFFINTERDD
1021 DYAGFVFGYQ SSSRFYVVMW KQVTQSYWDT NPTRAQGYSG LSVKVVNSTT GPGEHLRNAL
1081 WHTGNTPGQV RTLWHDPRHI GWKDFTAYRW RLSHRPKTGF IRVVMYEGKK IMADSGPIYD
1141 KTYAGGRLGL FVFSQEMVFF SDLKYECRDP
```

Table 3.1 Derived amino acid sequence of human TSP1 P07996 without any mutations or engineered alterations within the protein sequence. The top score, in the table above, using Mass Spectometry was revealed to P07996, human TSP1, according to UniProtKB-SwissPort. The data were obtained from PNACL, Core Biotechnology Services, University of Leicester.

Protein	Matching peptide sequences		Observed Mass (Da)	Calculated Mass (Da)
	Peptide position	Peptide sequences		
Properdin	353-360	RKFDGHRC	759.3790	758.3824
	67-77	RSGGLCQPCRS	1034.4630	1033.4433
	359-369	RCAGQQQDIRH	1075.5160	1074.4876
	66-77	KRSGGLCQPCRS	1190.5920	1189.5444
	103-105	RCVGWNGQCSGKV	1252.5340	1251.5125
	368-380	RHCYSIQHCPLKG	1442.7030	1441.6595
	333-347	KSISCQEIPGQQSRG	1489.7400	1488.6991
	282-303	RTCNHPVPQHGGPFCAGDATRT	2178.9860	2177.9484
	359-380	RCAGQQQDIRHCYSIQHCPLKG	2499.2080	2498.1366
	44-68	KGLLGGGVSVEDCCLNTAFAYQKRS	2515.2050	2514.1995
	223-247	KCSAPEPSQKPPGKPCPGLAYEQRR	2554.2360	2553.2104
	222-247	RKCSAPEPSQKPPGKPCPGLAYEQRR	2682.3280	2681.3054
	44-67	KGLLGGGVSVEDCCLNTAFAYQKR	2359.1410	2358.0984

```
1   MITEGAQAPR LLLPPLLLLL TLPATGSDPV LCFTQYEESS GKCKGLLGGG
51  VSVEDCCLNT AFAYQKRSGG LCQPCRSPRW SLWSTWAPCS VTCSEGSQLR
101 YRRCVGWNGQ CSGKVAPGTL EWQLQACEDQ QCCPEMGGWS GWGPWEPCSV
151 TCSKGTRTRR RACNHPAPKC GGHCPGQAQE SEACDTQQVC PTHGAWATWG
201 PWTPCSASCH GGPHEPKETR SRKCSAPEPS QKPPGKPCPG LAYEQRRCTG
251 LPPCPVAGGW GPWGPVSPCP VTCGLGQTME QRTCNHPVPQ HGGPFCAGDA
301 TRTHICNTAV PCPVDGEWDS WGEWSPCIRR NMKSISCQEI PGQQSRGRTC
351 RGRKFDGHRC AGQQQDIRHC YSIQHCPLKG SWSEWSTWGL CMPPCGPNPT
401 RARQRLCTPL LPKYPPTVSM VEGQGEKNVT FWGRPLPRCE ELQGQKLVVE
451 EKRPCLHVPA CKDPEEEEL  His His His His His His
                          Polyhistidine tag
```

Table 3.2. Derived amino acid sequence of human Properdin P27918 with His 6X tag peptide sequence. The top score, in the table above, using Mass Spectrometry was revealed to P27918, human Properdin, according to UniProtKB-SwissPort. The data were obtained from PNACL, Core Biotechnology Services, University of Leicester**.**

3.3. Discussion

The plasmid DNA containing the complete coding sequence for human TSP1 was transfected into both the Chinese hamster ovary (CHO-K1) cell line and the Human Embryonic Kidney cell line (HEK)-293. Mouse monoclonal and goat polyclonal antibodies have been used for Dot and Western blot analyses to identify the expression of TSP1 either from the CHO-K1 or HEK-293. While recombinant TSP1 expression could not be detected in CHO-K1 cells, recombinant TSP1 was produced and successfully purified from transfected HEK cells. The failure to express recombinant of TSP1 from the CHO-K1 may probably be due to the binding activity of some ligand receptors of CHO-K1 cells towards TSP1 preventing recombinant TSP1 to be released into the supernatant of cell culture media. The physiological function of one of TSP1 ligand receptors (thrombospondin cell adhesion receptor) is to provide cell adhesion support. It may be that recombinant TSP1 may bind to the heparin sulphate proteoglycan on the cell wall of CHO-K1 cells (Kaesberg et al., 1989). It has been reported in 1997, that mutations of glycosaminoglycans (GAGs) within the cell membrane of CHO-K1 cells dissociated the binding of the N-terminal of TSP1 domain to GAGs. GAGs do not bind to any other TSP1 domain (Clezardin et al., 1997). I therefore believe that the failure to purify recombinant TSP1 from the supernatant of transfected CHO-K1 cells is caused by the binding of TSP1 to GAGs in this cell line. This was not observed in the transfected HEK293 cell line which allowed me to purify large quantities of recombinant TSP1 using this cell line.

The concentration of TSP1 in plasma under normal physiological conditions fluctuates between 60-300ng/ml (Switalska et al., 1985). However, the plasma concentration of TSP1 can vary considerably as a consequence of massive platelet activation during thrombotic events or pathophysiological conditions such as in peripheral arterial disease (PAD) (Smadja et al., 2011). TSP1 is a secreted protein and represents approximately 3% of the total protein concentration found in alpha granules of platelets 30–100µg in 10^9 platelets (Bonnefoy et al., 2008). I expressed recombinant human TSP1 in HEK293 cells and determined this recombinant protein to have a molecular weight of 540kDa under non-reducing and 180kDa under reducing conditions, respectively. These variations in sizes would illustrate the presence of disulphide bonds in TSP1 which are capable of connecting the three polypeptide chains between the amino (NH_2)-terminal region

and procollagen homology domain, resulting in the structure of a homo-trimeric glycoprotein.

The transfected CHO-K1 cell line producing recombinant human properdin was kindly provided by Dr M. Y. Ali (Ali et al., 2014). Recombinant human properdin was purified using a HisGravi Trap column. The addition of a His-tag sequence at the N-terminal region of properdin (Ali et al., 2014), allowed the recombinant properdin to be purified by Ni-sepharose column chromatography (Bornhorst and Falke, 2000).

A heparin binding domain (HBD) is located within the amino terminal region of TSP1 and is responsible for binding to heparin, sulfatide and heparin sulphate proteoglycan, while the central domain was found to bind heparin due to the presence of the heparin binding sequence in type-I TSR repeat (Dixit et al., 1984, Murphy-Ullrich and Mosher, 1987, Guo et al., 1992, Takagi et al., 1993, Yu et al., 2000). It has also been reported that properdin is capable of binding to heparin (Yu et al., 2005). Therefore, heparin sepharose chromatography was utilised to purify properdin after enzymatic cleavage of the His-tag sequence by TEV. The application of heparin chromatography to purify HBD containing proteins allows the use of buffers with high sodium chloride content to dissociate the binding of HBD containing proteins to heparin (Yee et al., 2003). The sodium chloride concentration required to elute properdin from heparin sepharose columns was relatively low compared to those reqired to elute TSP1 after heparin column chromatography.

Since the overall degree of similarity between the TSR repeats in TSP1 and the TSR repeats in properdin is approximately 47% (Goundis and Reid, 1988) with highly conserved positions of amino acids the differences between these highly related proteins in their ability to bind to heparin are quite remarkable and unexpected.

The normal plasma concentration of properdin varies within the range 5-25 µg/ml (Nolan and Reid, 1993). Properdin is essentially generated and secreted by leukocytes, including monocytes (Whaley, 1980, Schwaeble et al., 1994), T cells (Schwaeble et al., 1993) and neutrophils (Wirthmueller et al., 1997). The positive regulator properdin is released from secondary granules of neutrophils in response to pro-inflammatory stimuli, such as TNF-α and C5a. Properdin is present in plasma in different physiological forms; dimer, trimer and tetramer (Schwaeble and Reid, 1999b, Pangburn, 1989). In order to remove all possible contaminants, recombinant properdin was purified by Ni-sepharose column chromatography (which allows the use of repeated washing steps)

rather than just by gel filtration chromatography based on molecular weight of properdin cyclic forms (as described in Chapter-2). While I generated most of the recombinant properdin, some aliquots used were kindly given to me by Dr. M. Y. Ali (Ali et al., 2014), while serum preparations of native properdin forms were kindly provided by Dr Viviana P. Ferreira, University of Toledo, USA (Ferreira et al., 2010).

Chapter 4 (Results)

The role of human Thrombospondin (TSP1) and Properdin in Hemostasis or Thrombosis process

The role of human Thrombospondin (TSP1) and Properdin in Hemostasis or Thrombosis process

Human Thrombospondin 1 (TSP1) and Properdin are going to be investigated and identified with respect to determining the functional capability of interacting with and activating human platelets leading to expression of biomarkers or even secretions and eventually ending up with platelet clump formation as a result of activation. In thrombosis/hemostasis events, the extracellular matrix proteins associate with platelet activation by providing platelet adhesion support. Here in this chapter, Properdin interaction with different matrix proteins will be focused on besides the interactions with lipid; LDL, cholesterol and triglycerides, which are associated with such disease enhancing thrombotic events, like atherosclerosis.

4. Results

4.1 The role of human TSP1 and Properdin in Human platelets

Complement system synergistically promotes platelet activation at vascular injury sites to prevent blood loss and provide protection.

Platelet is an essential part of haemostasis through playing significant and effective function of forming platelet clump in vascular injury sites (Walsh, 2004). The role of TSP1 on platelets has already been established long time ago.

It has been suggested that the complement system may participate with haemostasis as a non-inflammatory process (Ricklin et al., 2010), also the activated platelets trigger complement activation by allowing pattern recognition molecules of CP and LP to bind and initiate complement activation, moreover, the AP can be initiated by properdin that binds to the hydrolysed of C3 ($C3_{H2O}$) or binds to C3b and fB and then forms a complex of C3 convertase of AP on the surface of activated platelet (Saggu et al., 2013), in order to increase the sufficiency of platelet activation by initiating the release of PMPs.

The aim of this chapter is to investigate the correlation of human Properdin besides TSP1. The aim identifies the functional activity of Properdin on human washed intact platelets by measuring the following biomarkers; P-selectin (CD62P), α-thrombin (FIIa) and thrombin generation by PMPs, as a result of activation. Moreover, the chapter aims to investigate the binding ability of Properdin toward ECMs proteins and atherogenic particles which may affect the platelet firm adhesion process leading to serious such as atherosclerosis disease or Thrombosis.

4.1.1. Binding activity of human TSP1 & Properdin on intact and activated platelets

Thrombospondin1 is, able to trigger platelet activation, a major molecule component released from platelet α-granules, and from certain human cells such as endothelial, epithelial cells (Isenberg et al., 2008b). Whereas, Properdin is released from certain immune cells like T-cell and granulocyte lineage (Schwaeble and Reid, 1999a), and is functioning as a positive regulator of AP of complement system. There are few scientific researches investigating the ability of human properdin to bind to human intact platelets which may result in platelet activation (investigating plasma properdin native

forms individually). The ability of Properdin alongside TSP1 to bind to intact platelets was investigated using ELISA by coating intact platelets on maxi-sorb 96-well plate (Nunc). Prostaglandin-E1 PGE1 (200ng/ml) was used for platelet activation prevention during washing process to ensure the only intact platelets are involved, while 1Unit/ml thrombin was used after washing process to obtain the activation as +ve control.

Both TSP1 and highly active oligomerised properdin were able to bind to intact and activated platelets comparing to the negative control human serum albumin (HSA) (Figure 4.1A, B). TSP1 was used as positive control known as agonist for platelet activation, shows dramatic attachment and subsequently leads to platelet aggregation (Isenberg et al., 2008b). In the current study, however, resting platelets were surprisingly observed to bind to highly active oligomerised properdin. P-selectin (CD62p) was targeted in ELISA as a biomarker to determine the response of platelet activation during extraction and washing process, by which biomarker levels could clearly point out the binding of platelets before and after activation (Figure 4.1C).

According to the statistical analysis (Table 4.2) revealed that highly active oligomerised Properdin showed highly significant difference in binding ($P<0.0001$) to either intact or thrombin-activated platelet comparing with binding to HSA as the same as TSP1 (Table 4.1). TSP1 showed no significant difference in binding between intact and activated platelets, whereas, Properdin showed significant difference ($P=0.001$). That would indicate the binding capability of highly active oligomerised Properdin either to intact or activated platelet, besides; the slight binding differences of platelets would reveal to increase the binding capacity on activated platelets possibly due to platelet expressing receptors and secretions during activation. It requires more investigation to identify properdin ligand receptors on platelet.

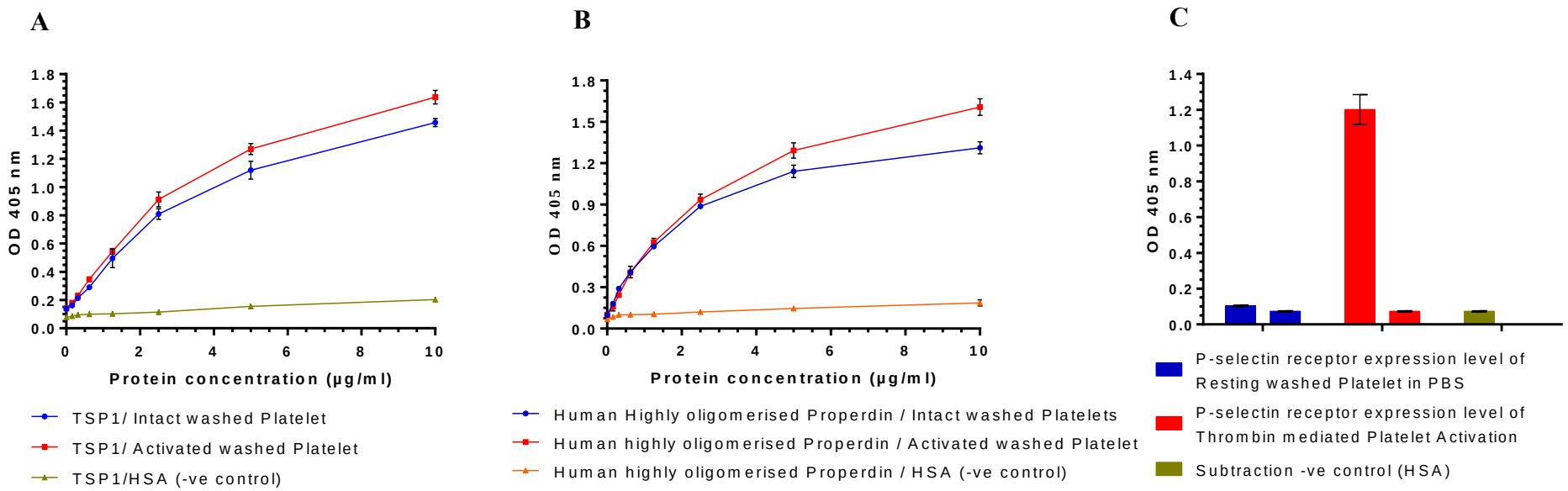

Figure 4.1. Thrombospondin1 (TSP1) and highly active oligomerised Properdin bind to the surface of intact and activated platelets. (A) Serial dilution starting from 10µg/ml recombinant human TSP1 shows binding on the surface of both human intact and activated washed platelets (150,000 platelets/µl). (B) Moreover, highly active oligomerised Properdin also binds to both intact platelets and thrombin-mediated platelet activation. The negative control of human serum albumin (HSA), served to block the residual plastic surface of micro-titration plate, show no binding to both recombinants. However, the last wells in the plate were only receiving buffer instead of diluted proteins from which the value of optical density (OD) of diluted proteins was subtracted. (C) Mouse monoclonal anti-human P-selectin (Santa Cruz Biotechnology) was used to determine the expression levels of P-selectin (CD62p) on outer membrane of platelet as biomarker for determining active or intact platelets. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM

Two Way ANOVA		
Binding TSP1	**Significant? P < 0.05?**	**P value Summary**
Intact Platelet vs HSA	Yes P= <0.0001	****
Activated Platelet vs HSA	Yes P= <0.0001	****
Intact Platelet vs Activated Platelet	No P= 0.068	NS

Table 4.1 The statistical significance results of (Figure 4.1A) binding TSP1 to Intact platelets and Activated platelets compared with binding to HSA (-ve control). The binding of TSP1 on either intact or activated platelets is significantly distinguishable from the negative control. However, no difference found between the binding on intact and activated platelets. The significant P value was statistically calculated by using Two Way ANOVA (Ordinary). Significant values=P<0.05. Key words; (*); significant. NS; Not significant.

Two Way ANOVA		
Binding Properdin	**Significant? P < 0.05?**	**P value Summary**
Intact Platelet vs HSA	Yes P= <0.0001	****
Activated Platelet vs HSA	Yes P= <0.0001	****
Intact Platelet vs Activated Platelet	Yes P= 0.001	**

Table 4.2 The statistical significance results of (Figure 4.1B) binding Properdin to Intact platelets and Activated platelets compared with binding to HSA (-ve control). The binding of Properdin on either intact or activated platelets is significantly distinguishable from the negative control. There is also a significance difference between the binding on activated and intact platelets. The significant P value was statistically calculated by using Two Way ANOVA (Ordinary). Significant values=P<0.05. Key words; (*); significant. NS; Not significant.

4.1.2 Binding human plasma Properdin native forms to resting and activated human platelets

It has been reported that Properdin native forms; dimer, trimer and tetramer, bind to activated platelets but not resting platelets (Saggu et al., 2013). The binding ability of properdin native forms and oligomerised forms toward the intact and activated human platelets was achieved by ELISA. As in previous ELISA, the intact and activated platelets were treated with Prostaglandin-E1 PGE1 to prevent the activation during washing process and then platelets were coated in ELISA plates. Platelet activation was evaluated by targeting P-selectin (CD62p) using mouse monoclonal antibody against P-selectin (CD62p) (Santa Cruz Biotechnology). P-selectin would not be expressed in the outer membrane of resting platelets (Figure 4.2D).

Recombinant highly active oligomerised properdin and highly oligomerised form surprisingly showed significant binding to both intact and activated platelets, whereas, the other properdin native forms were observed with less binding ability comparing to the higher forms (Figure 4.2 A,B). According to the statistical analysis (Table 4.3), the highly oligomerised form showed a highly significant difference in means (0.921) from other forms in binding to intact platelet. On the other hand, the significant difference in binding of other properdin forms to activated platelets was dramatically increased in means, while, the means of properdin forms were not higher than highly oligomerised form. All together were compared to the –ve control HSA. That would indicate the significant role of properdin oligomerised form in the platelet activation process. Oligomerised active form of Properdin may exist in serum after complement activation, or generated after secretion from inflammatory cells with lower ratio levels comparing to the most other native forms.

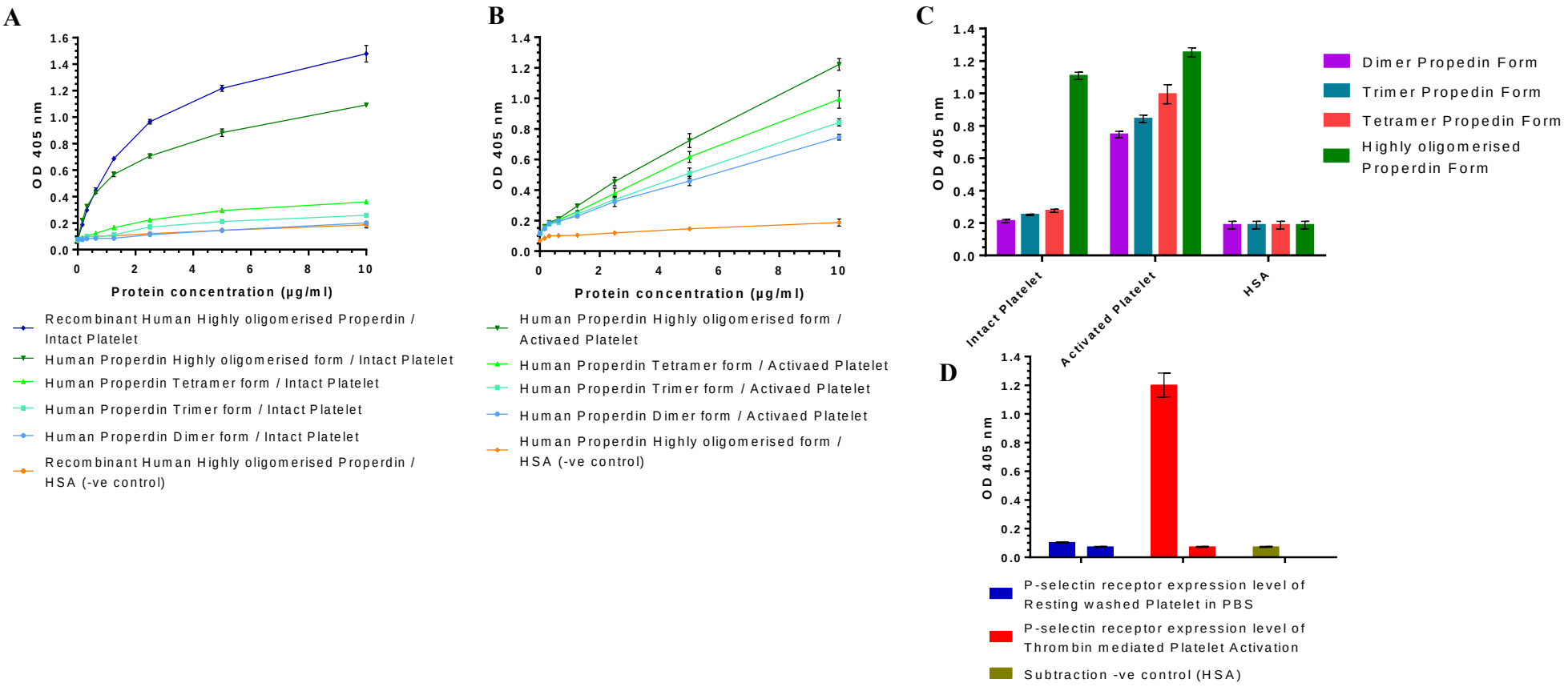

Figure 4.2. The binding capability of properdin forms to the surface of human intact and activated platelets. (A) Serial dilutions starting from 10µg/ml each native form of properdin (Dimer, Trimer and Tetramer) show no or less binding on the surface of human intact washed platelets (150,000 platelets/µl) but only the highly oligomerised form comparing with the recombinant highly active oligomerised properdin. However, (B) all properdin forms bind on the surface of thrombin-mediated platelet activation. The last wells in the plate were only receiving buffer instead of diluted proteins by which the value of optical density (OD) of diluted proteins being subtracted. (C) Comparing between properdin forms into two groups; intact and activated platelets using one single concentration of each form (10µg/ml). (D) Mouse monoclonal anti-human P-selectin (Santa Cruz Biotechnology) was used to determine the expression levels of P-selectin (CD62p) on outer membrane of platelet as biomarker for determining active or intact platelets. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM

Two Way ANOVA (Bonferroni's multiple comparisons test)		
Binding Properdin forms to; Intact platelets vs HSA	**Significant? P < 0.05?**	**Mean Diff.**
Properdin Dimer form	No (NS)	0.025
Properdin Trimer form	No (NS)	0.063
Properdin Tetramer form	No (NS)	0.088
Properdin Highly oligomerised form	Yes (****)	0.921
Two Way ANOVA (Bonferroni's multiple comparisons test)		
Binding Properdin forms to; Activated platelets vs HSA	**Significant? P < 0.05?**	**Mean Diff.**
Properdin Dimer form	Yes (****)	0.559
Properdin Trimer form	Yes (****)	0.655
Properdin Tetramer form	Yes (****)	0.808
Properdin Highly oligomerised form	Yes (****)	1.065
Two Way ANOVA (Bonferroni's multiple comparisons test)		
Binding Properdin forms to; Activated platelets vs Intact platelets	**Significant? P < 0.05?**	**Mean Diff.**
Properdin Dimer form	Yes (****)	0.534
Properdin Trimer form	Yes (****)	0.582
Properdin Tetramer form	Yes (****)	0.718
Properdin Highly oligomerised form	Yes (**)	0.144

Table 4.3 The statistical significance results of (Figure 4.2C) binding Properdin forms to Intact and Activated platelets compared with each other and with binding to HSA (-ve control). The binding of Properdin forms is significantly distinguishable only through the binding to activated platelets except the highly oligomerised form. The significant P value=<0.0001 was statistically calculated by using Two Way ANOVA (Bonferroni's multiple comparisons test). Significant values= P<0.05. Key words; (*); significant. NS; Not significant.

Two Way ANOVA (Bonferroni's multiple comparisons test)		
Binding to Intact platelets; Properdin Highly oligomerised form vs Properdin forms	**Significant? P < 0.05?**	**Mean Diff.**
Vs Properdin Dimer form	Yes (****)	0.896
Vs Properdin Trimer form	Yes (****)	0.858
Vs Properdin Tetramer form	Yes (****)	0.833
Two Way ANOVA (Bonferroni's multiple comparisons test)		
Binding to Activated platelets; Properdin Highly oligomerised form vs Properdin forms	**Significant? P < 0.05?**	**Mean Diff.**
Vs Properdin Dimer form	Yes (****)	0.506
Vs Properdin Trimer form	Yes (****)	0.410
Vs Properdin Tetramer form	Yes (***)	0.257

Table 4.4 The statistical significance results of (Figure 4.2C) binding Properdin Highly oligomerised form to Intact and Activated platelets compared with other properdin forms in all cases. The binding of Highly oligomerised form to intact platelet is significantly higher than the other forms while in case of activated platelets the significant comparisons is getting lower by mean diff. The significant P value=< 0.0001 was statistically calculated by using Two Way ANOVA (Bonferroni's multiple comparisons test). Significant values=P<0.05. Key words; (*); significant. NS; Not significant.

4.1.3 Binding TSP1 and Properdin to platelet receptors

After observing the capability of both recombinants to bind to either intact or activated platelets, it is essential to investigate which receptor that properdin binds to on platelets while TSP1 ligand receptors have been established for long time such as, GPIb/V/IX, CD36, CD47 and GPIIb/IIIα. Therefore, certain of TSP1 ligand receptors were purchased from different commercial companies.

4.1.3.1. Biding to GPIbα and GPV of GPIb/V/IX complex

After vessel injury, ECMs proteins such as collagen, TSP1 and vWF as adhesive molecules, exposed by sub-endothelial layer required for platelet adhesion. The GPIb/V/IX complex is an essential adhesion receptor, interacts with vWF, Fibronectin, TSP1 and other matrix proteins (Jurk and Kehrel, 2005, Andrews and Berndt, 2004). GPIb/V/IX consists of four leucine-rich repeats GPIbα (CD42b), GPIbβ, GPIX and GPV (CD42d) (Andrews et al., 2003). CD42b and CD42d were chosen for binding assay to represent platelet adhesion complex receptor.

Both TSP1 and Properdin showed an obvious and clear binding to GPIbα (CD42b) and GPV (CD42d) (Abcam) using ELISA by coating the repeats in 96-well plates. Suggesting, properdin could be a new ligand as an adhesive protein for GPIb/V/IX complex and might be associated in platelet adhesion process. The binding was justified by comparing the binding of HSA to TSP1 and Properdin, resulted with obviously no binding, similar to background (Figure 4.3).

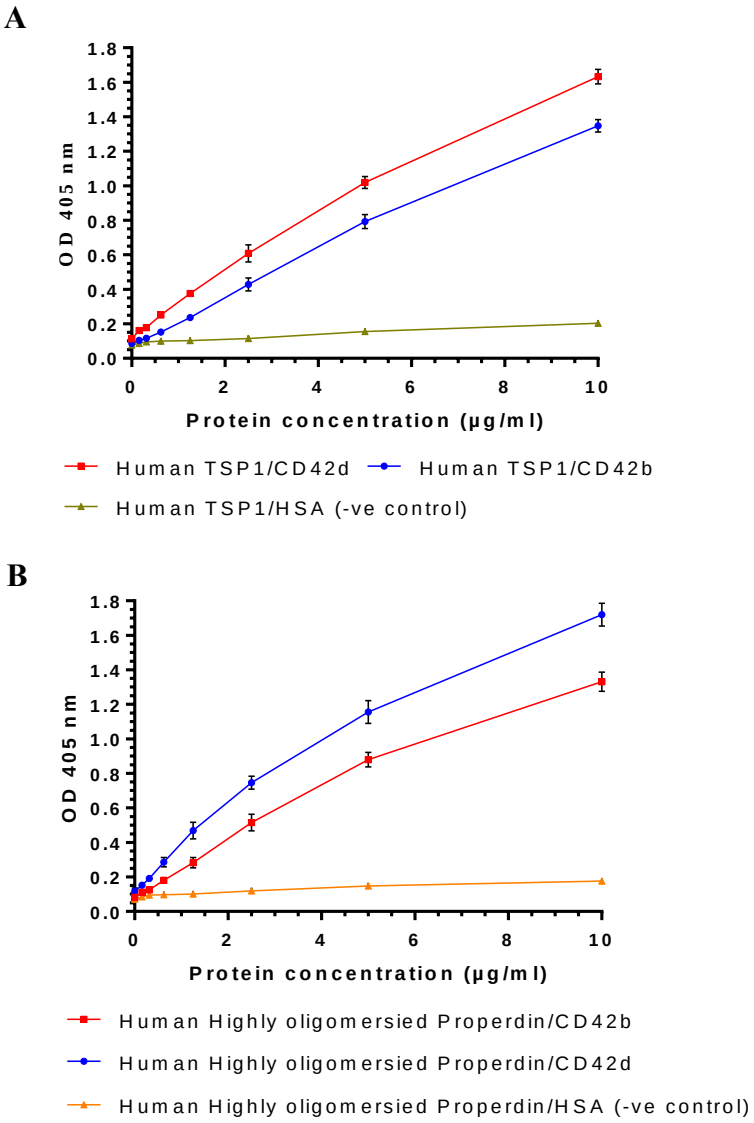

Figure 4.3. Binding capability of TSP1 and highly oligomerised Properdin toward platelet receptors GPIbα (CD42b) and GPV (CD42d) of platelet GPIb/V/IX complex. The binding assay was established using ELISA assay. 3µg/ml (300ng/well) of each repeat was coated into Maxisorb 96-well plate. 1% HSA was used as a negative control as it is used for blocking buffer. Serial dilutions starting from 10µg/ml both TSP1 (A) and Properdin (B) show the ability to bind to platelet receptors while no binding was observed toward the negative control (HSA). The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

4.1.3.2. Binding to CD41α (Integrin α2b)

Platelet integrin α2bβ3 (GPIIb/IIIα) (CD41/CD61) is considered to be a central platelet receptor for platelet accumulation in association with fibrinogen (Fg) by linking platelets together and forming platelet aggregating (Jurk and Kehrel, 2005). Integrin α2b binds to its ligands such as Fg, vWF and TSP1, in active state to increase platelet activation through raising GPIIb/IIIα expression levels and also influx Ca^{2+} that is required for conformational changes on the complex to become attachable (Jurk and Kehrel, 2005). Platelet activation occurs firstly via GPVI and GPIb/V/IX (Andrews and Berndt, 2004). CD41α was chosen for binding assay toward TSP1 and Properdin to identify other ligands.

Both TSP1 and Properdin showed an obvious and clear binding to integrin α2b (CD41α) (Abcam) using ELISA by coating the integrin in 96-well plates. Suggesting, properdin could be a new ligand for GPIIb/IIIα complex and might be associated with Fg providing support for platelet clumps formation. The binding was justified by comparing the binding of HSA to TSP1 and Properdin, resulted with obviously no binding, similar to background (Figure 4.4).

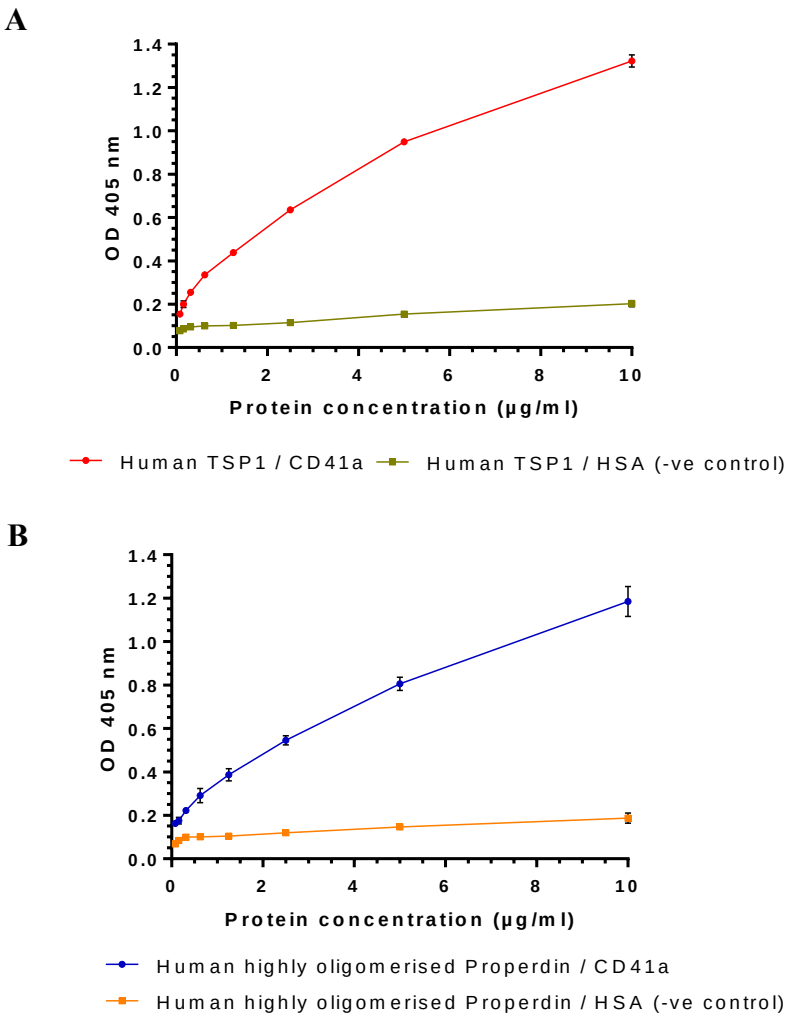

Figure 4.4. The binding capability of TSP1 and highly oligomerised Properdin toward platelet receptor CD41α (Integrin α2b) (GPIIb/IIIα complex) was established using ELISA. 3µg/ml (300ng/well) of the integrin was coated into Maxi-sorb 96-well plate. 1% HSA was used as a negative control as it is used for blocking buffer. Serial dilutions starting from 10µg/ml both TSP1 (A) and Properdin (B) show the ability to bind to platelet receptor while no binding was observed toward HSA. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

4.1.3.3. Binding to GPIV (GPIIIb) (CD36)

CD36 is known as TSP1 receptor. TSP1 triggers platelet activation through CD36 (Roberts et al., 2010, Isenberg et al., 2007). The responsible domain within TSP1 that binds to CD36 is located within the TSR repeat. However, Properdin has been found to share identical similarities in TSR repeats with TSP1. Thus, it is vital to identify properdin binding to CD36 alongside with TSP1 in comparison with negative controls. Whole molecule of CD36 (R&D) and 100-200aa peptide sequences (Abcam) of CD36 were commercially provided. 100-200aa peptide sequence called CLESH-1 domain of CD36 (CD36, LIMP-2, Emp sequence homology), and located between peptide sequence 93-155aa, and consider the binding site for TSP1 (Park, 2014, Klenotic et al., 2013). Comparatively, properdin was able to bind to CD36 as strongly as TSP1. CD36 was prepared as a chimera receptor attached with IgG-Fc peptide. Thus, the attached peptide was used as a negative control alongside with HSA. Properdin binding to CD36-IgG-Fc was clearly observed comparing to IgG-Fc (-ve control) (Figure 4.5). The binding was confirmed toward 100-200aa peptide sequence of CD36 (Figure 4.5). The binding was justified by comparing the binding of HSA to TSP1 and Properdin, resulted with obviously no binding, in similar to subtract background.

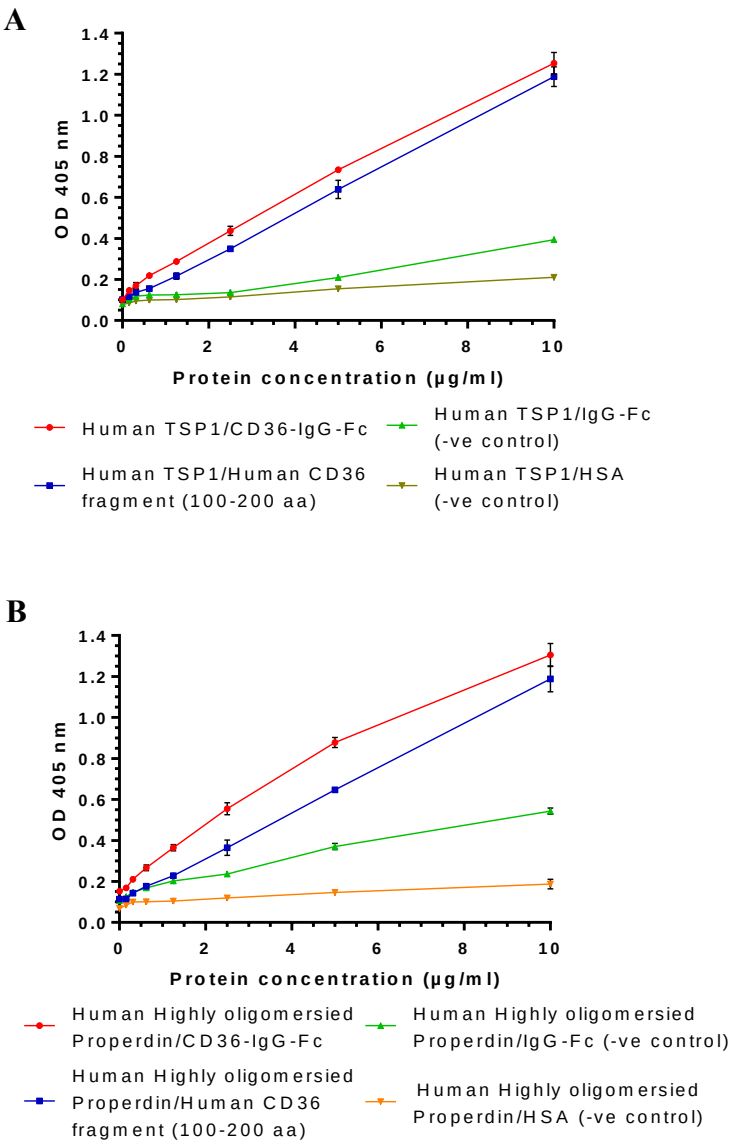

Figure 4.5. Binding TSP1 and highly oligomerised Properdin to Platelet receptor GPIV (CD36). CD36 was provided from (R&D) as whole molecule but chimaera CD36-IgG-Fc (A), while the binding was confirmed using CD36 peptide sequence 100-200aa provided from (Abcam) (B). Both recombinants; 10µg/ml TSP1 and Properdin, are able to bind to the coated 5µg/ml (500ng/well) chimaera CD36-IgG-Fc in 96-well plate (A) and the binding was confirmed with ability of binding toward the coated (5µg/ml) CD36 peptide sequence 100-200aa (B). 5µg/ml IgG-Fc of the chimaera molecule was used as a negative binding control showed a slight binding activity toward TSP1 and Properdin in comparison with the whole molecule (A, B). However, both TSP1 and Properdin showed no binding to blocking buffer 1% HSA served as a negative control (A, B). The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

4.1.3.4. Binding to CD47 integrin associated protein (IAP)

It has been found that the cell binding domain CBD in TSP1 binds to CD47 and triggers platelet activation leading to activating the GPIIb/IIIa complex (integrin α2b/β3) (Lagadec et al., 2003). As stated previously concerning the similarity of TSR repeats between TSP1 and properdin, it is also important to investigate the binding ability of properdin to CD47 causing platelet adhesion. CD47 was provided commercially from R&D, and prepared as a chimera receptor attached with IgG-Fc peptide. Thus, the attached peptide was used as a negative control alongside with HSA.

Properdin binding to CD47-IgG-Fc was clearly observed comparing to IgG-Fc (-ve control) (Figure 4.6). The binding was justified by comparing the binding of HSA to TSP1 and Properdin, resulted with no obvious binding, similar to background.

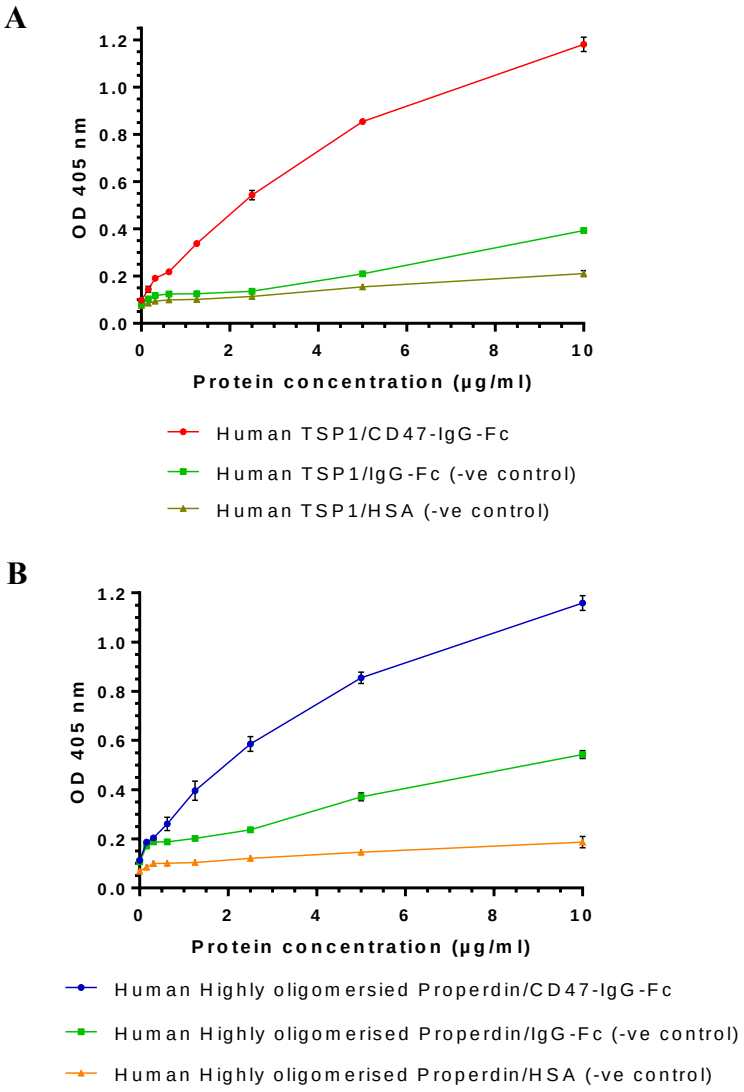

Figure 4.6. Binding TSP1 and highly oligomerised Properdin to Platelet receptor integrin associated peptide CD47 (IAP). CD47 was provided from (R&D) as whole molecule but chimaera CD36-IgG-Fc..Both recombinants; 10µg/ml TSP1 and Properdin, are able to bind to the coated 5µg/ml (500ng/well) chimaera CD36-IgG-Fc in 96-well plate. 5µg/ml IgG-Fc of the chimaera molecule was used as a negative binding control showed a slightly binding activity toward TSP1 and Properdin in comparison with the whole molecule. However, both TSP1 and Properdin showed no binding to blocking buffer 1% HSA served as a negative control. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

4.1.4 Function Activity

4.1.4.1. Platelet Adhesion by TSP1 and Properdin

At vessel injury, circulated platelets start a haemostatic plug initiation to prevent blood loss by first adhering onto the ECMs, exposed on sub-endothelial layer. The first step of adhesion process is known as tethering step, and then secondly platelets change their shape required for adhesion which lead to secretion, and lastly platelets start to aggregate by linking other platelets resulting in clumps known as aggregation process. Platelet adhesion process mainly begins on binding ECMs to GPIb/V/IX complex (Jurk and Kehrel, 2005). The adhesion process was applied in 96-well plate (Maxisorb/Nunc) by coating TSP1, Properdin alongside with positive controls such as collagen type-I and fibronectin (Fn), whereas, 1% HSA or BSA served as negative controls.

Washed intact platelets (200,000-150,000 platelet/µl) were able to adhere on the coated materials including TSP1, and surprisingly platelets were able to adhere on highly active oligomerised properdin comparing with negative controls to justify the binding observance of the platelet adherence on them.

As can be seen from (Figure 4.7A), platelets were clearly and obviously adhered onto the coated proteins when observed under microscope as small and stained tiny fragments. Platelet adhesion can be distinguished clearly by observing aggregated platelets on collagen type-I as the same as TSP1. Moreover, the process was initiated by observing clumps on highly oligomerised properdin, whereas, Fn showed tiny platelet clumps and many adhered individual platelets comparing to other molecules and negative controls. Every adhesive molecule including negative controls was pictured five times under microscope using 20x magnification power lens, and then treated in ImageJ program for counting adhered platelets numbers. The calculated values were represented as mean±SEM using GraphPad Prism6 (Figure 4.7B).

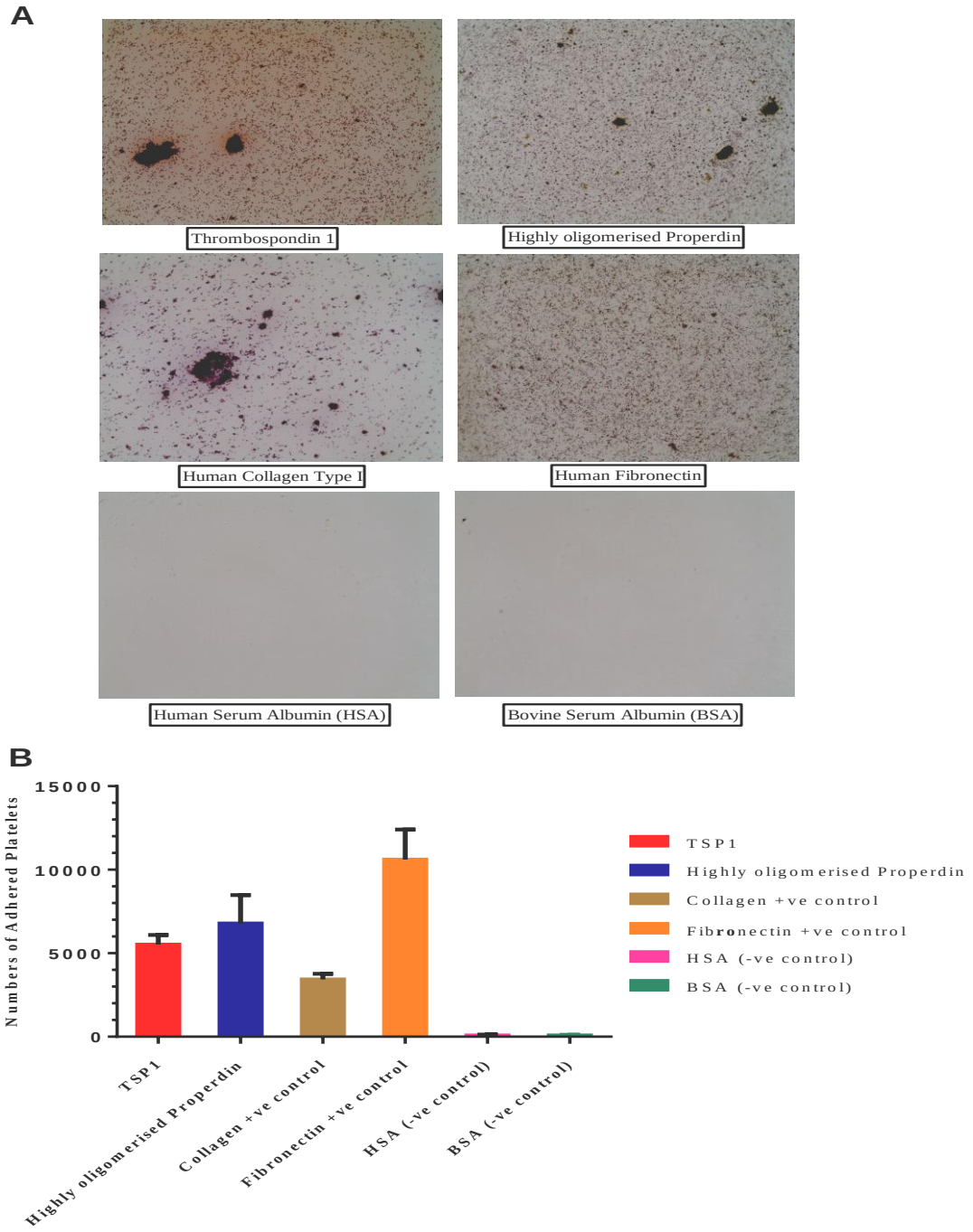

Figure 4.7. Platelet Adhesion Assay. Washed resting platelets 150,000-200,000 platelet/µl was incubated in 96-well plate already coated with extracellular matrix proteins; collagen type I and fibronectin (Fn) and also including TSP1 and highly oligomerised Properdin. Compared with the Human and Bovine Serum Albumin (HSA, BSA) served as negative controls. All adhered platelets were fixed by 0.5% glutaraldehyde (Fisher) and then stained using 0.02% Toluidine blue (Sigma). (A) Adhered platelets were visualised using inverted microscope via 20x magnification power lens. 5 pictures were taken for each adhesive molecule. (B) All the pictures were analysed using ImageJ program to count the exact number of adhered platelet for each adhesive molecule. The experiment was repeated three times and the result was represented in quintuplicate. The relative values of all results are represented as mean±SEM..

4.1.4.2. Platelet Aggregation under Light Microscope by TSP1 and Properdin

In order to confirm platelet aggregation as previously noticed in the adhesion assay, platelets activation process was initiated for certain limited time 20minutes interaction at RT or 10minutes at 37°C. Three positive controls, are known as mediators of platelets aggregation, were used; human collagen type-I, TSP1, and Adenosine Diphosphate (ADP), whereas, HSA was used as a negative control. 10µg/ml of each protein was incubated with platelets comparing with highly active oligomerised Properdin.

The activated platelets were placed on Polysine microscopic adhesive slides to allow platelet adherence followed by fixation and staining process using Toluidine blue.

It is clear that all agonists were capable of triggering platelet aggregation via observing platelet clumps under light microscope using 40x0.6 magnification power lens. Surprisingly, properdin was also able to aggregate platelet while platelet clumps could not be observed with HSA (Figure 4.8). Platelet clumps were screened in different microscopic fields.

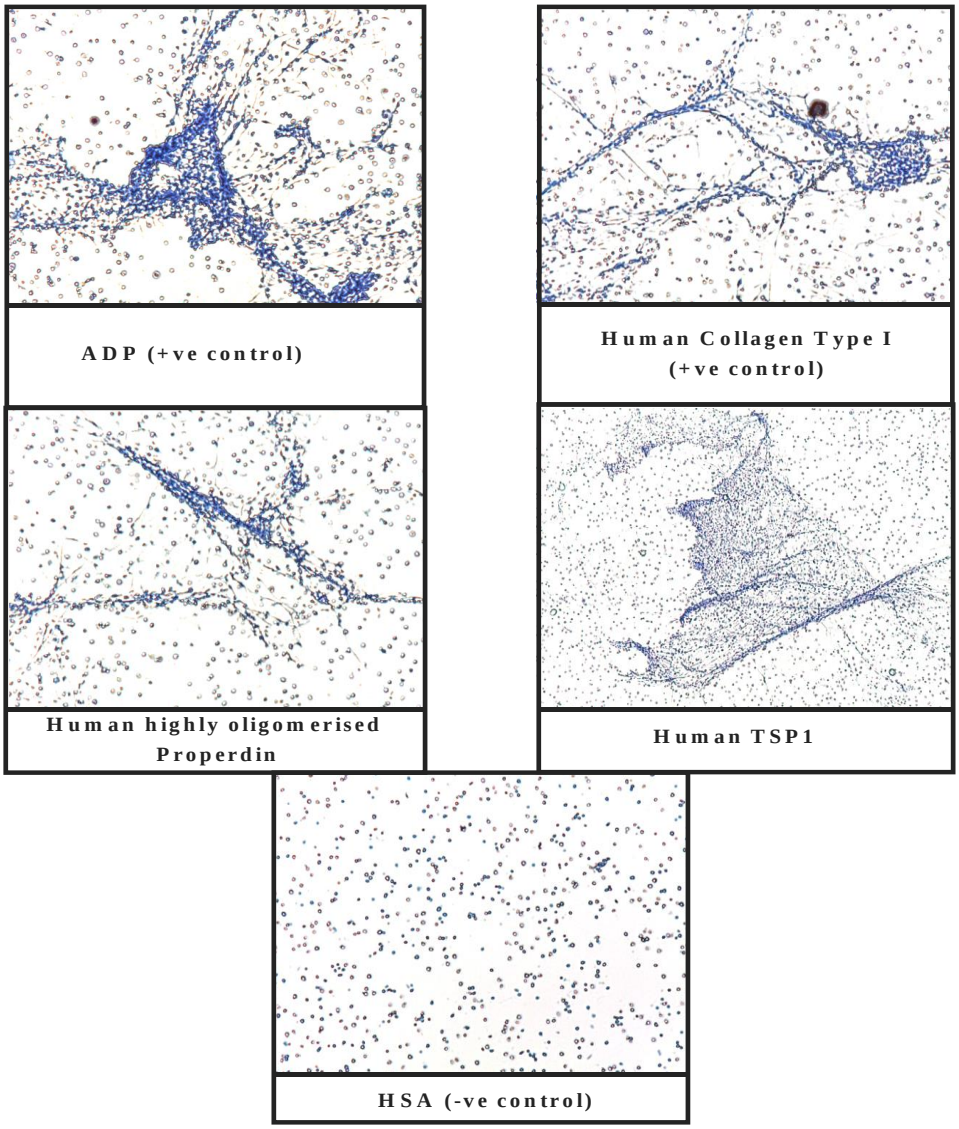

Figure 4.8. Screening platelet clumps formation under a light microscope using 40x0.6 magnification power lens. Three positive controls (above pictures) show clumping formation. The negative control (HSA) (lower right corner) shows intact platelets. All the recombinants show positive results of platelets accumulation. The experiment was repeated three times and the result was represented in five pictures for each.

4.1.4.3. P-selectin (CD62p) expression on activated platelets by TSP1 and Properdin

P-selectin (CD62p) is adhesion receptor which is expressed after tethering process by which platelets start to make pseudopods to adhere on ECMs. P-selectin is found and stored in platelets and endothelial cells in α-granules and Weible-Palade bodies, respectively (McEver et al., 1989, Wagner, 1993a). During cellular activation by the response to thrombotic agonists or inflammatory agents (Tedder et al., 1995, Metcalfe et al., 1997), CD62p translocation to outer plasma membrane of cell surface occurs within minutes (McEver et al., 1989, Wagner, 1993b) in order to act on rolling and capturing leukocytes (Giddings, 1999).

After activating platelets in adhesion and aggregation process, P-selectin biomarker was targeted using ELISA technique (Kamath et al., 2002) to identify the functional activity of TSP1 and Properdin, comparing with positive potent agonists and negative controls toward intact platelets that lastly were coated in 96-well plate.

In 10 minutes incubation at 37°C, TSP1 and Properdin were capable of triggering platelet activation by stimulating intact platelets to translocate P-selectin to plasma membrane. The surprising results were compared to positive agonists like Collagen type-I and ADP, while, HSA and phosphate buffer saline (PBS) served as negative controls (Figure 4.9). All positive controls including TSP1 served in maintaining the experiment to compare the expression levels on platelets alongside highly active oligomerised Properdin through quantifying P-selectin expression levels using mAb to P-selectin. The expression level of CD62p by Properdin was apparently significant comparing to positive agonists. More importantly, the experiment was justified by using negative controls were observed with baseline levels comparing with subtracted negative control. The results of negative controls revealed that the washed platelets were intact and were not activated but only the positive agonists showed the difference.

According to the statistical analysis (Table 4.5) revealed that highly oligomerised Properdin showed high significant difference in means (0.612) than ADP (+ve control) comparing to HSA (-ve control). Furthermore, TSP1 was the most significant agonist amongst the others, resulting in significant P-selectin expression levels. However, no significance differences were observed in means between the negative controls; HSA and PBS, on intact platelets.

The fact that leukocytes and other cells secrete properdin either in order to enhance the AP activation, suggesting, on activated platelets, through polymerising Properdin into higher forms that might able to trigger platelet activation during the activation of complement system.

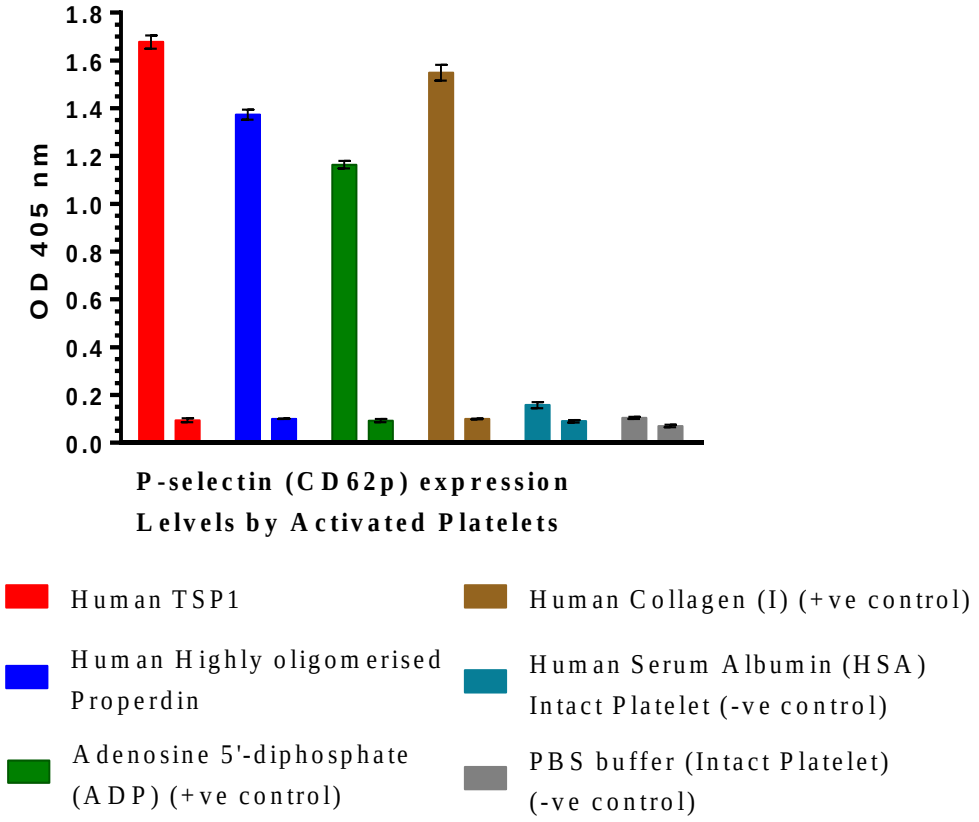

Figure 4.9. Evaluating the expression levels of P-selectin (CD62P) on TSP1 and Properdin-mediated platelet activation using ELISA based assay. Number of washed platelets was ranging between 150,000-200,000 platelets/µl. By using 30µM/ml ADP and 10µg/ml collagen-I as positive agonist controls to activate platelets, it was comparable by using 10µg/ml for both TSP1 and properdin. Following the incubation for 10 min at 37°C with or without agonists, fixed platelets were coated in maxi-sorb 96-well plate (Nunc). The levels of P-selectin (CD62p) were varied on using different agonists comparing with negative controls; Human Serum Albumin (HSA) and Phosphate buffer saline (PBS), show no or less amount of P-selectin considered to be baseline of the expression during platelets washing process. P-selectin was detected using mouse monoclonal anti-human P-selectin (Santa Cruz Biotechnology). The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

Two Way ANOVA (Bonferroni's multiple comparisons test)		
P-selectin expression levels on activated platelet	**Significant? P<0.05?**	**Mean Diff.**
TSP1 vs HSA (-ve control) Intact Platelet	Yes (****)	0.761
Properdin vs HSA (-ve control) Intact Platelet	Yes (****)	0.612
Collagen-I vs HSA (-ve control) Intact Platelet	Yes (****)	0.700
ADP vs HSA (-ve control) Intact Platelet	No (****)	0.504
HSA (-ve control) Intact Platelet vs PBS Intact platelet (-ve control)	No (NS)	0.036

Table 4.5 The statistical significance results of P-selectin (CD62p) expression levels from activated platelets using Two Way ANOVA (Bonferroni's multiple comparisons test) of comparing between all agonists including properdin and between the HSA intact platelet (-ve control). By mean differ. Properdin is significantly higher than ADP (weaker agonist). P value of all the significant results=P<0.0001. Key words; (*); significant. NS; Not significant.

4.1.4.4. Alpha-Thrombin secretion by TSP1 and Properdin mediated-platelet activation

α-Thrombin (FIIa) is a derivative active form from prothrombin (inactive form) located in platelet α-granules. Thrombin has a potential potent impact of triggering platelet activation, unlike other agonists, through binding to ligand receptors on platelets; Protease Activated Receptors (PAR1) and (PAR4), and also involved in binding to GPIbα (Broos et al., 2012) leading to platelet aggregation to express GPIIb/IIIα receptor (Andrews and Berndt, 2004).

The previous results of platelet adhesion and P-selectin expression on activated platelet by TSP1 and Properdin was confirmed by investigating further platelet secretion activity. Screening α-thrombin secretion levels was targeted using Sandwich ELISA KIT Thrombin (Abnova). The kit was designed for thrombin measurement levels in cell culture. 96-well plate pre-coated with mAb human α-thrombin.

Highly oligomerised properdin was compared with agonist controls collagen and ADP including TSP1. All the components were able to trigger platelets activation resulting in significant levels of α-thrombin. Several concentrations of standard thrombin (STD) (Provided by the kit) ranging from 20-0.15625ng/ml were used in order to determine the secreted thrombin levels. PBS, used to reconstitute the agonists, was not able to activate platelets, which is considered as a negative control (Figure 4.10).

According to the statistical analysis (Table 4.6) revealed that highly active oligomerised Properdin showed significant difference in means (0.087) more than the least ADP (+ve control) (0.070) comparing to PBS (-ve control). Furthermore, TSP1 was the most significant agonist amongst the others, resulting in thrombin release. The approximate α-thrombin concentration released by TSP1 mediated-platelet activation was 0.15625ng/ml compared to STD thrombin. However, the sub-traction negative control, used for measuring the non-specific binding of the antibodies to the background, was the same as the –ve control (PBS) that used to measure the effect of the buffer on platelet activation by showing no significance difference.

The current result is likely to prove the previous results and to confirm the potential activity of oligomerised properdin in triggering platelet activation.

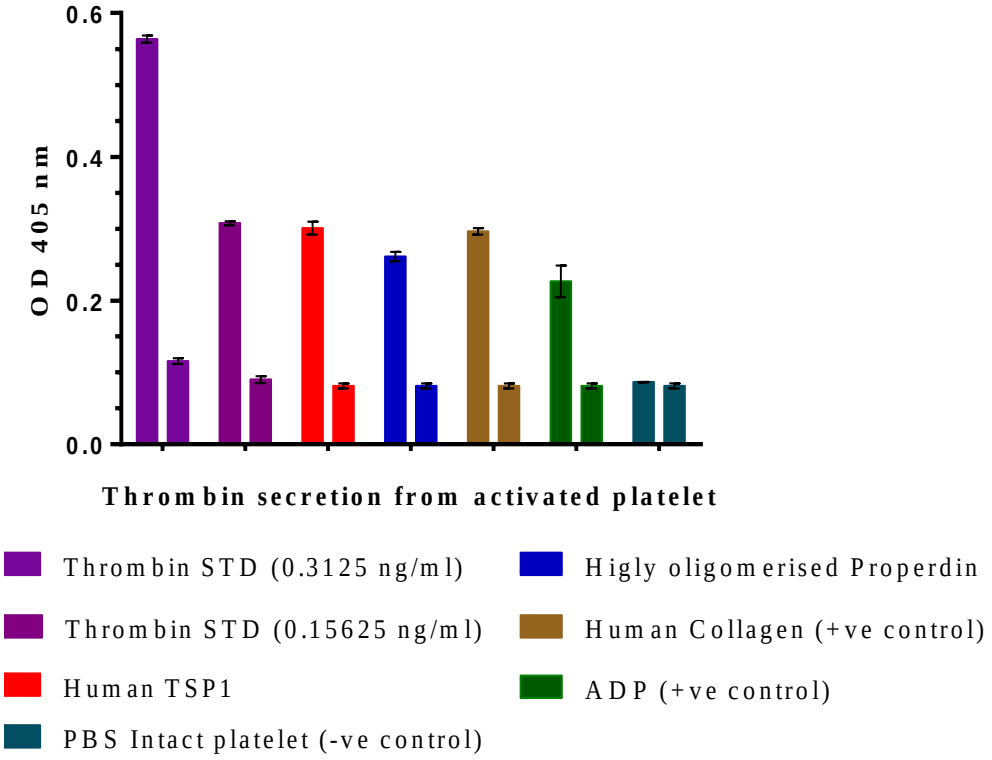

Figure 4.10. Measurement levels of α-Thrombin been secreted from activated platelet by different agonists; collagen and ADP, including TSP1 and highly oligomerised Properdin in comparison with known thrombin standard concentration (0.3125ng/ml and 0.15625ng/ml). Phosphate buffer saline (PBS) was incubated with intact platelet as a negative control. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

Two Way ANOVA (Bonferroni's multiple comparisons test)		
Thrombin secreted by activated platelet	**Significant? P < 0.05?**	**Mean Diff.**
TSP1 vs PBS Intact platelet (-ve control)	Yes (****)	0.107
Properdin vs PBS Intact platelet (-ve control)	Yes (****)	0.087
Collagen-I vs PBS Intact platelet (-ve control)	Yes (****)	0.105
ADP vs PBS Intact platelet (-ve control)	No (***)	0.070

Table 4.6 The statistical significance results of (Figure 4.10) secreting α-thrombin from activated platelet mediated by different agonists comparing to TSP1 and Properdin. According to the significance results, Properdin is considering to be significantly much more potent activator than ADP (weaker agonist) while almost in parallel to TSP1. The significant P value was statistically calculated by using Two Way ANOVA (Bonferroni's multiple comparisons test). Significant values=P<0.05. Key words; (*); significant. NS; Not significant.

4.1.4.5. Thrombin Generation resulted from Procoagulant Activity of Platelet Microparticles (PMPs) released by TSP1 and Properdin mediated-Platelet Activation

The ability of TSP1 and highly active oligomerised properdin to bind and activate intact platelet was performed and confirmed by targeting another important factor called platelet microparticles (PMPs) that increase platelet activation and lead to generate further activity resulting in thrombin generation as a result of coagulation cascade activation. The best and the easy way to evaluate the PMPs release is through using the thrombin generation functional assay for measuring the pro-coagulant activity of PMPs in plasma or washed platelets. The functional assay is provided with whole set using ZYMUPHEN MP-Activity KIT (HYPHEN BioMed). It is based on ELISA technique and was used because of the kit sensitivity which allows measuring and detecting all PMPs sizes from 0.05-1µm while, for example, flow cytometry fails to measure or detect PMPs lower than 0.2-0.4 µm (Gelderman and Simak, 2008). Therefore, ELISA is more reproducible and accurate assay than flow cytometry (Nomura and Fukuhara, 2004) and it was also recommended as an alternative promising tool due to the efficiency of detection in the presence of standards (Miyamoto et al., 1998).

The kit is designed to measure generated thrombin levels by PMPs. 96-well plate precoated with streptavidin and captured by Biotinylated Annexin-V. Basically, MPs inherit the surface antigens which originated from mother cells. One of the inherited molecules is a Phosphatidylserine (PS) which is a phospholipid surface cell membrane. PS exposed on outer membrane of platelets and PMPs in activation or apoptosis events (Zwicker et al., 2012).

It seems that TSP1 and highly oligomerised properdin are capable of triggering platelet activation resulting in PMPs release that had significant ability to generate thrombin. The assay principle is basically based on enzymatic reaction between FXa-FVa in the presence of calcium. Factor Xa binds directly to PS while Va is attached to it and also binds to tissue factor (TF). The Xa with cofactor Va synergise to activate exogenous prothrombin into thrombin, which is easy to detect by adding thrombin-specific chromogenic substrate. The negative control (PBS) did not activate platelets through detecting thrombin generation while ADP served as a positive control representing the lowest level of thrombin generation. The other positive controls, thrombin and collagen were

observed with significant result including TSP1 in thrombin generation. Highly oligomerised properdin was observed with significant higher thrombin generation level mediated by PMPs. Calcium-ionophore A23187 is strong agonist that disturb platelets to release more PMPs able to bind to annexin-V, used as non-physiological positive control to maintain the assay and also to obtain 100% PMPs from whole platelet population.

By comparing the thrombin generation by the force of the positive physiological agonists with Calcium-ionophore, the physiological agonists seemed to enhance approximately quarter of whole platelet population on releasing PMPs (Figure 4.11).

According to the statistical analysis (Table 4.7), highly active oligomerised Properdin was continuously showing a remarkable activity toward platelet on thrombin generation through observing significant difference in mean (0.273) more than thrombin (0.185) and the least ADP (0.047) comparing to PBS. Properdin showed mostly equal impact in thrombin generation compared with collagen type-I resulting in no different in means comparing to PBS. Furthermore, TSP1 was the most significant agonist in thrombin generation by PMPs release. The approximate concentration of the generated thrombin by PMPs of activated platelets was between 5.6nM and 2.8nM compared to MPs STD.

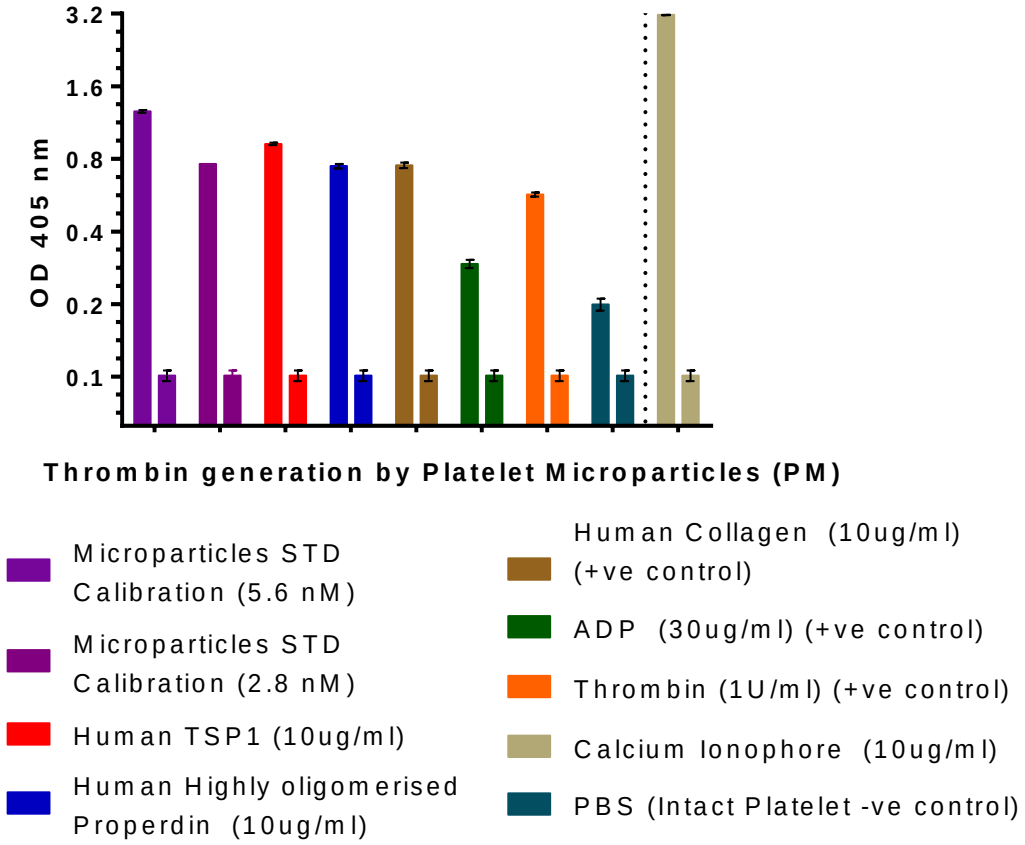

Figure 4.11. The Measurement levels of Thrombin generation by Microparticles release from platelet using ZYMUPHEN MP-Activity KIT (HYPHEN BioMed). 10µg/ml both TSP1 and highly oligomersied Properdin were able to activate the release of Microparticles that generate thrombin in the presence of FXa-FVa and Calcium ions. The concentration of Microparticles were released upon of using TSP1 according to this assay was between 5.6nM and 2.8nM. Highly oligomerised Properdin assists to release Microparticles in concentration 2.8nM. The PBS showed no activity toward washing platelet with respect to Microparticles release and thrombin generation which represent the negative control in comparison with positive controls. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

Two Way ANOVA (Bonferroni's multiple comparisons test)		
Thrombin generation by MPs	**Significant? P < 0.05?**	**Mean Diff.**
TSP1 vs PBS Intact platelet (-ve control)	Yes (****)	0.361
Properdin vs PBS Intact platelet (-ve control)	Yes (****)	0.273
Collagen-I vs PBS Intact platelet (-ve control)	Yes (****)	0.276
Thrombin vs PBS Intact platelet (-ve control)	Yes (****)	0.185
ADP vs PBS Intact platelet (-ve control)	Yes (***)	0.047

Table 4.7. The statistical significance results of (Figure 4.11) generating thrombin by MPs from activated platelet mediated by different agonists comparing to TSP1 and Properdin. According to the significance results, Properdin is significantly much more potent activator than ADP (weaker agonist) and thrombin while almost in parallel to collagen. The significant P value was statistically calculated by using Two Way ANOVA (Bonferroni's multiple comparisons test)..Significant values = $P < 0.05$. Key words; (*); significant. NS; Not significant.

4.2. The role of human TSP1 and Properdin in binding to initiator materials in platelet adhesion process or atherosclerosis

In this section, the relation of Properdin and TSP1 with respect to initiating platelet adherence was tested regarding binding to the extracellular Matrix (ECM) proteins and also associating with lipid that cause fatal serious disease by initiating atherosclerosis is going to be presented in binding assay using ELISA.

4.2.1. Binding to Extracellular Matrix proteins (ECM)

The matrix proteins interact with each other forming structural grid firmly enough to maintain cells and tissue organisations.
The binding of TSP1 and Properdin to ECM was evaluated using ELISA by coating the following ECM proteins; human Collagen type-I, III, IV (Figure 4.12), and human von Willebrand Factor (vWF) (Figure 4.13) and human Fibronectin (Fn) (Figure 4.14) in 96-well plates.

4.2.1.1. Binding to human Collagens

Collagen is member of ECM that supports sub-endothelial layer. Collagen was found to activate resting platelet probably through collagen receptor GPIb-α (Kehrel et al., 1988). TSP1 considered to be one of thrombospondin family that involved in binding to extracellular matrix proteins, it has been reported that TSP1 binds to collagen I, III, IV, V (Galvin et al., 1987). In addition, C-, N-terminals and stalk region (Procollagen homology domain) of TSP1 were reported to be in association with collagen (Galvin et al., 1987).
All binding reactions were provided with Ca^{2+} and Mg^{2+} for either TSP1 or Properdin to bind Collagen due to allowing conformational changes of domain signature of TSP1 or Collagen. As the binding of TSP1 to collagen has been established, the assay was used to explore the binding of properdin to several types of human collagen. Three types of human collagen were used and all provided commercially; type-I, III and IV.
It is only collagen Type-I noticed to significantly bind highly oligomerised Properdin and other forms of properdin but lesser and gradual binding activity, suggesting, depends on cyclic higher form or on the activity of the oligomerisation process. Whereas, other types of collagen showed no binding comparing to negative control (Figure 4.12).

The variation among the results of Properdin forms to collagen type-I might reveal to the binding site of collagen or to the conserved TSR repeat of the higher active polymerised or oligomerised forms that apparently lead to higher binding affinity.

As reported by Galvin et al., (1987) that TSP1 bind to types of collagen, the current study confirm the binding to collagen types; I, III, IV.

Due to the structural homology between the type-I repeat of Properdin and TSP1, the name has been changed to TSR (Silverstein, 2002).

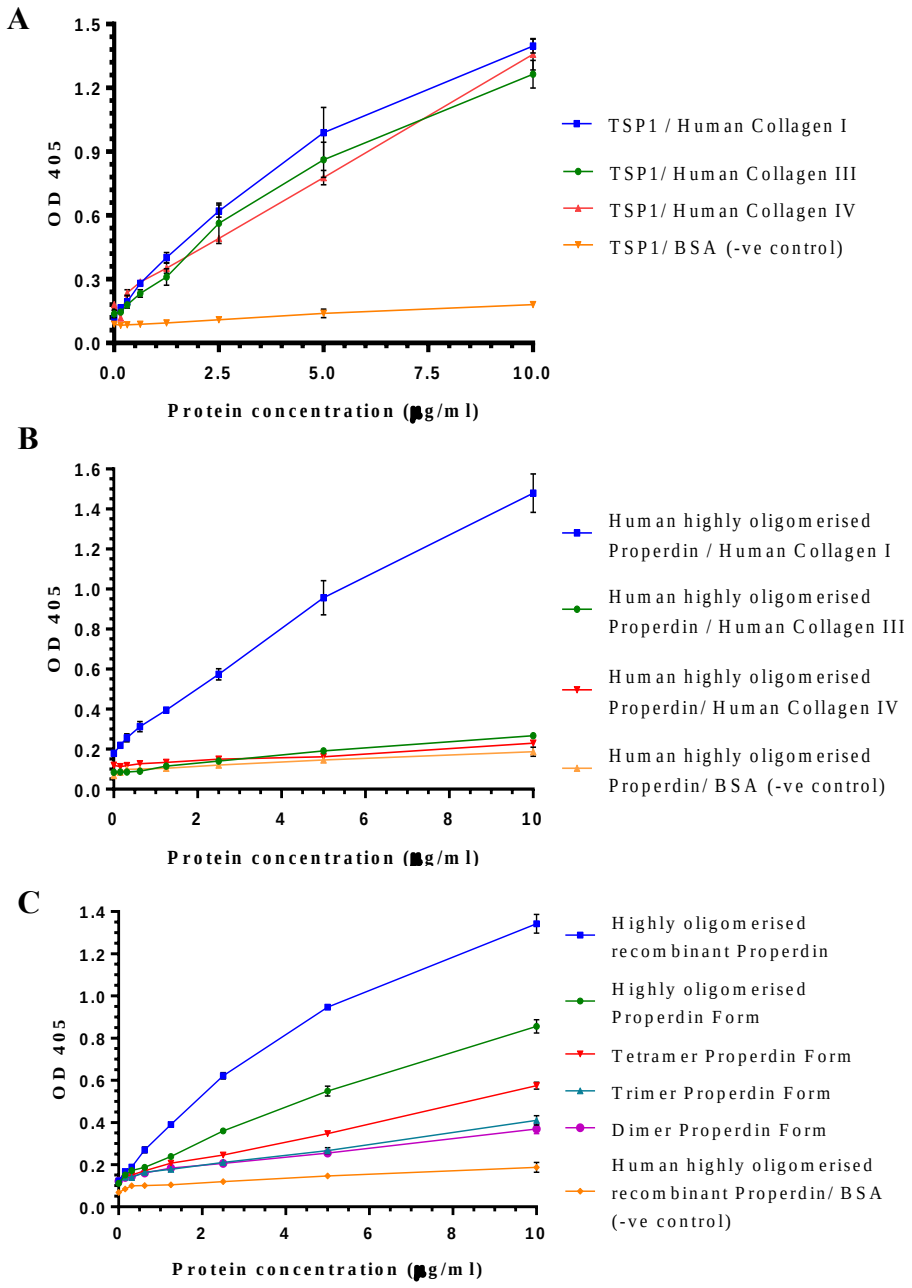

Figure 4.12. ELISA based assay was used for binding TSP1 and Properdin to human collagens. 10µg/ml of human collagen type-I, III and IV (Sigma) were coated in Maxisorb 96-well plate (1µg/well) (Nunc) in order to investigate the binding capability to (A) TSP1 and (B) Properdin. After observing the binding of Properdin to collagen type-I, collagen type-I was used in further to investigate the binding toward Properdin forms in order to distinguish the variable binding between Properdin forms. Thus, (C) Properdin forms show gradual increase in binding toward collagen type-I. The higher form of properdin obtains greater binding affinity than smaller form. The binding activity was detected using 1:1000 mouse polyclonal antibody against TSP1 (Sigma) and 1:10,000 rabbit polyclonal antibody against Properdin (kindly provided by Dr. M.A Youssef, University of Leicester). The positive control was considered to be TSP1. However, both TSP1 and Properdin showed no binding to the blocking buffer 1% HSA served as a negative control. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

4.2.1.2. Binding to human von Willebrand Factor (vWF)

vWF is member of ECM that support and mediate platelet adhesion process starting from tethering, rolling and lastly adhesion (Ware and Ruggeri, 2001). vWF interacts with GPIb/IX/V complex and integrin $\alpha IIb\beta_3$ resulting in platelet aggregation (Bonnefoy et al., 2006). The A3 domain of vWF was found to be the binding site for TSP1 in the same binding manner for ADAMTS13, suggesting, the vWF proteolysis process by ADAMTS13 might be inhibited (Pimanda et al., 2004). It has been reported that the binding of TSP1 to vWF was found in participating of protection from proteolysis by ADAMTS13 (Bonnefoy et al., 2006).

The current study shows that highly active oligomerised Properdin, like TSP1, is able to bind to vWF comparing to binding to BSA (Figure 4.13), suggesting, it might be the binding of Properdin to vWF involved in the protection from degradation process.

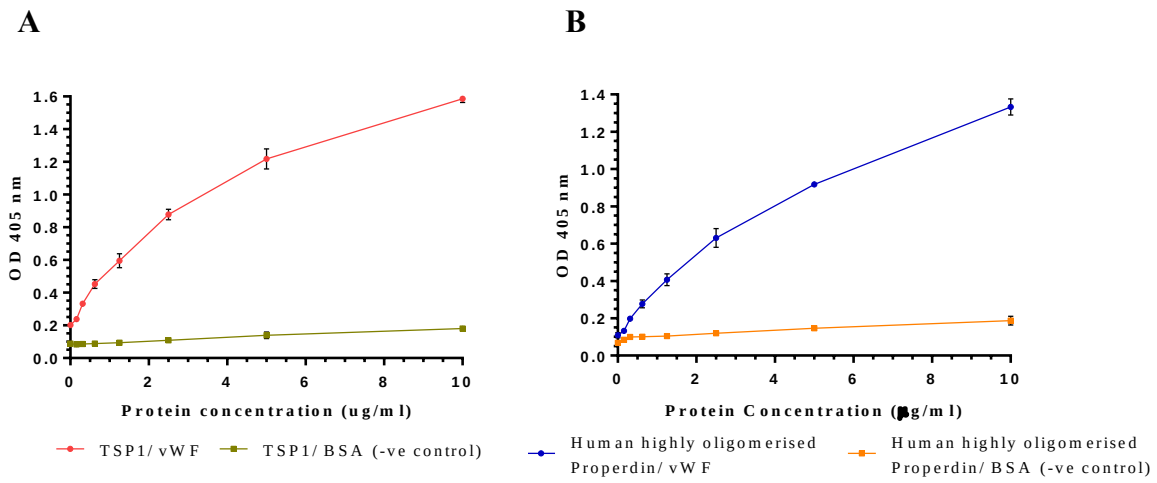

Figure 4.13. ELISA based assay was used for binding TSP1 and Properdin to human vWF. 10µg/ml of human vWF (HTI) was coated in Maxisorb 96-well plate (1µg/well) (Nunc) in order to investigate the binding capability to (A) TSP1 and (B) Properdin. The binding activity was detected using 1:1000 mouse polyclonal antibody against TSP1 (Sigma) and 1:10,000 rabbit polyclonal antibody against Properdin (kindly provided by Dr. M.A Youssef, University of Leicester). The positive control was considered to be TSP1. However, both TSP1 and Properdin showed no binding to the blocking buffer 1% HSA served as a negative control. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

4.2.1.3. Binding to human Fibronectin (Fn)

Fibronectin (Fn) is member of ECM that required for triggering platelet activation causing tethering process to platelet. TSP1, was reported by many researchers, bind to Fn, suggesting the interaction might be critical for ECMs assembly (Lahav et al., 1984).
Moreover, the peptide sequence GGWSHW in the 2nd TSR repeat of TSP1 is consider to be an essential binding site for Fn (Sipes et al., 1993). Basically, by matching the protein sequence of TSP1 with Properdin through UniprotKB databases, the peptide sequence GGWSGW was recognised in similar to the binding site for Fn. Technically, that was led to a concept of the capability of Properdin to bind Fn and subsequently may lead to platelet firm adhesion. The current study observed the ability of oligomerised Properdin to bind Fn, as well as the other forms of properdin but with lesser and gradual binding activity, suggesting, depends on cyclic higher form (Figure 4.14).
The variation among the results of Properdin forms to Fn might reveal to the peptide sequence in the conserved TSR repeat of higher polymerisation forms that apparently lead to higher binding affinity.
Upon the interaction of TSP1 with Fn may suggest to role in platelet adhesion by linking platelets to Fn.

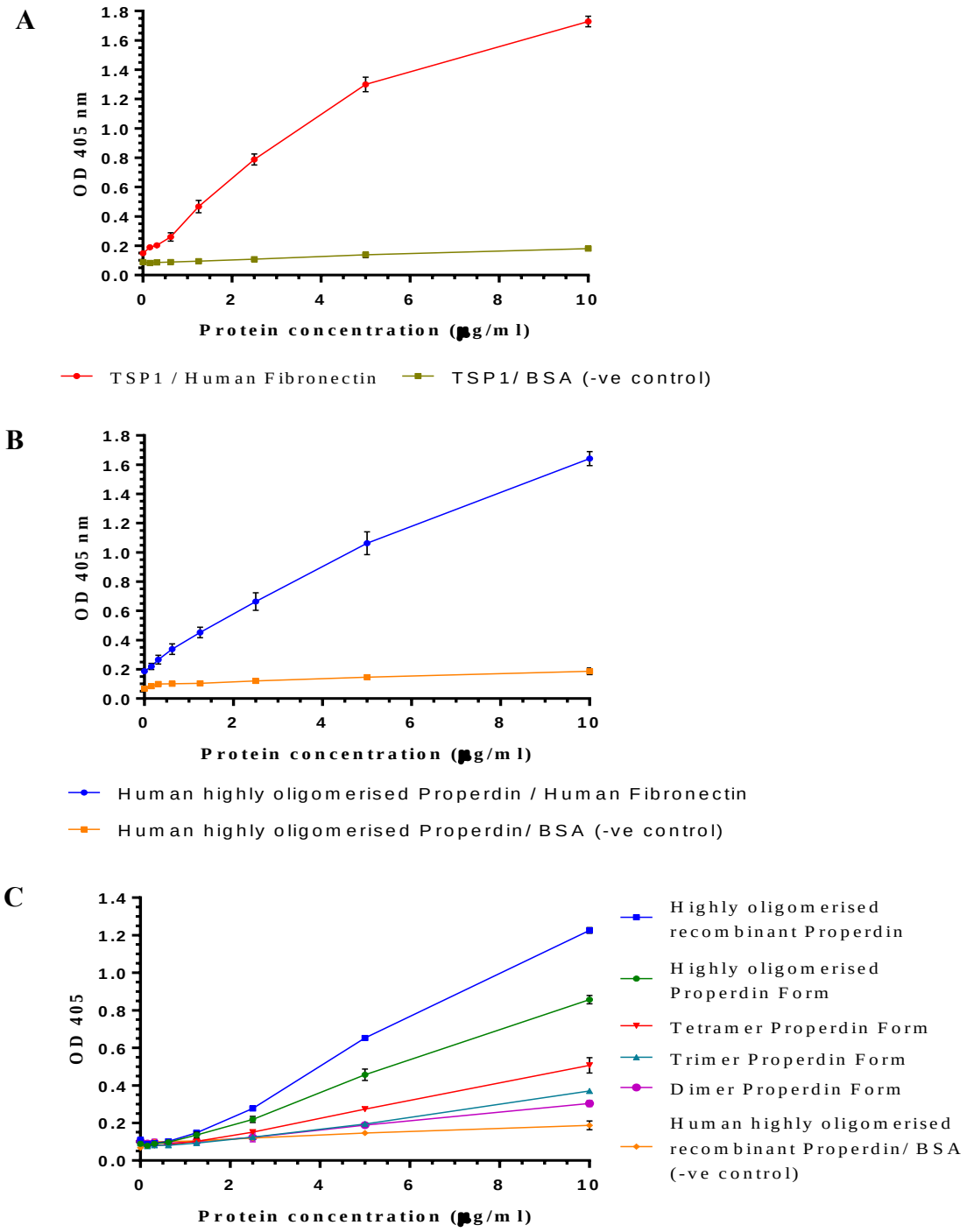

Figure 4.14. ELISA based assay was used to investigate the binding of TSP1 and Properdin to human Fibronectin (Fn). 10µg/ml human Fn (Sigma) was coated in Maxisorb 96-well plate (1µg/well) (Nunc) in order to investigate the direct binding capability of (A) TSP1 and (B) Properdin. In addition, (C) Properdin forms were used in further investigation toward Fn interaction in order to distinguish the binding variety between Properdin forms. Thus, Properdin forms show gradual an increase binding toward Fn (C). The higher form of properdin obtains greater binding affinity than smaller form. The binding activity was detected using 1:1000 mouse polyclonal antibody against TSP1 (Sigma) and 1:10,000 rabbit polyclonal antibody against Properdin (kindly provided by Dr. M.A Youssef, University of Leicester). The positive control was considered to be TSP1. However, both TSP1 and Properdin showed no binding to the blocking buffer 1% HSA served as a negative control. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

4.2.2. Binding to human Fibrinogen (Fg)

Despite the fact that Fg is required for platelet aggregation by linking platelets and forming platelet plug through GPIIb/IIIa complex (α2bβ3) (Savage et al., 1996, Jurk and Kehrel, 2005), it is also an essential molecule released from activated platelet and forming complex interaction with TSP1 on platelet membrane surface (Leung and Nachman, 1982).

The current study showed the ability of oligomerised Properdin, like TSP1, to bind immobilised Fg in 96-well plate as well as the other forms of properdin but with lesser and gradual binding activity, suggesting, depends on cyclic higher form. The assay was used BSA as negative control for comparison (Figure 4.15).

The variation among the results of Properdin forms to Fg might reveal to the higher polymerisation forms that apparently lead to higher binding affinity.

These results would suggest the involvement and the support of Properdin in platelet aggregation process by interaction with Fg as same as TSP1 interaction of strengthen and support Fg to platelet.

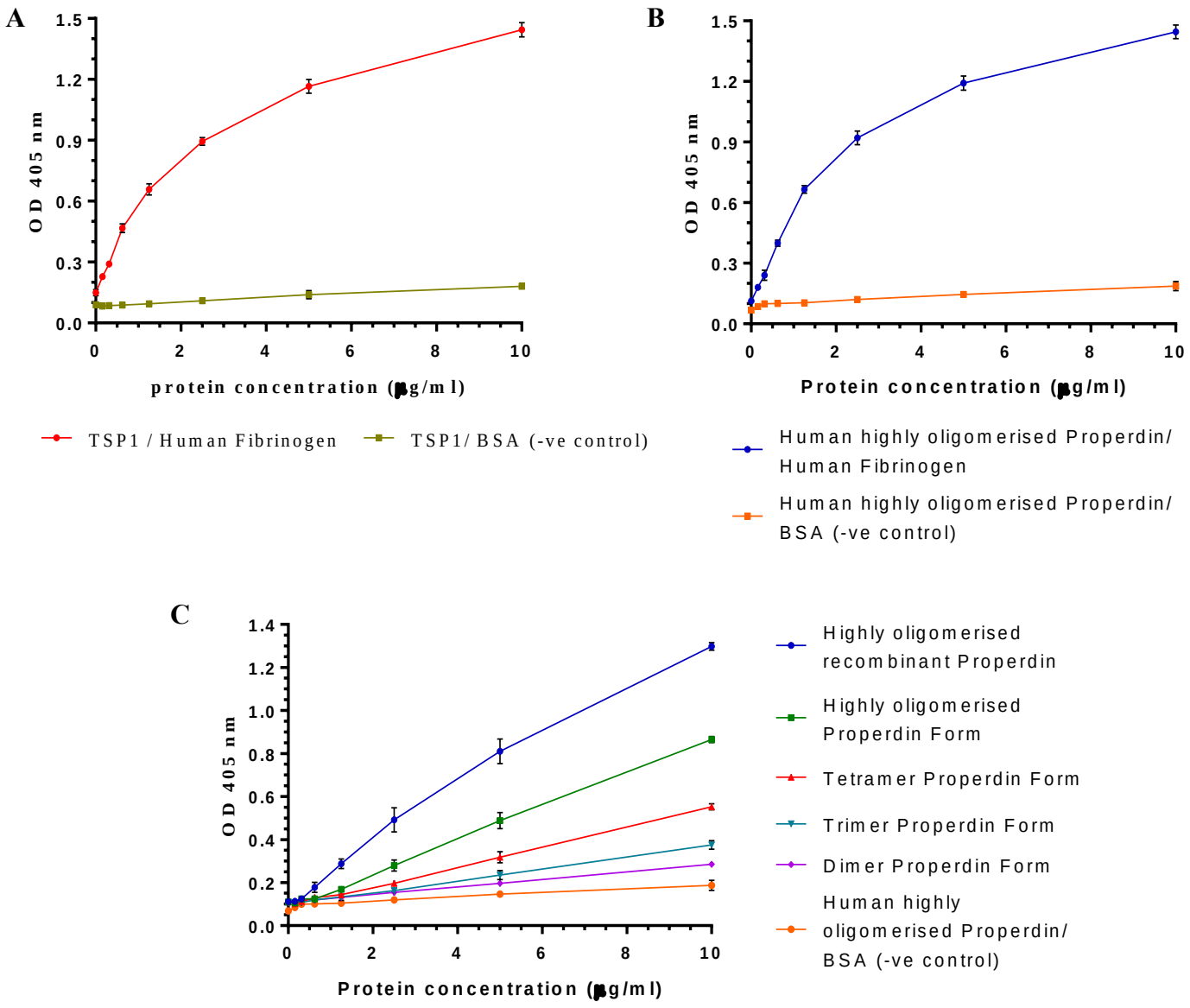

Figure 4.15. ELISA based assay was used to investigate the binding of TSP1 and Properdin to human Fibrinogen (Fg). 10µg/ml human Fg (Sigma) was coated in Maxisorb 96-well plate (1µg/well) (Nunc) in order to investigate the direct binding capability of (A) TSP1 and (B) Properdin. In addition, (C) Properdin forms were used in further investigation toward Fg interaction in order to distinguish the binding variety between Properdin forms. Thus, Properdin forms show gradual an increase binding toward Fg (C). The higher form of properdin obtains greater binding affinity than smaller form. The binding activity was detected using 1:1000 mouse polyclonal antibody against TSP1 (Sigma) and 1:10,000 rabbit polyclonal antibody against Properdin (kindly provided by Dr. M.A Youssef, University of Leicester). The positive control was considered to be TSP1. However, both TSP1 and Properdin showed no binding to the blocking buffer 1% HSA served as a negative control. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

4.2.3. Binding to Lipids; Cholesterol, Triglycerides and Low density lipoprotein (LDL)

The common factor associated with atherosclerosis disease is the increased levels of lipids like cholesterol, triglycerides and low-density lipoprotein (LDL). In addition, obesity is a crucial factor which may cause cardiovascular disease (CVD). Many studies have referred to the association between TSP1 and CVD (Krishna and Golledge, 2013, Stenina et al., 2004). However, the higher levels of TSP1 in plasma have been noticed in patients with coronary artery disease (CAD) (Choi et al., 2012).

The current study showed the binding capability of TSP1 and properdin toward cholesterol, triglycerides and LDL, based on a theory that TSP1 could participate in atherosclerosis disease by attaching to precipitated lipids such as LDL, by which platelet adherence could be initiated (Figure 4.16) and then lead to the formation of local cellular accumulation besides fibrin formation known as blood clotting. However, due to the ability of properdin of adhering platelets, it could participate in atherosclerosis disease by interacting with lipids.

TSP1 and oligomerised properdin significantly bind to cholesterol, triglycerides and LDL, whereas, Fg was observed with no binding to the lipid which served as negative control. All binding proteins to lipids were justified comparing with binding to HSA as subtracting negative control.

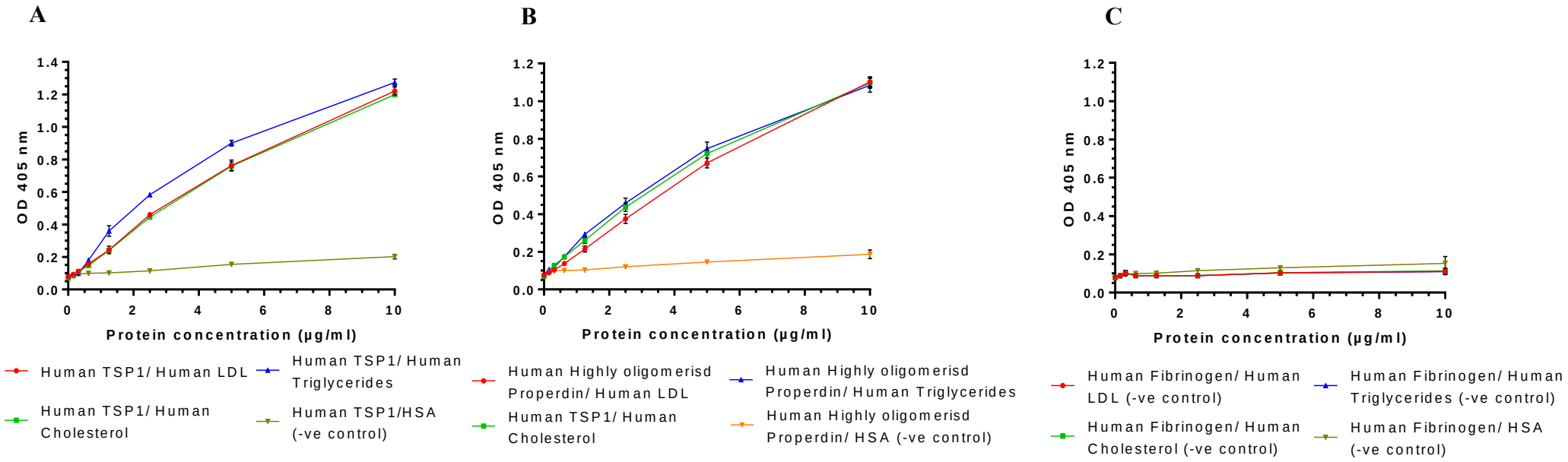

Figure 4.16. ELISA based assay was used to investigate the binding of TSP1 (A) and Properdin (B) to Lipid.in comparison with binding of fibrinogen (Fg) (C) served as negative control. The human lipid was included the following; Cholesterol, Triglycerides and Low density lipoprotein (LDL). All of which were coated in 96-well plate (Maxisorb) (Nunc) with 10µg/ml (1µg/well). Serial dilutions of TSP1, Properdin and fibrinogen starting from 10µg/ml were incubated with the coated materials. 1% Human serum Albumin (HSA) (Blocking buffer) was incubated with each tested protein to serve as subtracting negative control. The binding activity was detected using the following antibodies; 1:5000 mouse polyclonal antibody against human TSP1 (Sigma) and 1:10,000 rabbit polyclonal antibody against human Properdin (kindly provided by Dr. M.A Youssef, University of Leicester) and 1:5,000 mouse monoclonal IgG antibody against human Fibrinogen (Santacruz biotechnology). The positive control was considered to be TSP1. However, both TSP1 and Properdin showed no binding toward the blocking buffer 1% HSA. Moreover, Fg shows no binding capability to the coated materials. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

4.3 Discussion

The hemostasis/thrombosis process where the activated platelets are involved in the events, which generates plug formation for the host protection from blood loss, has been highlighted by a number of researchers regarding correlating the activation of the complement system for protection from pathogenic invaders in such events. For instance, the association of such microorganisms as *Streptococcus pneumoniae* with activated platelets or clotting events at the site of vessel injury has been classified for microbial pathogenesis as an essential part for colonisation and dissemination (Rennemeier et al., 2007, Sjobring et al., 2002) while the complement system plays an essential role as a first-line defence of innate immunity for pneumococcal infection clearance (Tu et al., 1999, Brown et al., 2002, Yuste et al., 2005). There are also examples of diseases like haemolytic uraemic syndrome (HUS) and paroxysmal nocturnal haemoglobinuria (PNH) where the stimulated platelets are involved by the activation of complement system which implies the raising of thrombus formation in tissues (Del Conde et al., 2005). In addition, platelets have been involved in abnormal events such as atherosclerosis plaque, arterial thrombosis, and cardiovascular diseases pathogenesis (Ulutin, 1986, Yardimici, et al., 1995). The atherosclerotic plaque caused by occlusive thrombosis is likely to cause rupture or restenosis of blood supply leading to two serious causes of death-ischemic stroke and myocardial infarction-in developed countries (La Bonte et al., 2012). Precisely, many factors of the complement system molecules trigger platelet activation which, in turn, initiates the promotion of thrombus formation. One of the complement proteins, called factor properdin, which is a positive regulator of the alternative pathway (AP), has been recently studied with respect to platelet interaction (Saggu et al., 2013, Ruef et al., 2008). From the other side, Thrombospondin1 (TSP1) is a homo-trimeric glycoprotein, essential for platelet activation leading to platelet accumulation by adhering platelets onto a matrix layer at the site of vessel injury. Some researchers have found some identical positions between TSP1 and properdin and also other proteins within the complement system. It has been found that properdin shares similarity in a conserved amino acid sequence of TSR domain of TSP1 (Goundis and Reid, 1988, Robson et al., 1988) about 47% of identity (Goundis and Reid, 1988). Therefore, the prospective advantages of this project provides and defines the evidences of Properdin, where the TSR repeat domains are involved similarly to TSP1, could play

a crucial role of leading to burst either the haemostasis process or the thrombotic events via increasing procoagulant activity via thrombin generation, thus leading to clot formation.

The discussion will look at the following points; first of all, characterising the role of highly oligomerised active properdin and TSP1 on the interaction with human washed platelets which may result in platelet adhesion and then activation leading to a possible procoagulant microparticles' release by which thrombin generation possibly occurs which, in turn, initiates the promotion and the activation of coagulation cascade in haemostasis or thrombosis events. Secondly, determining the role of TSP1 and properdin forms; dimer, trimer, tetramer and oligomerised/polymerised form including the recombinant highly oligomerised active properdin, with respect to binding to extracellular matrix proteins (ECM); human collagen type-I, III and IV, and fibronectin (Fn), von Willebrand factor (vWF), and also to human fibrinogen (Fg). Lastly but certainly not least, defining the role of TSP1 and properdin to bind to atherogenic particles; low density lipoprotein (LDL), cholesterol and triglycerides, whereby in certain levels of these particles have been noticed to increase the incidence rate of atherosclerosis disease by accumulating platelets and inflammatory cells at the inflammable site. All of these are crucial substances that interfere and interact with platelet causing platelets adhesion and accumulation, resulting in the activation of coagulation cascade.

The findings of the current exploration establish *in vitro* that the activation of platelets caused by both TSP1 and properdin can synergise in the haemostasis process and also may promote wound healing and, therefore, may cooperate in such uncontrolled events as atherosclerosis disease, resulting in thrombus formation and leading to myocardial infarction.

4.3.1 Oligomerised Properdin and TSP1 Play an Important Role on the Surface of Human Intact Platelets Resulting Platelets Activation and Platelets Adhesion via Monitoring P-selectin (CD62p) Expression Receptor

TSP1 is a protein secreted from most cells, and represents 3% of total proteins in alpha granules of platelets 30-100µg/10^9 platelets (Bonnefoy et al., 2008, Legrand et al., 1997), but the concentration in normal plasma in physiological conditions fluctuates between 60 and 300ng/ml (Switalska et al., 1985). However, the abnormal concentration varies in the case of the thrombosis or haemostasis process at the site of injury where activated platelets secrete TSP1, resulting in an increase of concentration locally up to 15-40µg/ml within minutes (Legrand et al., 1997, Lamy et al., 2007), while the α-granules contents of TSP1 have been measured to contain 59mg/L (Mosher et al., 1985). There are some diseases rise the concentration of TSP1 which is associated with such as peripheral arterial disease (PAD) by which the concentration of TSP1 is raised up to 1.250µg/ml in plasma in patients (Smadja et al., 2011). Therefore, TSP1 has been considered as a potential plasmatic biomarker in PAD patients (Smadja et al., 2011). In addition to examples of abnormal TSP1 concentrations, TSP1 was found to correlate with bronchoalveolar lavage fluid (BALF) in human patients by raising the concentration of TSP1 up to 175µg/ml in serum in patient suffering with non-specific interstitial pneumonia (NSIP) and usual interstitial pneumonia (UIP) and idiopathic interstitial pneumonia (IIP) due to fibrotic responses leading to an expression of TSP1 from alveolar epithelium and macrophages (Ide et al., 2008). Despite the fact of low quantities of secreted levels of TSP1, it is likely substantial due to being found in patients deficient in TSP1, especially of Gray platelet syndrome by which the endogenous TSP1 was found to develop the α-granules of megakaryocytes (Mosher, 1990, Booth and Berndt, 1987, Rosa et al., 1987).

On the other hand, the normal concentration of factor properdin of alternative pathways in plasma varies from 4-25µg/ml (Nolan and Reid, 1993). Properdin is essentially generated and secreted by leukocyte, including monocytes (Whaley, 1980, Schwaeble et al., 1994), T-cells (Schwaeble et al., 1993) and neutrophils (Wirthmueller et al., 1997). Properdin is released when the chemotaxis inflammatory markers, such as TNF-α and C5a, are released in the bloodstream by which the inflammatory cells are stimulated. Proper-

din presents in plasma in different physiological polymerised forms; dimer, trimer and tetramer (Schwaeble and Reid, 1999b, Pangburn, 1989) while non-physiological highly active higher forms (artefact) can be generated through/after thawing and freezing, and from a lengthy period of storage (Saggu et al., 2013, Ferreira et al., 2010, Farries et al., 1987). In contrast, it has been observed by Smith (1984) that the higher forms of properdin; pentamers and hexamers, were relatively lower than the other polymerised forms; cyclic dimer, cyclic trimer and cyclic tetramer, by 80% in case of complement activation using zymozan. This suggests the putative artefact theory (thawing/freezing) (Farries et al., 1987) of generating oligomerised higher forms of properdin is also likely to occur during complement activation.

The function of factor properdin in the alternative pathway (AP) of the complement system is essential for stabilising the C3bBb or ($C3_nBb$) convertase on certain target surfaces, for example, necrotic and apoptotic cells and pathogenic invaders like bacteria.

In order to define which separated cyclic form of human properdin is initiating the activation of the haemostasis/thrombosis process via activating platelet, beside human TSP1, through binding onto intact platelet surfaces and also to platelet receptors, leading to an expression of a biomarker receptor P-selectin (CD62p) as a result of platelet activation. During the current study, direct ELISA-based assays were established, which revealed that both human TSP1 and highly active oligomerised properdin are capable of binding to both washed human intact and thrombin-activated platelets. The current study confirms that the highly oligomerised properdin form is likely to be the only polymerised molecule of Properdin forms that is capable of binding and triggering platelet activation at 37°C for 10 minutes incubation in comparison with different agonists including TSP1.

A recent study by Saggu et al. (2013) has shown that human properdin only interacts with human platelets under activation conditions, but not to intact platelets using the flow cytometry technique. Virtually, washing intact platelets have not bound to the following serum physiological forms of properdin; dimer, trimer and tetramer, unless the intact platelets were treated by such agonists as thrombin or arachidonic acid (Saggu et al., 2013). Non-separated properdin forms and the highly oligomerised form of properdin were not included in the previous study, suggesting, the whole story cannot be un-

derstood unless defining the role of properdin containing low and high cyclic polymerised forms together.

However, it has been shown that pentameric and hexameric forms of purified human properdin from plasma could be involved with the physiological forms, but with low concentrations in complement activation using zymozan (Smith et al., 1984). Moreover, the findings of the current study show the ability of the recombinant highly active oligomerised properdin, mostly representing the highly formed polymer, to bind to washed intact platelets, while the other separated forms; dimer, trimer and tetramer, were in less or no interaction to intact platelets, which is compatible with Saggu et al. s' (2013) results. On the other hand, all the forms of properdin including non-separated forms within the recombinant properdin were certainly binding to thrombin-activated platelets. Although, it is still controversial whether the oligomerised higher forms of properdin are naturally involved in human plasma/serum, generated during complement activation (Smith et al., 1984, Chapitis and Lepow, 1976), or just formed in laboratory artefact conditions, either during protein purification or repeating cycles of freezing and thawing the protein (Saggu et al., 2013). This suggests that during the activation of the complement system, the oligomerised highly active forms of properdin might be generated in order to drive the activation of the AP, or even the released properdin from activated leukocytes and other cells at the site of vessel injury would initiate the formation of active forms of properdin even with slight concentrations. That would bring to understand the correlation between haemostasis/thrombosis and the complement system in thrombus events in order to comprehend the molecules cause cross-talk which could be targeted for a therapeutic approach. This suggests the way of forming physiological forms of properdin in physiological conditions would also initiate a theory of speculating that the activated properdin beside the released properdin from leukocyte cells probably generated after activation of the complement system at the site of vessel injury, where properdin starts to polymerise into higher forms by which platelet activation may be initiated, leading to activation of the controlled haemostasis process or uncontrolled thrombus formation. For example to the interference of complement activation molecules with thrombosis, it has been found that MBL/MASP-1 plays a crucial role in such events of thrombus formation by which MASP1 is found to slice the major substrates for thrombin, fibrinogen (Fg) and coagulation factor XIII (FXIII), with slow enzymatic sufficiency in comparison with thrombin *in vitro* (Hess et al., 2012, Megyeri et al., 2009, Krarup et al., 2008, Hajela et al., 2002) and *in vivo* (La Bonte et al., 2012). The

role of MASP-1 has also been demonstrated in correlation with coagulation pathways through MBL or MASP-1 deficient mice, which have been noticed to have a longer bleeding time on removing the tail tip (Takahashi et al., 2011). Therefore, using non-separated forms of properdin, including the highly polymerised/oligomerised form, is crucial to conclude the relevance of properdin including higher forms to the process of haemostasis or thrombosis with regard to platelet activation.

In addition, the formation of platelet-leukocyte aggregation in whole blood was stimulated using a properdin dose-dependent manner while the platelet in the whole blood was not activated through measuring P-selectin and GPIIb/IIIa (Ruef et al., 2008). It is more important to investigate the role of highly active oligomerised properdin on washing platelets rather than using whole blood to prevent the interference of natural inhibitors of platelets like prostaglandins. Also, in chapter-5 of the current study, the results showed that TSP1 was able to inhibit the activity of highly active oligomerised properdin via measuring the C3b deposition on the target surface. That would suggest the possibility of the natural TSP1 in whole blood to be an inhibitor for the exogenous properdin. Moreover, the current study also confirms the direct binding capability of TSP1 toward properdin by which may form complexity and prevent activity of the molecule on the platelet surface. Moreover, the observations of Ruef et al. (2008) did not provide information about the activity of the purchased commercial properdin on the activation of the alternative pathway (AP), as being it is believed that the activity of properdin might be related to the high activity of higher cyclic forms of polymerised/oligomerised properdin (Ali et al., 2014). More precisely, it has been stated that the activated properdin was in charge of binding surface-bound C3b, leading to formation of C3 convertase of the fluid phase, while the native forms of properdin bind just to C3bBb (Smith et al., 1984). That would explain the functional activity of the higher oligomerised forms of properdin on platelet stimulation. Therefore, the activity of native properdin is likely dependant on complement activation, either resulting in inflammatory cells stimulation by chemotaxis leading to properdin release, or through polymerising higher forms in case of complement activation, according to Smith et al. (1984). Additionally, all forms of oligomerised properdin were able to bind to C3b but in variable degrees of avidity, as the higher oligomer form showed aggressive higher binding compared to lower oligomer forms (Smith et al., 1984). All these evidences would indicate that purchasing commercial properdin by Ruef et al. (2008) was likely to be native but certainly not an oligomerised active molecule with the possibility of stimulating platelet activation.

Nevertheless, the findings of the current study of binding human properdin to human washed intact platelets conflict with Saggu's results. The findings were established and approved by using direct ELISA which indicates the binding of properdin and TSP1 to the intact platelets. The use of a semi-quantitative direct ELISA tool was the first time regarding detecting the binding of TSP1 and properdin to whole platelet and for quantifying expression levels on fixed platelet surface rather than platelet lysate. The binding assay of TSP1 and properdin to intact platelets (resting) revealed that platelets were not activated during the coating step on the microtitration plates, and that can be seen obviously through the P-selectin (CD62p) expression levels. Moreover, properdin was able to bind to the surface of intact platelets, the same as TSP1. The binding results of TSP1 to resting platelets totally agrees with the results of Wolff et al. (1986), when the latter observed the binding activity using a radio immune assay by incubating $_{125}$I-TSP1 with unstimulated and thrombin-stimulated platelets (Wolff et al., 1986). The activity of the coated intact platelets was measured by detecting the expression levels of P-selectin (CD62p) using monoclonal anti-human CD62p, which shows non-significant and very low in expression levels of P-selectin in comparison with the thrombin-mediated platelet activation (positive control) and with the non-specific binding of the antibody to the background (subtraction negative control). The event of low level of expression was normally due to the process of withdrawing blood in the anticoagulant sodium citrate. It has been demonstrated that the whole blood taken into sodium citrate causes expression of P-selectin in intact platelets but with a low percentage compared to anticoagulant K_3 EDAT using flow cytometry (Ritchie et al., 2000). Therefore, in order to control the activation by inhibiting platelets during the washing process, prostaglandin E_1 (PGE_1) was required as an antagonist binding through adenylyl cyclase P2Y receptor by competing the agonist ADP resulting in the mediation of cyclic adenosine 3',5' mono-phosphate (cAMP) formation and stimulating both protein kinase-A (PKA) and vasodilator stimulated phosphoprotein (VASP) in resting platelets (Kloeze, 1967, Geiger et al., 1999, Thijs et al., 2010, Roberts et al., 2010, Gharehbaghian et al., 2015). That low level of P-selectin in intact platelets was considered as a baseline. Therefore, the platelets which were used for binding assays toward TSP1 or properdin were obviously intact platelets (not activated) which can also be confirmed through the fact that the physiological native forms of properdin could not bind to resting platelets while bound to activated platelet, according to the current study.

It has been suggested that the P-selectin marker of the activated platelet in various diseases is the potential and the most abundant and reliable receptor considered for such screening diagnosis (Amrani et al., 1995, Goldsmith et al., 2000, Kamath et al., 2002). In 2002, platelet cell lysate was used to quantify the majority of P-selectin (CD62p) per platelet using ELISA in order to compare between healthy volunteer controls and atrial fibrillation (AF) patients (Kamath et al., 2002). Furthermore, it has been quantified that the soluble sP-selectin was monitored using polyclonal antibody in patients, and that the soluble sP-selectin of the total P-selectin was not co-participated to platelet membranes in the lysate, compared to 90% of the total P-selectin bound to the membrane (Semenov et al., 1999). Therefore, in this study the attention was focused on quantifying the majority of binding P-selectin to the membrane of activated platelets stimulated by different agonists; collagen type-I and adenosine 5'-diphosphate (ADP), compared with TSP1 and the highly oligomerised properdin by coating fixed platelets to 96-well microtitration plates instead of using platelet lysate, due to preventing the cross-reaction of the monoclonal antibody with non-secreted P-selectin in α-granules of platelets. Therefore, the binding of TSP1 and properdin to intact platelets resulting in P-selectin (CD62p) expression levels were quantified using direct ELISA.

As a result of binding, the current study of P-selectin revealed that the recombinant human TSP1 was capable of triggering activation of intact platelets by expressing a significantly higher level of CD62p in comparison with collagen-I and ADP agonists, including highly oligomerised properdin. In addition, the highly oligomerised properdin was also found to sufficiently induce platelet activation by monitoring the expression levels of CD62P, which was significantly more than ADP induction. It is suggested that the binding of properdin to the intact platelet, subsequently leading to triggering platelet activation, may be due to the activity of unseparated recombinant of the highly active oligomerised properdin by which it has been observed that the functional activity is substantially more than the equal amount of purified native properdin in alternative pathway of complement activation (Ali et al., 2014). Therefore, the highly oligomerised properdin seems to effectively act as an agonist for triggering platelet activation. There are several possible suggested reasons behind that action; one of them is that due to a degree of homology between the TSR repeats of both TSP1 and properdin. Moreover, the more significant levels of P-selectin expression using TSP1 than properdin could refer to the N-terminal and C-terminal of TSP1. In 2001, it was found that the sequence peptide RFYVVMWK in the C-terminal region of TSP1 is critical for platelet activation

regarding signalling pathway via an Fc receptor γ-chain, and also is independent of αIIb/β3 and GPIba receptors stimulation (Tulasne et al., 2001). In 2003, it was agreed that the C-terminal region can activate platelets independently. However, it has been suggested that the previous sequence in the C-terminal is partially dependant on binding fibrinogen to aIIb/β3 receptor (Trumel et al., 2003).

The aggregation process of platelets depends on fibrinogen (Fg) binding to GPIIb/IIIa complex linking and providing a bridge between platelets to form platelet accumulation. GPIIb/IIIa was found on the surface of intact platelets between 40,000 and 50,000 as complexes. All the following ligand components of GPIIb/IIIa like fibrinogen, TSP1 and vWF etc., cannot bind to the receptor with high affinity, but only in activation conditions by which the receptor and calcium ion influx are released from α-granule and dense granule to the surface of the platelet leading to conformational change on the receptor structure (Jurk and Kehrel, 2005). Hence, augmenting the binding affinity of the Fg to GPIIb/IIIa complex was observed upon the activation and receptor conformation via disulfide changes (Lahav et al., 2002). In addition of providing more evidences of the substantial activation of properdin and TSP1 to platelet aggregation, the fibrinogen (Fg) was able to bind to activated platelets, mediated by different agonists including TSP1 and properdin, compared to intact platelets in 96-well plates (Figure 7.1). The current study of binding Fg to activated platelets would indicate the activation of αIIb/β3-integrin (GPIIb/IIIa complex) (CD41/CD61). Therefore, it means that the αIIb/β3 integrin was induced during the activation conditions via TSP1 and properdin by which the tyrosine kinase and the G-coupled receptors of platelets might be activated following the signal transduction of transferring phosphor and calcium (Golden et al., 1990, Broos et al., 2012), which are very important for processing the activation.

The binding of highly oligomerised properdin and TSP1 to platelets can be clearly seen through stimulating living intact platelets for adhesion process where the process is firstly initiated in haemostasis/thrombosis events. After the adhesion process (tethering) onto the exposed extracellular matrix layer, the platelets shapes start to change, resulting in adhesion, secretion and aggregation (clumps formation) which were observed after using oligomerised properdin and TSP1 compared with positive agonists. Initially, the adhesion process basically begins from the binding of the extracellular matrix molecules to GPIb/V/IX complex receptor on platelets (Adam et al., 2003, Andrews and Berndt, 2004, Jurk and Kehrel, 2005, Broos et al., 2012).

Therefore, the binding investigation of TSP1 and properdin to commercial recombinant platelet receptors like GPIbα (CD42b) and GPV (CD42d), where involved in the complex receptor GPIb/V/IX, and also CD36 (GPIV or GPIIIb) and the integrin associated protein (IAP/CD47). The mentioned receptors and more are involved in platelet adhesion, activation and proliferation. For instance, either von Willebrand Factor (vWF) or thrombin binds to platelet receptor GPIbα (CD42b) but in different binding sites leading to platelet activation (Dumas et al., 2003, Adam et al., 2003), while TSP1 has been identified as an alternative adhesive molecule promoting platelet adhesion resulting in clumps formation under a high share rate, but the share resistance not higher than vWF, considering the predominant molecule for platelet activation through that receptor (Jurk et al., 2003). However, that would not mean disregarding the impact of TSP1 on either static or flow condition to platelets because many examples of thrombotic events have been investigated due to the association of TSP1 by considering the importance of the interaction in initiating the process of platelet activation. Therefore, it has been suggested as a therapeutic target beyond the interaction under high share condition of TSP1 to GPIb-α (CD42b) (Jurk et al., 2003). The binding of the N-terminus and TSR1 domains of TSP1 to GPIbα (CD42b) has been theoretically investigated that these binding sites might share similar identity to the binding sites on vWF A1 domain. Therefore, a shearing similarity on TSP1 in two different domains has been reported; N-terminus and TSR1, and between A1 domain of vWF which are relatively identical in around 33 amino acids by 27.3% and in 50 amino acids by 24%, respectively (Jurk et al., 2003). However, the current study observed the binding of oligomerised properdin to the platelet adhesion receptor GPIbα (CD42b) compared to the negative control. This suggests the ligand receptor would be recognised through the TSR1 of properdin, by which around 47% identity was found with TSP1 mainly in TSRs domain (Goundis and Reid, 1988). That would explain how such a positive regulator protein, which is involved in the complement system, could overlap or cross-react into another system causing platelet adhesion. In addition to binding to GPIb/V/IX complex, the current study also illustrates the binding of properdin and TSP1 toward GPV (CD42d), which is one of four leucine-rich repeats consistencies performing the complex (Andrews et al., 2003), by which platelets activation could be triggered through in a static condition. Other platelet receptors like CD36 and CD47 were not considered as platelet adhesion receptors under high share condition. Especially when CD36 was blocked by antibody OKM5, the adhered platelet onto TSP1 under high share condition was partially inhibited (Jurk et al., 2003).

It seems that TSP1 bind to another receptor than CD36 for adhesion process. Moreover, it has been suggested that TSP1 regulates the inhibitory pathway caused by PGE1 in platelets instead of direct activation through CD36 and causing aggregation by which the mechanism of cAMP/protein kinase A (PKA) formation modulated under physiological flow condition rates using capillary tubes coated with fibrinogen (Roberts et al., 2010). The preventing formation of cAMP/PKA was noticed using a peptide sequence of TSR1 of TSP1 VTAGGVQKRSRL which led to preventing VASP phosphorylation (Roberts et al., 2010). However, the binding site of TSP1 to CD36 was located within the TSR repeat consisting of the conserved sequence CSVTCG. That sequence of TSR1 was found in an identical position with TSR1 of properdin. Therefore, both TSP1 and properdin were found to interact with the commercial chimera protein structure CD36-IgG-Fc and also 100-200aa peptide sequence of CD36 (GPIIIb) of the current study. The peptide sequence 93-155aa of CD36 considered the action binding site for TSP1 known as the CLESH-1 domain (CD36, LIMP-2, Emp structural homology-1) (Klenotic et al., 2013, Park, 2014) which is involved within the commercial peptide sequence 100-200aa. In addition, the conserved sequence of the CLESH-1 domain of CD36 is identical to gp120 in HIV, which confirms that this sequence CSVTCG of the TSR1 type-I completely interacted with both CLESH-1 and gp120 *in vitro* (Crombie et al., 1998). This suggests the necessity for further work to identify the role of oligomerised properdin in binding to HIV in future. Hence, the activation of platelets by properdin possibly occurs through the TSR motifs via binding to CD36.

It has been found that the cell-binding domain (CBD) in TSP1 binds to CD47 and triggers platelet activation, leading to conformational change in activating the GPIIb/IIIa complex (integrin α2b/β3) (CD41/CD61) (Chung et al., 1999, Lagadec et al., 2003). The platelet activation was observed from the derived peptide sequence of the CBD (KRFYVVMWKK) by increasing the signalling activation of the integrin α2β1 (Chung et al., 1999, Frazier et al., 1999). Despite the fact that no identical peptide sequences were found for properdin with TSP1 regarding the binding sites to CD47 using the alignment option on the website www.uniprot.org, the current study revealed that properdin binds to CD47 through using commercial chimera protein structure CD47-IgG-Fc compared with the negative control IgG-Fc. This suggests the use of CD47 as a receptor for properdin might be involved in platelet interaction.

The consequences of platelet adhesion and activation would lead to platelet aggregation mainly through the binding of the predominant activated receptor αIIb/β3-integrin (GPIIb/IIIa complex) (CD41/CD61) on the activated platelet to fibrinogen thereby crosslinking other platelets together, forming clumps (Golden et al., 1990, Broos et al., 2012). The current study found that besides the binding of TSP1 to the commercial platelet subunit receptor CD41 (αIIb), that the oligomerised properdin was capable of binding too, compared to the negative control used in the assay. Moreover, both recombinant molecules found to bind the immobilised fibrinogen (Fg) in plastic surface. As the binding of TSP1 to Fg has already been established for many years but properdin is new in the field, it might be associate with clot formation. The binding of properdin forms; tetrameric, trimeric and dimeric forms including oligomerised form, to Fg were observed in dramatic order with respect to forms sizes. This suggests the function of properdin is likely to associate with Fg and fibrin mesh by crosslinking platelets and mediating adhesion to monocyte and leukocytes, from where Properdin secreted, through the adhered platelets at the site of vascular injury. For instance of adhering immune inflammatory cells onto activated platelets, the bound P-selectin (CD62p) to eosinophils was found to crosslink the immune inflammatory cells to the adhered platelets through the integrin subunit αIIb (CD41) (Larsen et al., 1989, Rinder et al., 1991, Johansson et al., 2012). In addition, binding properdin forms to Fg may provide a strength to the fibrin mesh (polymerised fibrin) according to their sizes, similar to TSP1 that binds to fibrin through the disulphide bound as co-polymerise (Bale et al., 1985). However, it has been believed that TSP1 also interacts with Fg, as molecule to molecule, for cooperating on the cytoskeleton membrane of activated platelets to operate platelet aggregation (Leung and Nachman, 1982). It has been inferred that because of the differences of TSP1 concentration in plasma before and after platelet activation, this would mainly assess to function in co-polymerising fibrin and also cooperate with fibrin on platelet aggregation (Bale et al., 1985). However, the significant differences between TSP1 concentrations before and after activation is likely to refer not only to cooperate with fibrin but also to corporate with Fg as following; Fg-TSP1-Fg, which results in strengthening the efficiency for crosslinking platelets via GPIIb/IIIa complex, which would go along with the theory stated by (Leung and Nachman, 1982, Bonnefoy et al., 2001). Additionally, the same manner may be implicated for Properdin. It has been proved by Panetti, et al. that expressing TSR type-I, including all or part of the following repeats; 1st, 2nd and 3rd repeat, of TSP1, was found to be responsible for binding to

the α-chain of un-polymerised Fg indicating the involvement of the 2nd repeat and the 3rd only in the crosslinking to fibrin (Panetti et al., 1999). This suggests platelet adhesion onto highly oligomerised properdin or even secreted active properdin under high share condition requires more investigation including measuring properdin levels before and after platelet activation in whole blood or plasma.

4.3.2 Oligomerised Properdin and TSP1 Play an Important Role of Activating Coagulation Pathways through Thrombin and Pro-coagulant Activity of Microparticles Release

What is more, as a way to define the role of either properdin or TSP1 in activating platelets, certain components of platelet secretion were targeted as biomarkers to define the activation process of platelets in haemostasis/thrombosis. There are several blood components, vascular and blood cells modulated by the components released from platelet granules to interact with activated platelets. The α-granule of platelets secretes α-thrombin in the process of activation, as low levels of the secreted thrombin are not capable of forming a firm clot, but the concentration is able to trigger other platelets activation (Jurk and Kehrel, 2005) specifically through thrombin ligand receptors called protease-activated receptors; PAR1, PAR-4 (Zhang and Colman, 2007, Jarvis et al., 2003, London, 2003) and also via GPIb of the complex GPIb/IX/V that shows high binding affinity toward thrombin (Mazzucato et al., 1998, London, 2003). It has been reported that PAR-4 receptor initially requires high concentration of α-thrombin leading to platelet aggregation, but not PAR-1, in association with the release of calcium and the enhancement of P_2Y_{12} (ADP receptor) (Holinstat et al., 2006). The required concentration of α-thrombin for platelet activation has been measured separately for each receptor to be 1nM (high sensitive) for RAR-1 and 30nM (low sensitive) for RAR-4 (Greco et al., 1996, Kahn et al., 1999, London, 2003). Furthermore, the trace amount of secreted thrombin is able to convert factor FV to FVa (active form) in the coagulation cascade of the extrinsic pathway. Moreover, thrombin can be generated by the enzymatic serine proteases combination of FVa-FXa that attach to tissue factor (TF) on the surface of platelets to convert prothrombin (FII) to active thrombin (FIIa), but in a small amount (Tracy et al., 1983, Monroe et al., 1996). The experiment of Sandwich ELISA using KIT Thrombin (Abnova), coated with monoclonal antibody, used for targeting α-thrombin released from washed activated platelet defined the sensitivity of the washed platelets amongst exogenous oligomerised properdin and TSP1 compared to agonists. Both highly oligomerised properdin and TSP1 were capable of triggering platelet activation through thrombin secretion, and subsequently may lead to coagulation cascade activation. The ultimate concentration of the soluble thrombin for $150x10^6$platelets/ml is approximately 0.156nM for TSP1, and less for properdin. However, the counted platelet

number used for thrombin experiment was less than the normal platelet number in blood in physiological conditions that found to be 200-400x10^6 platelet/ml (Hoffbrand et al., 2001). This suggests the differences of platelet count number would possibly increase the secreted levels of α-thrombin up to 0.3nM, which is enough for GPIb activation (Greco et al., 1996) at the site of vessel injury. These few amounts of thrombin are likely to be propagated or generated by the same process or even by microparticles (MPs), especially in conditions where they may be assessed to improve such thrombotic events as atherosclerosis disease. Platelet microparticles (PMPs) are small particles derived from platelet cytoskeleton membrane during activation, leading to extending the functional activity of coagulation by having all mother cell membrane receptors like a receptor for coagulation factor Va which induces the prothrombinase reaction. These PMPs can be produced by thrombin, collagen and complement components of C5-9 (Sims et al., 1988, Wiedmer et al., 1989, Abrams et al., 1990). It has been found that PMPs expose GPIb, GPIIb/IIIα, P-selectin (CD62p) and TSP1 (George et al., 1986, Gawaz et al., 1996b). Therefore, it has been demonstrated that PMPs provide a mtarix grid for platelet adhesion in particular through GPIIb/IIIα onto the sub-endothelial layer (Merten et al., 1999) and also possibly through TSP1. Most of the exposed receptors and proteins on the surface of PMPs can be used as biomarkers for identification (Horstman and Ahn, 1999). However, the current study for identifying the presence or released PMPs from washed platelets used thrombin generation assay instead. Truly, thrombin was generated from platelets by the released microparticles using thrombin generation assay by ELISA. Basically, the assay depends on the combination of the serine proteases FXa-FVa captured by phosphatidylserine (PS) or TF exposed on MPs resulting α-thrombin (IIα) propagation from the exogenous prothrombin (II) by which the process required Ca^{+2}. On the other hand, the level amount of the generated IIα reverts to the significant values of PMPs which expose PS, due to the fact of the relation between IIα propagation levels and the PS. Therefore, it has been reported that PMPs seriously induced thrombin propagation, as well as coagulation pathway activation by refereeing to an exhibition of the negatively charged phospholipids (i.e., phosphatidylserine) (PS) on the outer membrane (Wolf, 1967, Horstman and Ahn, 1999, Berckmans et al., 2001, Zwicker et al., 2012). Increasing levels of PMPs have been noticed in patients with serious diseases like disseminated intravascular coagulation (Holme et al., 1994), myocardial infarction (Gawaz et al., 1996a) and cardiovascular disease (VanWijk et al., 2003). The current study implemented the use of ZYMUPHEN MP-Activity KIT (HYPHEN BioMed) by which

thrombin generation from MPs of activated platelets was basically identified by measuring the prothrombinase activity generated from the captured PMPs by annexin V already labelled with biotin that also captured by coated streptavidin in 96-well plates (Hugel et al., 1999, Mallat et al., 2000). It has been well characterised by determining the most potent agonist activating the production of PMPs as following from potency to weaker agonist; calcium-ionophore (A23187), (collagen+thrombin), collagen, thrombin, ADP and epinephrine (Heinz Joist et al., 1974, Bode and Miller, 1986, Sims et al., 1989, Horstman and Ahn, 1999). Therefore, that was exactly the outcomes of thrombin generation by PMPs mediated in serial order upon the agonist potency as previously mentioned, besides TSP1 being the most physiological potent agonist, while properdin and collagen were almost equal followed by thrombin then ADP. This suggests cooperation between TSP1 and properdin or with collagen, or all together, might create a dust-storm of PMPs.

The results of thrombin generation caused by TSP1 and highly oligomerised properdin would indicate the importance of the molecules in thrombotic events through promoting platelet-derived pro-coagulant activity. The ultimate goal of this project, in future work, is to inhibit or target those molecules -TSP1 and properdin- by which may decrease the levels of pro-coagulation caused by PMPs which may result in down-regulating the activity of such diseases like atherosclerosis disease, myocardial infraction, suggesting a therapeutic approach.

4.3.3 Oligomerised Properdin and TSP1 Play an Important Role of Binding Extracellular Matrix Proteins (ECM) Possibly Resulting in Platelet Adhesion

The haemostasis process requires extracellular matrix (ECM) adhesive molecules like collagen, vWF, TSP1 and fibronectin, etc., for triggering platelet activation and also allowing platelets to adhere on the exposed surfaces. This process was imitated in the current study of adhesion assay using most of the mentioned adhesive molecules, including highly oligomerised properdin compared to human and bovine serum albumin already coated into 96-well microtitration plates. Intact washed platelets were obviously adhered into all the adhesive molecules including TSP1 and, surprisingly, to the highly oligomerised properdin, while intact platelets could not adhere to the negative controls; BSA and HSA. The current findings might confirm the ability of oligomerised properdin to bind intact platelet membrane through one or more of the adhesive receptors; CD41/CD61, CD36, CD47 and GPIb/V/IX complex, which were shown on the previous results. This promising result and the previous results would indicate a significant role of the highly oligomerised/polymerised properdin in the process of platelet adhesion, which is likely in turn to have an effect on the following process of thrombotic events besides TSP1.

The current study also identified the role of properdin forms, including the highly oligomerised form, besides TSP1, in the first step of the haemostasis process through binding to the exposed ECM proteins at the vascular injury. Immobilised collagen type-I, fibronectin (Fn) and vWF were all observed to interact with soluble highly oligomerised properdin, while no interaction was observed with the other types of collagen like type-III and IV. However, the current results observed the binding of other native physiological forms; tetramer, trimer and dimer, of properdin with collagen type-I and Fn but the binding was increased in gradual affinity in serial order upon, suggesting, properdin forms sizes (the largest form followed by smaller form, etc.) compared to the highly oligomerised form. Basically, the differences in the binding affinity between properdin forms with ECM might indicate a dependency on the higher polymerisation molecules. However, it has been established for many years that TSP1 is involved in the interaction with ECM in order to crosslink between platelet interaction and ECM for cooperating platelet adhesion process. The binding site of TSP1 to Fn was confirmed to be not lo-

cated in the TSR repeats (Panetti et al., 1999) by which the binding sites of Fg and Fn in TSP1 has been indicated in various regions (Homandberg and Kramer-Bjerke, 1987), whereas, the peptide sequence GGWSHW in the second TSR repeat is considered to be an essential binding site in TSP1 for binding with Fn (Sipes et al., 1993). It has been found that the interaction between Fn and TSP1 assesses TSP1 to interact with $\alpha 3\beta 1$ integrin (Rodriguez-Manzaneque et al., 2001). In addition, the interaction performed against TSP1 degradation process was due to stabilising the calcium-dependent structure of TSP1 (Dardik and Lahav, 1999). By matching the protein sequence of TSP1 with properdin using the UniprotKB databases, the peptide sequence GGWSGW was found in properdin to match the TSP1 peptide sequence in TSR1 for Fn binding. This suggests the binding interaction of properdin and Fn maybe processed through the matched peptide sequence.

Moreover, it has been reported that TSP1 interacts with collagen-I, III, and IV (Galvin et al., 1987) by characterising the binding sites of TSP1 in association with collagens located in the C-, N-terminals and stalk region (Procollagen homology domain) (Galvin et al., 1987).

Furthermore, vWF interacts with the GPIb/IX/V receptor complex and also the integrin $\alpha_{II}b/\beta_3$ resulting in platelet aggregation (Bonnefoy et al., 2006) and is also considered as one of the ECMs performing platelet adhesion onto the sub-endothelial exposed layer. However, the A3 domain of vWF was found to be the binding site of TSP1 in the same binding manner with ADAMTS13, suggesting the vWF proteolysis might be processed in slower interaction (Pimanda et al., 2004). It has been reported that the TSP1 was found to participate in vWF protection from proteolysis by ADAMTS13 (Bonnefoy et al., 2006). As a result of binding highly oligomerised properdin to vWF, the current study suggests that the properdin might also interfere in the interaction between ADAMTS13 and vWF and provide some protection to the exposed vWF from the proteolytic process. It requires more invistgation to find out the involvement of Properdin in vWF proteolysis protection.

The differences between TSP1 and properdin regarding binding to ECM is that TSP1 is a Ca^{2+} dependent molecule requiring calcium for conformational change to certain subunits (Calmodulin) (Dardik and Lahav, 1999) while suggesting that properdin may require to polymerise into higher forms in order to crosslink and interact with ECMs, especially when active properdin is secreted from leukocytes, monocytes and endothelial cells at the vascular injury site.

The results would indicate the involvement of the native physiological forms of properdin, including highly oligomerised forms, in the process of supporting the ECMs in order to strengthen the interaction of platelet adhesion. It is also suggested that properdin is likely either to serve as an adhesive molecule, or make a bridge between the exposed matrix and the adhered platelet in the thrombotic events.

4.3.4 Oligomerised Properdin and TSP1 Possibly Play an Important Role in Atherosclerosis Disease via Truly Binding to Low-density Lipoprotein (LDL)

Atherosclerosis is defined as an inflammatory disease due to inflation of an increase in the plasma concentration of cholesterol and low-density lipoprotein (LDL), which are found to be the vital factors as atherogenic potential particles (1993, Ross, 1999, Nicholson et al., 2000) and triglycerides. Triglycerides have been found to be a significant biomarker in cardiovascular disease (CVD). Triglycerides are associated with the atherogenic materials such as LDL, and also accumulate on endothelial cells ready for scavenging by macrophages that are later converted into foam cells (Gianturco et al., 1998, Botham et al., 2007) by which atherosclerotic plaque is promoted (Talayero and Sacks, 2011). There are several factors associated with atherosclerosis disease, such as the expression of P-selectin or E-selectin on activated adhered platelets or activated endothelial cells, respectively. Moreover, the integrins of the β3 family eventually expressed on activated platelets like GPIIb/IIIa (α2b/β3 integrin) are found to play a role in fibrin formation (Schwartz et al., 1999). It has been found that the isolated GPIIb/IIIa-coated micro beads interacted with LDL resulting in an inhibition of the fibrinogen interaction while using high concentrations of LDL which activated the washed platelets by stimulating the binding site of fibrinogen on platelets (Tetik et al., 2008). Thrombospondin has been observed to be a critical component of atherosclerotic plaque (Wight et al., 1985) and arterial basement membrane (Arbeille et al., 1991). The interaction of TSP1 with human plasma lipoprotein has been shown in order to figure out how TSP1 is incorporated into atherosclerosis plaque (Muraishi et al., 1993). A theory that TSP1 may transfer lipoproteins into the atherosclerotic lesions has been supported by a result showed a significant binding of TSP1 to the very-low-density lipoprotein (VLDL) (Muraishi et al., 1993). Surprisingly, the current result observed a significant binding of either TSP1 or highly oligomerised properdin toward cholesterol, triglycerides and LDL, while fibrinogen is observed as a negative control which could not bind to the atherogenic particles. The results would indicate that properdin is likely to incorporate to function as a transfer protein, the same as TSP1 for lipoprotein. Another suggestion is that properdin may adhere to the atherosclerotic plaque by participating to

the atherogenic particles in atherosclerotic lesions, and may cause a firm adhesion for platelets.

That would suggest the highly oligomerised properdin and TSP1 might be incorporated negatively in patients suffering from atherosclerosis disease or coronary artery disease by participating with the lipids for establishing the fundamental base for accumulating the inflammatory immune cells into the adhered platelet as a result of response to these molecules.

To sum up, the result of human properdin in the haemostasis or thrombosis system by activating platelets implies the important function of highly oligomerised properdin in preventing blood loss. Moreover, the role of highly oligomerised properdin likely goes beyond the alternative pathway of the complement system to be associated with thrombosis by acting on platelet receptors transforming platelets to burst their essential contents, including microparticles' procoagulant activity to function on triggering activation of the coagulation cascade as a cross-talk between those systems. Moreover, highly oligomerised properdin requires more investigation about the possibility of this highly oligomerised molecule to associate with most serious diseases like atherosclerosis disease by, for example, measuring the concentration of properdin in such diseases, especially after observing properdin interacting with LDL, cholesterol and triglycerides.

Chapter 5 (Results)

(The role of TSP1 in complement system of innate immunity)

The role of TSP1 in complement system of innate immunity

The functional activity of human TSP1 on complement system of innate immunity is going to be identified by screening the levels of C3b and C4b deposition on activated platelets or even on Mannan, which represents microorganisms, and then identify the targeted molecules in complement system.

5. Results

5.1. Binding of TSP1 to different bacterial pathogens referring to bind to peptidoglycan of cell wall composition including chondroitin sulphate (CS) of Platelets

Bacterial adhesion process is the first and initial mechanism that bacterial pathogens tend to exploit via some extracellular matrix (ECM) proteins in infection process initially through such molecules as TSP1 which was found to act as bridge between host cell and gram positive bacterial pathogen (Rennemeier et al., 2007). It has been reported that TSP1 binds peptidoglycan of gram positive bacteria providing such microbial protection from phagocytosis by blocking the binding site on bacterial cell wall from peptidoglycan's receptor of phagocyte cells, while that opportunity of binding has been reported to cover only the capsulated and non-capsulated gram positive bacteria but not capsulated gram negative bacteria (Rennemeier et al., 2007).

Rennemeier's result showed the binding of TSP1 to G+ve bacteria, such as, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Listeria monocytogenes* causing significant binding, while, TSP1 appeared to bind to G-ve bacteria, such as, *Escherichia coli*, *Legionella pneumophila*, *Haemophilus influenzae* and *Neisseria meningitidis*, causing slight interaction. Here, the current findings conflict with Rennemeier's result. The current results confirmed that TSP1 bind to both Gram- negative and Gram-positive bacteria and to either capsulated or non-capsulated strains comparing with negative control from capsulation point of view, whereas, Rennemeier's results lack of comparing the binding to capsulated and non-capsulated gram negative bacteria for the same species, and instead of that Rennemeier pointed out the binding of TSP1 to peptidoglycan of gram positive bacteria as an issue (Rennemeier et al., 2007).

The binding capability of TSP1 was clearly observed through ELISA. The study demonstrated the binding of TSP1 to either G+ve or G-ve bacteria that were capsulated or non-capsulated. *Staphylococcus aureus* (DSM20233), *Neisseria meningitides* (group $_{\text{wild type}}$ A-Z2491, $_{\text{wild type}}$ B-MC58) and *Streptococcus pneumoniae* ($_{\text{wild type}}$ D39) were chosen to distinguish in binding to TSP1 between G+ve and G-ve pathogenic bacteria (Figure 5.1).

Moreover, *N.meningitides* group B was genetically modified in the capsulation ($MC58_{\Delta}antS_{\Delta}SiaD$) and also ($MC58_{\Delta}SiaDcay$) (Unkmeir et al., 2002) in order to illus-

trate the difference of TSP1 interaction with and without capsule. Other strains of *Neisseria meningitides* e.g. N176.1 and N199.1 were also targeted in the capsulation gene (Oldfield et al., 2013) (Figure 5.2). All of which, were provided by Dr Christopher D.Bayliss (University of Leicester) were fixed and coated into 96-well plates (Maxisorb) (Nunc) after measuring the turbidity and setting up the optical density (OD) wave length for all the strains in OD_{550}=0.6. Besides, mannan and zymosan from *Saccharomyces cervisiae* (Sigma), *N*-acetylated BSA (Invitrogen) and Chondroitin sulphate (CS) (Sigma) were coated to investigate the binding affinity of TSP1 toward different polysaccharide residues from different sources.

The condition of interaction between TSP1 and bacterial pathogens, self or pathogenic materials was provided in tris hydrochloride buffer containing Ca^{2+} and Mg^{2+}. The binding activity of TSP1 was increased in a dose-dependent manner to all bacterial pathogens including gram-positive and –negative bacteria with slightly variations in binding affinity between pathogens. TSP1 was able to bind strongly to *Neisseria meningitides* group A and *Staphylococcus aureus* more than other organisms (Figure 5.1). These results suggest that the TSP1 interact with either peptidoglycan and polysaccharide capsule of G+ve bacteria and G-ve bacteria, respectively. Therefore, the binding affinity toward different strains of *N.meningitides* has shown significant binding to both group A and B (capsulated) including the binding to the non-capsulated strains, where the capsule is knocked out, but with less affinity comparing with capsulated strains (Figure 5.2). All of which were compared with negative control of binding TSP1 to BSA which served as blocking material in ELISA. This suggests that the capsulation is very important ligand for TSP1 attachment in order to initiate bacterial adhesion process for capsulated bacteria, whereas, the binding to non-capsulated *N.meningitidis* could refer to binding some of virulence factors on the bacterial surface which subsequently lead to impairment complement activation as later on will be discussed in this chapter.

Moreover, the current results have observed the favourite binding affinity of TSP1 toward glycosaminoglycan (GAG) containing chain of polysaccharides consisting of an amino sugar either *N*-acetylgalactosamine (GalNAc) or *N*-acetylglucosamine (GlcNAc), which represented in Chondroitin sulphate (CS) as sulphated sugar consisting of GalNAc more than mannan and zymozan which contain polysaccharide of repeating sugar units of mannose and glucose, respectively. Following mannan, binding to *N*-acetylated-BSA was downward and differentiated from the other polysaccharides. That may due to

representing the monosaccharide ligands of *N*-acetylmuramic acid (MurNAc) (Figure 5.3).

The interaction of TSP1 can cross beyond ECM grid to attach with different bacterial pathogens; Gram-positive or –negative bacteria, not only through the residual peptidoglycan cell wall content but also the composition of capsule by which would increase the binding opportunity even if the bacteria are classified as G-ve species. However, that would lead to other considerations about the ability of such virulence factors on the surface of bacterial pathogens to bind TSP1 against complement activation as later on will be discussed in this chapter.

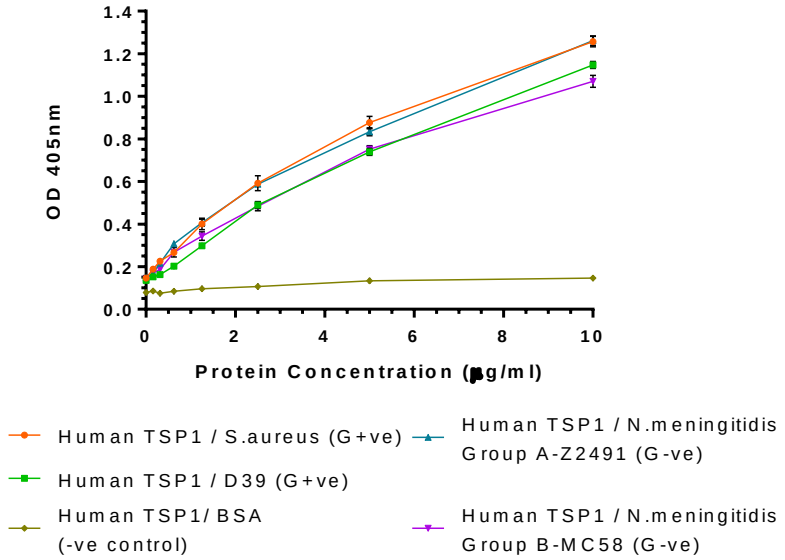

Figure 5.1. Direct ELISA based assay for TSP1 binding to Gram positive and Gram negative bacteria. Gram positive bacteria; *S.aureus* (DSM20233) and *S.pneumonia* ($D39_{WT}$ capsulated), and Gram negative bacteria; *N.meningitides* (A-Z2491, B-MC58 capsulated) were coated in 96-well micro-titration plate (Maxisorb) (Nunc). The optical density (OD) was measured for all coated bacteria in weave length OD_{550}=0.6. 1% Bovine serum Albumin (BSA) (Blocking buffer) was incubated with TSP1 to serve as subtracting negative control. The binding activity was detected using the following antibody; 1:1000 mouse polyclonal antibody against human TSP1 (Sigma) followed by 1:5,000 Goat anti-mouse polyclonal IgG antibody-Alkaline phosphatase conjugate (Sigma). The absorbance of colour substrate development was absorbed at 405nm. TSP1 showed no binding toward the blocking buffer 1% BSA. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

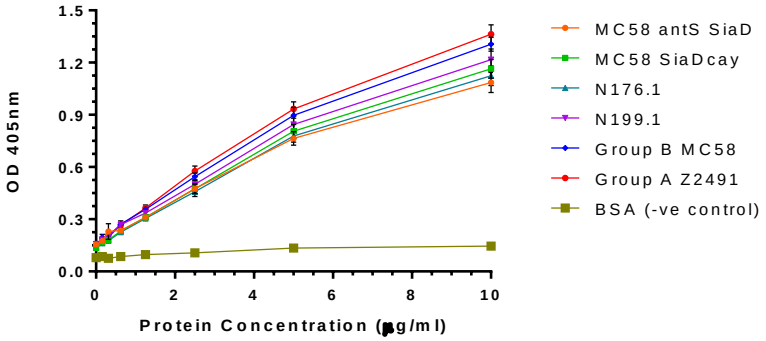

Figure 5.2. Direct ELISA based assay for TSP1 binding to different strains of capsulated and non-capsulated of *N.meningitides*. Capsulated meningococci Group A-Z2491, B-MC58, and non-capsulated meningococci $MC58_{\Delta}antS_{\Delta}SiaD$, $MC58_{\Delta}$ SiaDcay, N1761 and N199.1 (kindly provided by Dr Christopher D.Bayliss, University of Leicester) were coated in 96-well micro-titration plate (Maxisorb) (Nunc). The optical density (OD) was measured for all coated bacteria in weave length OD_{550}=0.6. 1% BSA (Blocking buffer) was incubated with TSP1 to serve as subtracting negative control. The binding activity was detected using the following antibody; 1:1000 mouse polyclonal antibody against human TSP1 (Sigma) followed by 1:5,000 Goat anti-mouse polyclonal IgG antibody-Alkaline phosphatase conjugate (Sigma). The absorbance of colour substrate development was absorbed at 405nm. TSP1 showed no binding toward the blocking buffer 1% BSA. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

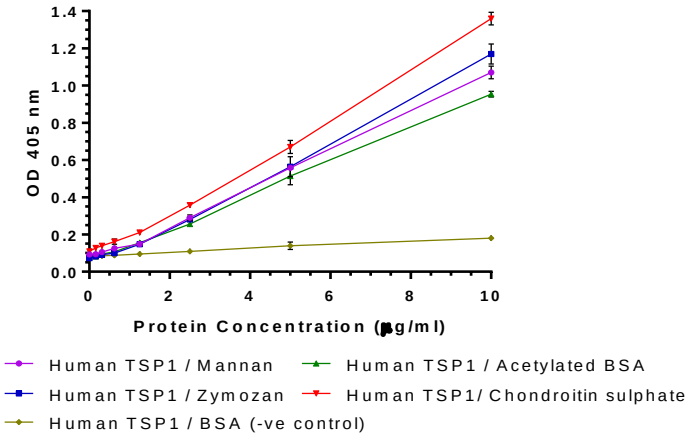

Figure 5.3. Direct ELISA based assay for TSP1 binding to different sugar ligands. The polysaccharide units of *N*-acetylglucosamine (GlcNAc) represents Mannan and Zymozan, while *N*-acetylgalactosamine (GalNAc) represents Chondroitin sulphate (CS), whereas, the monosaccharide *N*-acetylmuramic acid (MurNAc) represents *N*-acetylated-BSA. 10µg/ml (1µ/well) each of sugar ligand material was coated in 96-well micro-titration plate (Maxisorb) (Nunc). . 1% BSA (Blocking buffer) was incubated with TSP1 to serve as subtracting negative control. The binding activity was detected using the following antibody; 1:1000 mouse polyclonal antibody against human TSP1 (Sigma) followed by 1:5,000 Goat anti-mouse polyclonal IgG antibody-Alkaline phosphatase conjugate (Sigma). The absorbance of colour substrate development was absorbed at 405nm. TSP1 showed no binding toward the blocking buffer 1% BSA while observed significant binding toward all sugar ligands. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

5.2. The role of TSP1 in Complement system

In this section of study, we are going to investigate the role of TSP1 that is released by activated platelet to be used by diversity of pathogenic microorganisms during the activation of complement system. That would elucidate the cross talk between platelet activation or thrombotic events and between complement system.

5.2.1. The effective role of TSP1 in C3b deposition on activated platelets

In the case of vessel injury, it would allow to expose ECM proteins that are likely to be sticky to bind circulated or local platelet leading to platelet activation and release mediators that are able to activate other circulating platelets and then subsequently leading to platelet accumulation in order to reduce bleeding. That activation occurring on platelet surface would trigger complement activation by exposing chondroitin sulphate (CS) onto platelet cell wall. It has been shown that CS triggered complement activation (Hamad et al., 2008). Consequently, C1q, C4, C3 and C9 molecules of complement cascade were found to bind to activated platelet (Hamad et al., 2010), whereas, it has been observed that the activated C3 molecule (C3b) was certainly bound to stimulated platelets through P-selectin receptor (CD62p) by which they suggested the activation of AP is likely to be triggered on/by stimulated platelet surface (Del Conde et al., 2005). In addition, it has been indicated that the C3(H_2O) is recruited only by properdin on thrombin-activated platelet which may in consequence drive the formation of C3 convertase of AP; while on using arachidonic acid-mediated platelet activation, both C3b and C3(H_2O) were recruited by properdin (Saggu et al., 2013).

Therefore, by using Enzyme Linked Immune Sorbent Assay (ELISA), the levels of C3b deposition on thrombin-activated platelets were quantified in the presence of TSP1 using rabbit polyclonal anti-human C3c antibody (Dako) (Cat A0062). The experiment was based on coating the fixed thrombin-activated platelets in 96-well plates (Maxisorb) (Nunc). The procedure of C3 deposition assay was followed as described in the Materials and Methods section. All complement pathways were activated by preparing human serum in serial dilutions starting from 1:20 (5%) in BBS containing Ca^{2+} and Mg^{2+} with or without 10µg/ml of exogenous TSP1. During the dilutions, the prepared human serum was placed on ice in order to prevent the complement activation. After incubating

the diluted serum containing the exogenous TSP1 and compared to the positive and negative controls, the deposition of C3b levels were quantified.

According to ELISA of C3 deposition assay, TSP1 was instead of what it was thought to imitate the role of properdin of AP due to the similarity of TSRs that responsible for increasing the stability of C3(H_2O) deposition on the target surface, strikingly, the levels of C3b deposition on activated platelets were observed to be impaired comparing with the human serum served as positive control which was noticeably more in levels of C3b than human serum containing exogenous TSP1 (Figure 5.4). However, little C3b deposition were detected on the surface of intact platelets comparing with the C3b levels on BSA (background), both served as negative controls. That would indicate the inactivation of complement system which was intact to some extent from activation through monitoring C3b deposition levels on the surface of intact platelets compared with activated platelets. The impairment levels of C3b would indicate the binding of TSP1 to *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc), and the last repeating units of galactose was found to be involved as a conserved molecule represented in chondroitin sulphate (CS). Thus, TSP1 compete out the complement recognition molecules on binding to such ligands. I propose to investigate the activity of TSP1 on the proteolytic activity of complement system that enable C4b deposition on the targeted surface, as well as, I suggested the competition binding behaviour of TSP1 with Mannose-binding lectin (MBL) on CS and mannan as residual ligands where C3b deposition was inhibited in the presence of TSP1.

In addition, the minimum concentration of TSP1 that reduced the deposition of C3b was determined in dose dependent manner of TSP1 from 10-0µg/ml in a single serum concentration 1:20 (5%). The C3 deposition was gradually impaired in increasing TSP1 concentrations starting from the highest concentration (10µg/ml) and then the impairment levels were downward gradually with some reduction activity on C3b in the lowest concentration (2.5µg/ml) of TSP1. The levels of C3b deposition were gradually raised by decreasing the concentrations of TSP1 (Figure 5.5). 5µg/ml TSP1 was able to reduce the C3 consumption from conversion into C3 deposition on activated platelets. That concentration would be equal a the concentration of TSP1 in haemostasis events where activated platelets 150–400×10^9 release around 100µg/ml.

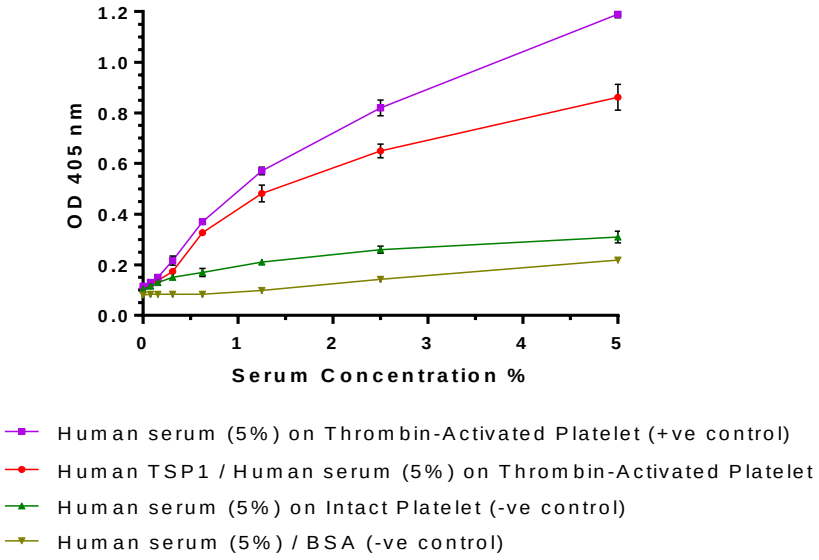

Figure 5.4. Quantifying C3b deposition levels of complement activation on thrombin-activated platelets in the presence of TSP1 using ELISA based assay. Human Washed platelets (150,000-200,000 platelet/µl) were activated by thrombin (1U/ml) while other washed platelets were not activated but fixed served as negative control (intact platelet). All together were coated in 96-well micro-titration plate (Maxisorb, Nunc). Barbital buffer saline (BBS) containing (Ca^{2+}, Mg^{2+}) was used to serially dilute human serum 1:20 (5%) pre-incubated with or without TSP1 at range of concentrations from 10-0 µg/ml. The inhibition of C3b deposition was certainly observed in the exogenous of TSP1 in comparison with human serum served as positive control while the negative control of measuring the amount of C3b deposition on intact platelets showed little C3b deposition compared to the C3 deposition on BSA. The negative control was used to observe the spontaneous of the C3b deposition on BSA that used as blocking buffer. The C3 deposition was detected using 1:5000 polyclonal anti-human C3c antibody (Dako). This result would indicate involvement of TSP1 in the complement system regulation. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

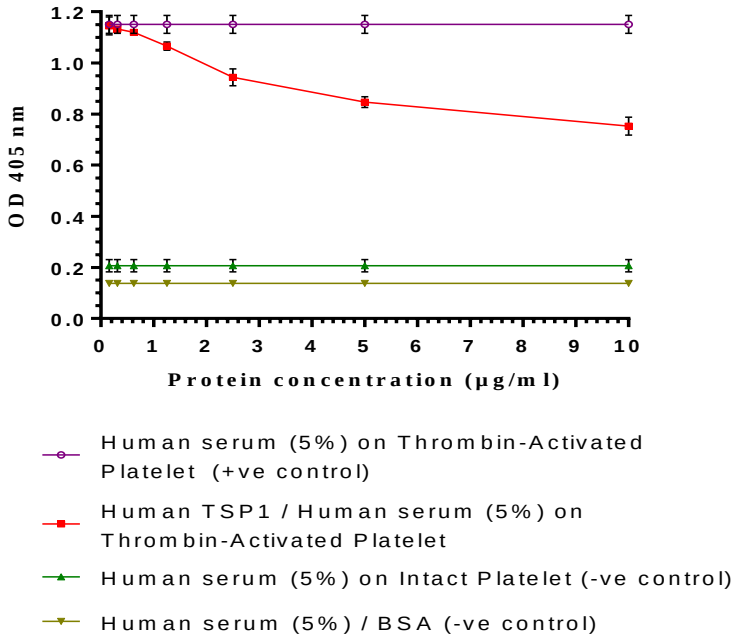

Figure 5.5. C3b inhibition ELISA assay using dose dependant manner of TSP1 on thrombin-activated platelets. Human washed platelets (150,000-200,000 platelet/µl) were activated by thrombin (1U/ml) while other washed platelets were not activated but fixed served as negative control (intact platelet). All together were coated in 96-well micro-titration plate (Maxisorb, Nunc). Barbital buffer saline (BBS) containing (Ca^{2+}, Mg^{2+}) was used to serially dilute TSP1 at range of concentrations from 10-0 µg/ml with a single concentration of human serum 1:20 (5%).
The positive control and the negative control were used only to quantify the C3b deposition occurs on activated and intact platelets, respectively, while BSA served as negative control used to observe the spontaneous amount of C3b deposition on the blocking surface. The C3 deposition was detected using 1:5000 polyclonal anti-human C3c antibody (Dako). P value for C3b deposition on activated platelets in the presence of TSP1 in comparison with in the absence of TSP1 using Two Way ANOVA (Bonferroni's multiple comparisons test) = P value = < 0.0001. P value for intact platelets (-ve control) in comparison with C3b deposition on BSA (-ve control) = P value = 0.4029. This result would indicate involvement of TSP1 in the complement system regulation. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

Two Way ANOVA (Bonferroni's multiple comparisons test)		
5% Human Serum (+ve control) vs 5% Human serum + TSP1	**Significant? P < 0.05?**	**Mean Diff.**
(10µg/ml) TSP1 concentration	Yes (****)	0.391
(5µg/ml) TSP1 concentration	Yes (****)	0.297
(2.5µg/ml) TSP1 concentration	Yes (***)	0.201
(1.25µg/ml) TSP1 concentration	No (NS)	0.078
(0.625µg/ml) TSP1 concentration	No (NS)	0.025
5% Human Serum (+ve control) vs 5% Human serum /BSA (-ve control)	Yes (****)	1.034
5% Human serum + TSP1 vs 5% Human serum /BSA (-ve control)	Yes (****)	0.643

Table 5.1. The statistical significance results of C3b inhibition ELISA assay using Two Way ANOVA (Bonferroni's multiple comparisons test) comparing between different concentrations of exogenous 10µg/ml TSP1 in single concentration of 5% human serum and between 5% human serum (+ve control), following incubation on Thrombin-activated platelets. P value of all the significant results = P < 0.05. Key words; (*); significant. NS; Not significant.

According to the statistical analysis (Table 5.1) TSP1 was showing a remarkable activity against the activation of complement system on activated platelets by decreasing C3b deposition in comparison with positive control (human serum incubated on activated platelet). The remarkable activity of TSP1 of impairing C3b deposition was significantly and gradually observed by increasing the concentration of TSP1 beginning from 2.5µg/ml till 10µg/ml. Statistically, by subtraction the Mean Diff of +ve control from the Mean Diff after using 10µg/ml TSP1, about 40% from the total C3b deposition levels on activated platelets has been remarkably cut off and down regulated by TSP1.

However, instead of using human platelets to investigate the role of TSP1 in the activation of complement system, the previous experiment was repeated by using 10µg/ml mannan instead of activated platelet, due to the similarity between mannan and chondroitin sulphate (CS) in activating complement system (Figure 5.6). The predicted impairment of C3b deposition levels on mannan using 1.25% of human serum containing

an exogenous TSP1 was found to be the same as down regulating C3 deposition on activated platelets experiment in the previous figure (Figure 5.5).

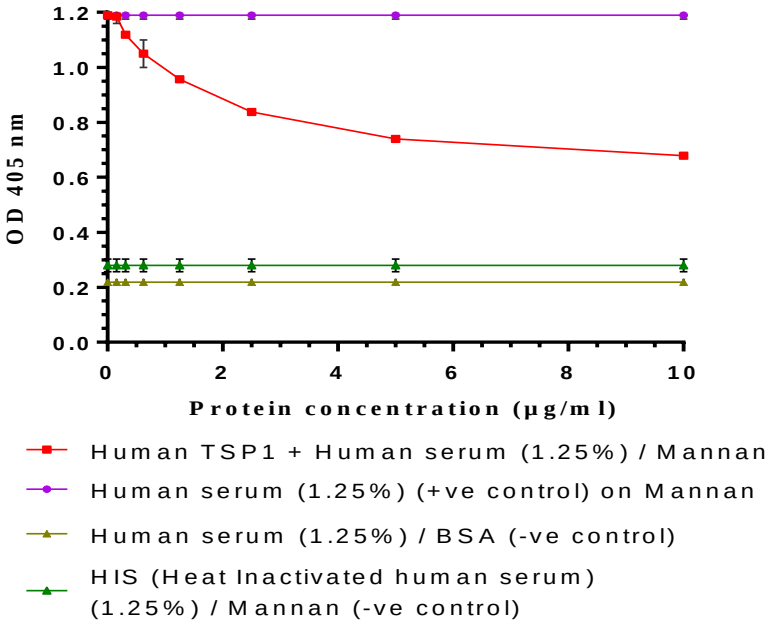

Figure 5.6. C3b inhibition ELISA assay using dose dependant manner of TSP1 on 10µg/ml mannan. Mannan was coated in 96-well micro-titration plate (Maxisorb, Nunc). Barbital buffer saline (BBS) containing (Ca^{2+}, Mg^{2+}) was used to serially dilute TSP1 at range of concentrations from 10-0 µg/ml with a single concentration of human serum 1:80 (1.25%). The positive control and the negative control were used only to quantify the C3b deposition occurs on mannan and BSA, respectively, while BSA served to observe the spontaneous amount of C3b deposition on the blocking surface. The C3 deposition was detected using 1:5000 polyclonal anti-human C3c antibody (Dako). This result would indicate involvement of TSP1 in the complement system regulation. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

5.2.2 The effective role of TSP1 in C4b deposition

After discovering the reduction ability of TSP1 on C3b deposition of complement activation on activated platelets, C4b deposition assay was the next step to follow and figure out which pathway of complement system was regulated by TSP1. C3 convertase C4b2a consists of two cleaved molecules; C4 and C2. C4 is only restored at the classical pathway (CP) and lectin pathway (LP). Due to the fact that C4 concentration is about 500µg/ml in serum which is lower than C3, about 1-1.5mg/ml (Abbas et al., 2010), human serum was diluted at higher concentration 1:20 (5%) in tris-hydrochloride buffer containing Ca^{2+},Mg^{2+}. That was done to activate the LP and CP in this experiment in order to figure out in general the functional activity of TSP1 in the two pathways. Therefore, if there is any activity of TSP1 to render the levels of C4b deposition on mannan, then the use of MBL binding buffer containing 1M NaCl would be crucial. However, by using high salt concentration would inactivated CP via dissociating C1 complex and leading to prevention of the activation of the endogenous C4. Also, high salt concentration would break down the bounds interaction between TSP1 and its ligands as can be observed through using high salt concentration in TSP1 purification for protein elution. Hence, the procedure was followed as described in the Materials and Methods section.

10µg/ml TSP1 was extraordinarily impairing the C4b deposition levels on mannan in comparison with human serum donated from a healthy volunteer used as a positive normal control. C4b was detected using chicken monoclonal anti-human C4c (Immunsystem AB) with a concentration of 1:2000 (Figure 5.7).

The minimum concentration of TSP1 that inhibits the deposition of C4 was determined using serial dilutions of TSP1 from 10-0µg/ml in a single serum concentration 1:20 (5%) (Figure 5.8). The levels of C4b deposition were gradually raised by decreasing the concentrations of TSP1. The C4b deposition was gradually inhibited by the highest concentration 10µg/ml and followed by 5µg/ml and 2.5µg/ml of TSP1. The 5µg/ml concentration would be equal to the concentration of TSP1 in haemostasis events where activated platelets $150–400\times10^9$ release TSP1 around 100µg/ml. Statistically, upon the OD of the +ve control (Figure 5.7-5.8) which represents the deposition levels of C4b on mannan, it was up to 50% of total C4b deposition levels has been inhibited and down regulated by using 10µg/ml TSP1.

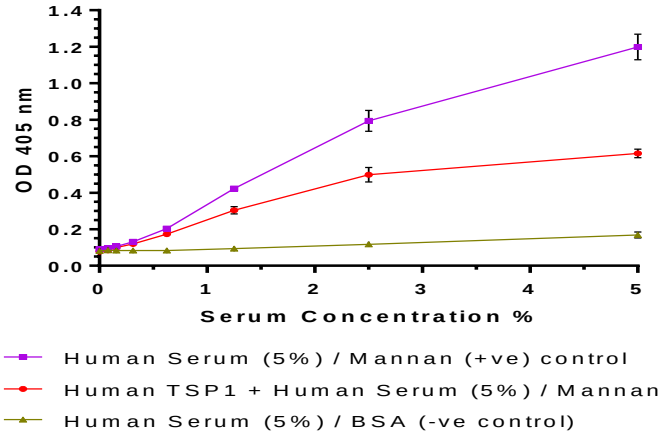

Figure 5.7. Quantifying C4b deposition levels of complement activation on Mannan in the presence of TSP1 using ELISA based assay. 10µg/ml Mannan was coated in 96-well micro-titration plate (Maxisorb, Nunc). Tris-hydrochloride buffer containing (Ca^{2+}, Mg^{2+}) was used to serially dilute human serum 1:20 (5%) pre-incubated with or without TSP1 at range of concentrations from 0-10µg/ml. The C4 deposition was detected using 1:2000 chicken monoclonal anti-human C4c (Immunsystem AB). The inhibition of C4b deposition was certainly observed in the exogenous of TSP1 in comparison with human serum served as positive control while the negative control served to observe the amount of exposing C4b deposition on BSA that used as blocking buffer. This result would indicate involvement of TSP1 in the complement system regulation. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

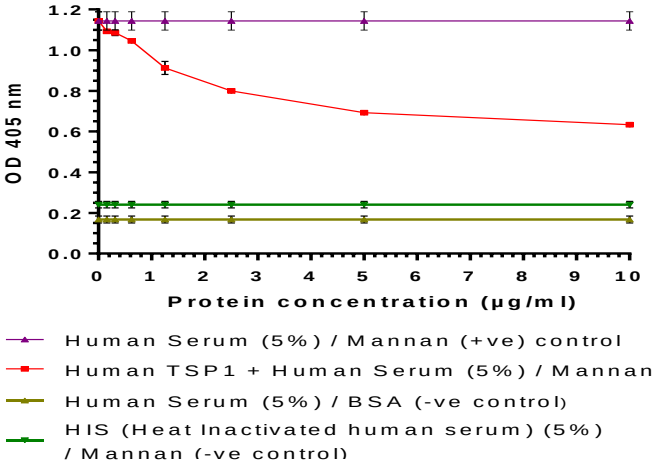

Figure 5.8. C4b inhibition ELISA assay using dose dependant manner of TSP1 on 10µg/ml mannan. Mannan was coated in 96-well micro-titration plate (Maxisorb, Nunc). Tris-hydrochloride buffer containing (Ca^{2+}, Mg^{2+}) was used to serially dilute TSP1 at range of concentrations from 10-0 µg/ml with a single concentration of human serum 1:20 (5%). The positive control and the negative control were used only to quantify the C4b deposition occurs on mannan and BSA which both giving steady higher and lower level of C4b deposition, respectively. The C4b deposition was detected using 1:2000 chicken monoclonal anti-human C4c (Immunsystem AB). P value for C4b deposition on Mannan in comparison with or without TSP1 using Two Way ANOVA (Bonferroni's multiple comparisons test) = P value = < 0.0061. This result would indicate involvement of TSP1 in the complement system regulation. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

Two Way ANOVA (Bonferroni's multiple comparisons test)		
5% Human Serum (+ve control) vs 5% Human serum + TSP1	**Significant? P < 0.05?**	**Mean Diff.**
(10µg/ml) TSP1 concentration	Yes (****)	0.509
(5µg/ml) TSP1 concentration	Yes (****)	0.450
(2.5µg/ml) TSP1 concentration	Yes (****)	0.344
(1.25µg/ml) TSP1 concentration	No (**)	0.230
(0.625µg/ml) TSP1 concentration	No (NS)	0.057
5% Human Serum (+ve control) vs 5% Human serum /BSA (-ve control)	Yes (****)	0.975
5% Human serum + TSP1 vs 5% Human serum /BSA (-ve control)	Yes (****)	0.466

Table 5.2. The statistical significance results of C4b inhibition ELISA assay using Two Way ANOVA (Bonferroni's multiple comparisons test) of comparing between different concentrations of exogenous 10µg/ml TSP1 in single concentration of 5% human serum and between 5% human serum (+ve control), following incubation on Mannan. P value of all the significant results = P 0.05. Key words; (*); significant. NS; Not significant.

According to the statistical analysis (Table 5.2) the TSP1 was showing a remarkable activity against the activation of complement system on Mannan by decreasing C4b deposition in comparison with positive control (5% human serum). The remarkable activity of TSP1 of impairing C4b deposition was significantly and gradually observed by increasing the concentration of TSP1 beginning from 1.25µg/ml till 10µg/ml. More Statistically, by subtraction the Mean Diff of +ve control from the Mean Diff after using 10µg/ml TSP1, about 50% from the total C4b deposition levels on mannan has been remarkably cut off and down regulated by TSP1.

5.2.3 The role of TSP1 in Lectin Pathway (LP)

5.2.3.1 The binding competition of TSP1 and Mannose binding lectin (MBL) to Chondroitin sulphate (CS) and Mannan

The function of TSP1 in complement system was suggested to be the same binding manner of MBL toward polysaccharides, especially when I found TSP1 binds to mannan and zymozan and is also capable of attaching to the capsulated bacterial strains, while it has not been found to bind to non-capsulated bacterial strains (Rennemeier et al., 2007). To determine whether regulating LP of complement system by TSP1 through MBL, mannan and chondroitin sulphate (CS) were used to serve as pathogenic associated molecular patterns (PAMPs) and molecular pattern of activated platelets, respectively. TSP1 and MBL were tested in competition assay for binding to mannan and CS in 96-well microtitration plate (Maxisorb, Nunc) (Figure 5.9A,B) respectively. The competition was by targeting MBL binding using mouse monoclonal anti-human MBL antibody (ANTIBODYSHOP). The results of the competition were astonishing by observing that 10µg/ml TSP1 was able to compete 10µg/ml MBL on binding to either mannan or chondroitin sulphate (CS) by almost 100% on mannan while 80% on CS (Figure 5.9A,B). Therefore, another confirmation observation was investigated by pre-incubating different serial dilutions of soluble mannan starting from 10µg/ml with one single concentration of 10µg/ml TSP1 in order to then, allow MBL to bind to immobilized mannan. In other words, the soluble mannan was precisely used to inhibit the binding of TSP1 to the immobilized mannan. Therefore, the equivalent concentration of soluble mannan to TSP1 was observed to allow 10µg/ml MBL to bind to coated mannan in comparison with positive control representing free 10µg/ml MBL incubated alone with immobilised mannan. More precisely, the low concentrations of soluble mannan were observed gradually to inhibit MBL binding (Figure 5.10). Thus, these observations would explain the role of TSP1 in competing MBL the recognition molecule of LP resulting in down-regulation of complement activation on such pattern recognition molecules either of activated platelets or bacterial pathogens. Suggesting, TSP1 manifests to passively regulate LP through competing MBL on binding to either *N*-acetylglucosamine GlcNAc or *N*-acetylgalactosamine (GalNAc), and might also compete other recognition molecules of LP like ficolins and collectins, and C1q of CP of complement system.

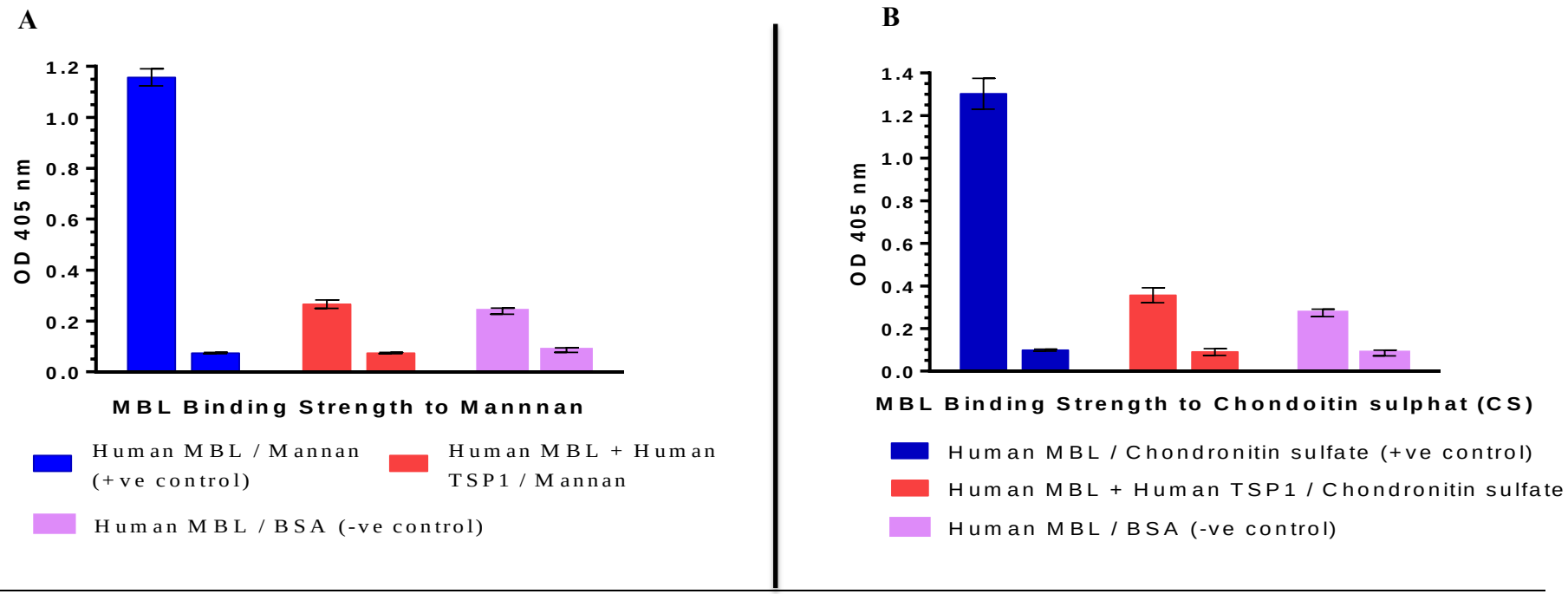

Figure 5.9. Direct competition assay of human TSP1 and MBL binding to *N*-acetylglucosamine GlcNAc of mannan and *N*-acetylgalactosamine (GalNAc) of chondroitin sulphate (CS). Both bar graphs were dealt in the same manner of targeting human MBL using mouse monoclonal anti-human MBL antibody (ANTIBODYSHOP). 10µg/ml mannan (A) and CS (B) were coated in 96 well microtitration plates (Maxisorb, Nunc). 10µg/ml human TSP1 was added into physiological buffer (Tris-hydrochloride containing Ca^{2+} and Mg^{2+}) already contain 10µg/ml human MBL (Kindly provided by Dr. Russel Wallis, University of Leicester). However, 10µg/ml human MBL alone was used for purpose of comparison as a positive control on mannan and also as negative control on BSA. Both experiments were dealt in the same time of 1 hour incubation at 37°C. The observation of the competition was the ability of TSP1 to prevent MBL binding mostly to Mannan and CS. That would indicate involvement of TSP1in complement regulation via competing MBL to bind either to bacterial pathogens or activated platelets. The development colour substrate was absorbed at 405nm and all reading values were recorded at same point of time for comparisons. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

Two Way ANOVA (Bonferroni's comparisons test)		
Binding to Mannan	**Significant? P < 0.05?**	**Mean Diff.**
MBL/Mannan (+ve control) vs MBL/BSA (-ve control)	Yes (****)	0.453
MBL/Mannan (+ve control) vs MBL+TSP1/ Mannan	Yes (****)	0.445
MBL+TSP1/ Mannan vs MBL/BSA (-ve control)	No (NS)	0.008

Table 5.3 The statistical significance results of (Figure 5.9A) human MBL binding to Mannan in the presence or absence of human TSP1 by using Two Way ANOVA (Bonferroni's comparisons test). The significant values = P < 0.05. Key words; (*); significant. NS; Not significant.

Two Way ANOVA (Bonferroni's comparisons test)		
Binding to Chondroitin sulphate (CS)	**Significant? P < 0.05?**	**Mean Diff.**
MBL/ Chondroitin sulphate vs MBL/BSA	Yes (****)	0.521
MBL/ Chondroitin sulphate vs MBL+TSP1/ Chondroitin sulphate	Yes (***)	0.477
MBL+TSP1/ Chondroitin sulphate vs MBL/BSA	No (NS)	0.043

Table 5.4 The statistical significance results of (Figure 5.9B) human MBL binding to Chondroitin sulphate in the presence or absence of human TSP1 by using Two Way ANOVA (Bonferroni's comparisons test). The significant values = P < 0.05. Key words; (*); significant. NS; Not significant.

According to the statistical analysis (Figure 5.9A) (Table 5.3) revealed that the MBL binding to mannan was completely competed with 100% significant inhibition by TSP1 comparing to BSA (-ve control). Statistically, by subtraction the Mean Diff of binding MBL to Mannan from Mean Diff in the presence of TSP1, it remarkably resulted in a complete inhibition by the use of equal concentration of TSP1 against MBL. Similarly, TSP1 was able to significantly compete the binding of MBL to CS (Figure 5.9B) (Table 5.4) in similar to the impact of competition occurred on Mannan (Figure 5.9A) (Table 5.3).

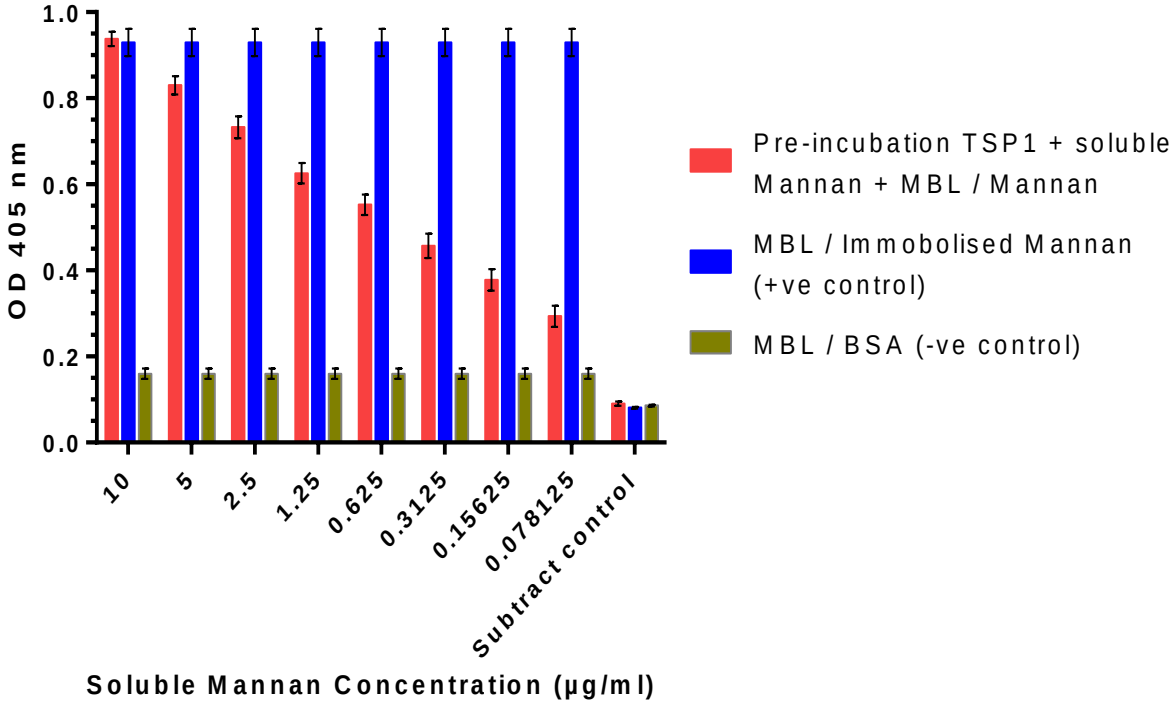

Figure 5.10. Indirect inhibition or competition assay of human TSP1 and MBL binding to *N*-acetylglucosamine (GlcNAc) of mannan. By mouse monoclonal anti-human MBL antibody (ANTIBODYSHOP) was used for targeting human MBL. 10µg/ml mannan was coated as immobilised molecule in 96 well microtitration plates (Maxisorb, Nunc). Meanwhile, different serial dilutions of soluble mannan starting from 10µg/ml were pre-incubated with single concentration of human TSP1 (10µg/ml) for 1 hour at 37°C. Then, single concentration of 10µg/ml human MBL (Kindly provided by Dr. Russel Wallis, University of Leicester) was incubated in the immobilised mannan (positive control), and mixed with, after all, the pre-incubated TSP1 and lastly incubated with BSA (negative control). The observation of the competition was the ability of the free molecules of TSP1, which was not blocked by soluble mannan, to prevent MBL binding to Mannan. That would indicate the involvement of TSP1in complement regulation via competing MBL to bind to bacterial pathogens or even activated platelets. The development colour substrate was absorbed at 405nm and all reading values were recorded at same point of time for comparisons. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

Multiple t test (Sidak-Bonferroni method)		
Soluble Mannan Concentration µg/ml	**Significant? P < 0.05?**	**P value summary**
10	Yes P= 0.0007	(*)
5	Yes P= 0.001	(*)
2.5	Yes P= 0.002	(*)
1.25	Yes P= 0.003	(*)
0.625	Yes P= 0.004	(*)
0.3125	No P= 0.010	NS
0.15625	No P= 0.015	NS
0.078125	No P= 0.039	NS

Table 5.5. The statistical significance results of (Figure 5.10) of inhibiting MBL binding on immobilised Mannan by TSP1. The competition of TSP1 with MBL was inhibited by pre-incubation TSP1 with different concentrations of soluble mannan. The comparison was by comparing the MBL binding in the presence of pre-incubated TSP1 and by the negative control (binding MBL to BSA). Therefore, the P value of binding MBL to immobilised mannan is significantly decreased by decreasing the concentration of soluble mannan which allows TSP1 to compete MBL on immobilised mannan, and vice versa. The significant P value was statistically calculated by using Multiple t test (Sidak-Bonferroni method). Significant values = $P < 0.05$. Key words; (*); significant. NS; Not significant.

By statistical analysis to (Figure 5.10) (Table 5.5) revealed that the binding competition of TSP1 and MBL to immobilised mannan was significantly inhibited in gradual concentrations of soluble mannan pre-incubated with single concentration of TSP1. The increase in the significance P value revealed the ability of binding MBL to immobilised mannan, and vice versa, by which means the free molecules of TSP1 were gradually capable of holding position on immobilised mannan before MBL binding. Suggesting, this competition is likely to be the reason behind the inhibition impact of TSP1 on the activation of complement system through screening the C3b or C4b deposition on the target surface.

5.2.4 The role of TSP1 and Properdin in Alternative Pathway (AP)

5.2.4.1. C3 deposition of AP in the presence of EGTA

The role of properdin in AP is to bind and stabilise the C3 and C5 convertase complexes (C3bBb) ($C3_nBb$), (C3bBbC3b), respectively, on the target surface (Schwaeble and Reid, 1999a). It has been reported that using an exogenous properdin augmented and restored the functional activity of the AP via C3 deposition in the serum of properdin knockout mice (Ali et al., 2014). As represented in properdin structure, the TSR domain does not require calcium in order to function in AP and vice versa, regarding TSP1 binding to targets (Figure 5.12, 5.13) due to having calmodulin domain.

For preventing classical and lectin pathways activation, it is vital to use EGTA in order to distinguish the function activation of alternative pathways *in vitro*. As the role of EGTA is to consume calcium, hence it is impossible to determine the role of TSP1 in the AP without the needs for calcium.

It was found that the purified TSP1 using heparin chromatography was contaminated with heparin which was detected by haemolytic assay of AP (Scott et al., 1997). Therefore, it is crucial to find out whether the purified TSP1 by heparin sepharose chromatography has been contaminated with heparin, as heparin is Ca^{2+} independent causing inhibition to complement system. Hence, the experiment was to determine the levels of C3b deposition of AP in the presence of EGTA using ELISA based assay for justification the existence or the absence of heparin as contaminant by which heparin may be or may be not involved with the function activity of TSP1.

10µg/ml mannan was coated into 96-well plates in order to initiate complement activation. Human serum from healthy volunteers was serially diluted starting from 1:20 (5%) with the use of EGTA buffer in the presence or absence of exogenous 10µg/ml highly oligomerised properdin (Figure 5.12), 10µg/ml TSP1, 10µM, 20µM and 30µM/ml heparin (Sigma) (Figure 5.11). The levels of C3b deposition was detected using rabbit polyclonal anti-human C3c. The levels of C3b were measured against the OD and compared with the positive control (human serum applied on mannan) and also with the negative control which represent the levels of C3b deposition on BSA (blocking buffer). All the serial dilutions of human serum were placed on ice in order to prevent the complement activation.

In figure 5.11, virtually no inhibition occurred to the levels of C3b deposition of the AP on mannan in the presence of 10mM EGTA using a dose-dependent manner of TSP1 which was purified by heparin chromatography, whereas the levels were significantly decreased using different concentrations of heparin (Sigma). By the use of EGTA, the zero impact of exogenous TSP1 on the C3b deposition levels of AP in parallel with the +ve control indicated the likelihood of purifying TSP1 being without or less contamination with heparin that could explain the non-effects above. Thus, using 1 ml heparin sepharose for TSP1 purification could be useful and might have no impact on the activity of TSP1. Moreover, that led me to a great discovery for confirming the results of functional activity of TSP1 in complement system by quantifying levels of C3b and C4b deposition in the presence of Ca^{2+}.

However, the impact of the exogenous highly oligomerised properdin was observed through restoring the C3b deposition on mannan using serial dilution of human serum starting from 1:20 (5%) (Figure 5.12). The result of the current study of the exogenous highly oligomerised Properdin agreed with Ali et al., (2014) observations.

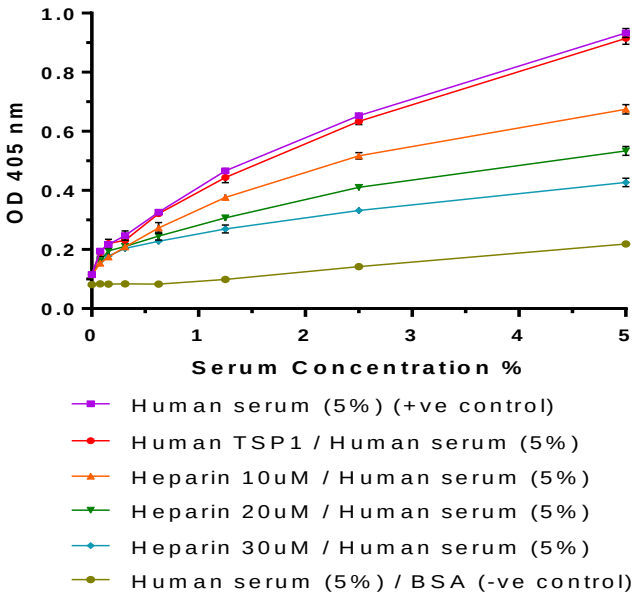

Figure 5.11. Quantifying the inhibition levels of C3b deposition of alternative pathway of complement system on mannan caused by the possibility of purified human TSP1 of containing heparin contamination using ELISA based assay. EGTA buffer contains 10mM EGTA and Mg^{2+}. Human serum was collected from human healthy volunteers and serially diluted using EGTA buffer starting from 1:20 (5%) in the presence or absence of 10µg/ml purified TSP1 (heparin chromatography column) compared with exogenous heparin 10µM, 20µM and 30µM (Sigma). The inhibition of C3b deposition was certainly observed in the exogenous heparin, whereas, in the presence of purified TSP1 (via heparin chromatography) was the same as the positive control that served as 5% human serum in the presence of EGTA+Mg^{2+} incubated on mannan with no addition materials. That would indicate the dependency of TSP1 on calcium and also observe no possible heparin contaminant was found by screening the C3b levels on Mannan for the purified TSP1 in comparison with different concentrations of heparin. The negative control was used to observe the spontaneous of the C3b deposition on BSA that used as blocking buffer. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

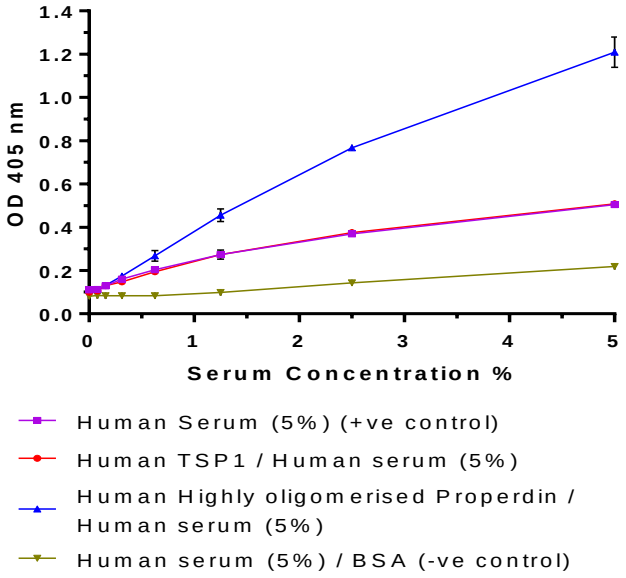

Figure 5.12. Quantifying levels of C3b deposition of alternative pathway activation of complement system on mannan using EGTA + Mg^{2+} buffer triggered by the presence of human highly oligomerised properdin using ELISA based assay. The EGTA buffer contains 10mM EGTA and Mg^{2+}. Human serum was collected from human healthy volunteer and serially diluted using EGTA buffer starting from 1:20 (5%) in the presence or absence of either 10µg/ml purified TSP1 or highly oligomerised properdin. The augmentation of C3b deposition levels was clearly observed by onward the levels from 2.5µg/ml properdin in 1.2% of diluted human serum, whereas, in the presence of TSP1 was not able to down-regulate the C3b deposition levels of alternative pathway in presence of EGTA + Mg^{2+} buffer. All of which were compared with positive control that served as 5% human serum incubated on mannan in the same conditions of using the buffer with no addition materials resulting the same quantity of C3b deposition levels as the exogenous TSP1. For properdin, that would indicate the ability of properdin to restore C3 levels in serum for deposition on the target surface up to 7 times more than the positive control even it was discriminated within low human serum concentration by which alternative pathway may not activated. Moreover, there was no activity of human TSP1 in alternative pathway using EGTA buffer. Suggesting, Ca ions are very important for TSP1 functionality in complement system. The negative control was used to observe the spontaneous C3b deposition on BSA that used as blocking buffer. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

5.2.4.2. The effective role of TSP1 on the activity of Properdin in the presence of Calcium

After following the role of TSP1 in the complement system via screening the reduction of C3b deposition levels on activated platelets and then determining the target ligands by which competing MBL of LP, it is exciting to find out how TSP1 function on mannan and also amongst the positive regulator Properdin of AP in the complement system. Thus, the C3b deposition assay using ELISA was repeated again to investigate whether TSP1 is capable of reducing the levels of C3 deposition on mannan, and also investigate the impact levels of C3b deposition in the presence of 10µg/ml properdin and 10µg/ml TSP1 all together on mannan using BBS containing Ca^{2+} and Mg^{2+} in order to provide the function requirements for TSP1.

It was predicted that TSP1 can impair the C3b deposition on mannan (Figure 5.13), as previously presented (Figure 5.6), as acting similarly on activated platelets (Figure 5.5). However, the highly oligomerised properdin was restoring the C3 deposition on mannan using serial dilution of human serum starting from 1:20 (5%) in the presence of Ca^{2+} and Mg^{2+} (Figure 5.13).

When mixing 10µg/ml of TSP1 with highly active oligomerised properdin in serial dilutions in human serum starting from 1:80 (1.25%) in the presence of Ca^{2+} and Mg^{2+}, surprisingly, TSP1 was able to inhibit the activity of the highly active oligomerised properdin on mannan through screening C3b deposition levels (Figure 5.14). The presence of Properdin with TSP1 manifested the functional activity of TSP1 in the AP by suggesting that either factor B or C3b can be targeted by TSP1 with more binding affinity which makes Properdin functionless in the environment of complement activation. Suggesting, that may explain how the activated platelets provide self-defence to their surfaces in the site of vessel injuries by manipulating TSP1, and other regulatory proteins, against the complement system. Also, Bacterial pathogens may use TSP1 for their protection besides colonisation and dissemination.

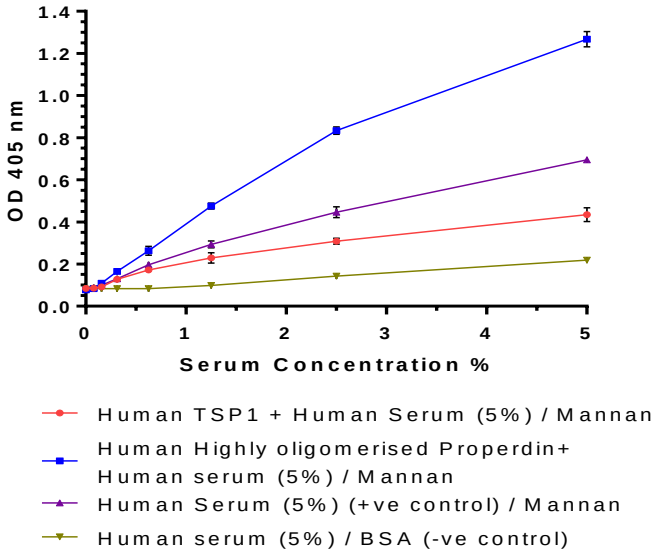

Figure 5.13. Quantifying levels of C3b deposition of complement activation on mannan by ELISA based assay using BBS containing Ca^{2+} Mg^{2+} triggered and down-regulated by human highly active oligomerised properdin and TSP1, respectively. 10µg/ml Mannan was coated in 96-well micro-titration plate (Maxisorb, Nunc). Human serum was collected from human healthy volunteer and serially diluted using Barbital buffer saline (BBS) containing (Ca^{2+}, Mg^{2+}) starting from 1:20 (5%) in the presence or absence of either 10µg/ml purified TSP1 or highly oligomerised properdin. The C3 deposition was detected using 1:5000 polyclonal anti-human C3c antibody (Dako). The augmentation of C3b deposition levels was clearly observed by onward the levels from 2.5µg/ml properdin in 1.2 % of diluted human serum, whereas, in the presence of TSP1 was able to down-regulate the C3b deposition levels of complement activation. All of which were compared with positive control that served as 5% human serum incubated on mannan in the same conditions of buffer usage with no addition materials resulting an increase of C3b deposition quantity comparing with exogenous TSP1. For properdin, that would indicate the ability of properdin to restore C3 levels of complement system in the presence of LP activation comparing with the positive control. Moreover, there was some activity of human TSP1 in the presence of calcium. Suggesting, Ca ions are very important for TSP1 functionality in complement system. The negative control was used to observe the spontaneous C3b deposition on BSA that used as blocking buffer. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

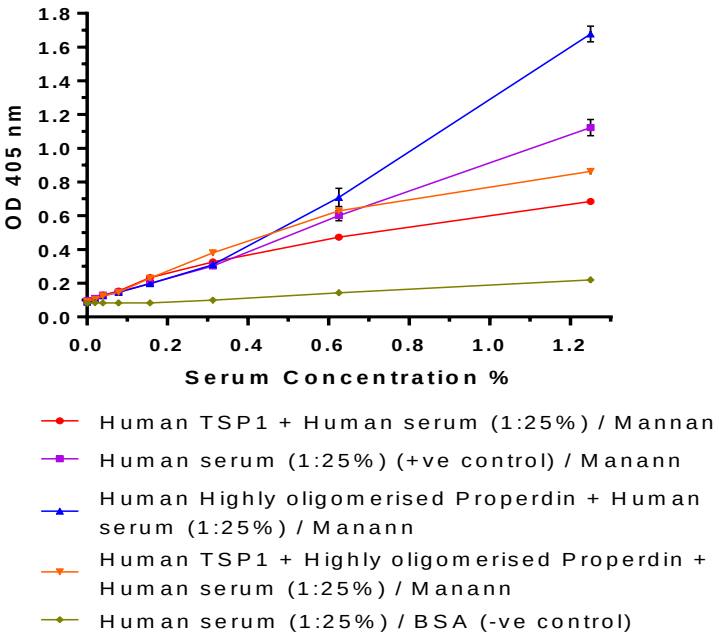

Figure 5.14. Down-regulating the activity of human highly active oligomerised Properdin by human TSP1 through quantifying levels of C3b deposition of complement activation on mannan by ELISA based assay using BBS containing Ca^{2+} Mg^{2+}. 10µg/ml Mannan was coated in 96-well micro-titration plate (Maxisorb, Nunc). Human serum was collected from human healthy volunteer and serially diluted using Barbital buffer saline (BBS) containing (Ca^{2+}, Mg^{2+}) starting from 1:80 (1.25%) in the presence, absence of both together 10µg/ml TSP1 and highly oligomerised properdin. The C3 deposition was detected using 1:5000 polyclonal anti-human C3c antibody (Dako). The augmentation of C3b deposition levels was clearly observed by onward the levels from 5µg/ml properdin in 0.6 % of diluted human serum, whereas, in the presence of TSP1 was able to down-regulate C3b deposition levels of complement activation. However, in the presence of both molecules, the activity of Properdin was inhibited in the presence of TSP1. All of which were compared with positive control that served as 1.25 % human serum incubated on mannan in the same conditions of buffer usage with no addition materials resulting an increase of C3b deposition quantity comparing with exogenous TSP1 or TSP1 and Properdin together. For TSP1, that would indicate the ability of TSP1 to down-regulate C3 levels of complement activation even in the presence of LP and AP activation comparing with the positive control. Suggesting, the results would indicate a new role of TSP1 involved in passive regulation of the complement system through down regulating the activity of Properdin while Properdin alone consider being a positive regulator. The negative control was used to observe the spontaneous C3b deposition on BSA that used as blocking buffer. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

5.2.4.3. The binding competition of TSP1 and Properdin to Chondroitin sulphate (CS)

Properdin was found to be one of glycoconjugates, which is a term used to describe the covalent interaction between proteins and carbohydrates, by binding to sulphated glycoconjugates through the motif (CPVTCG) which is similar to the amino acid sequence (CSVTCG) of TSR proteins (Holt et al., 1990, Cerami et al., 1992, Tuszynski et al., 1992, Higgins et al., 1995) including Properdin and TSP1 (Ginsburg and Roberts, 1988). Despite the fact that Properdin consists of three repeat motifs having similar amino acid sequences where localised in TSR1 and TSR2 (CSVTCG) and the amino acid sequence (CPVTCG) presented in TSR4 that are mainly responsible for interactions with carbohydrates, the current result observed the competition ability of TSP1 on binding to Properdin's carbohydrate ligand represented on CS, although, TSP1 consists of two motifs of (CSVTCG) localised in TSR2 and TSR3. That would suggest other motifs may involve in the interaction beside TSR domain of TSP1.

TSP1 and Properdin were tested in competition assay of ELISA for binding to CS in 96-well microtitration plate (Maxisorb, Nunc) (Figure 5.15). The competition was through targeting the binding of Properdin to CS using Rabbit polyclonal anti-human Properdin antibody (kindly provided by Dr. M.A, Youssif, University of Leicester). The astonishing results of the competition assay observed that 10µg/ml of TSP1 was capable of competing 10µg/ml Properdin on binding to chondroitin sulphate (CS) with mostly 80%.

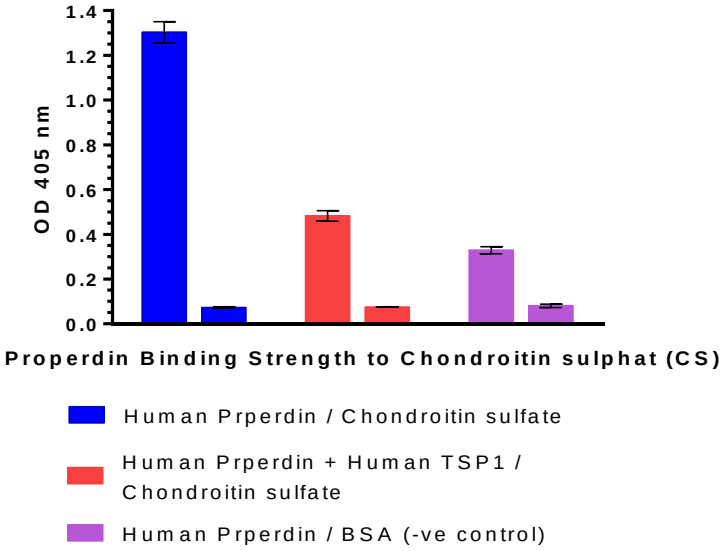

Figure 5.15. Direct competition assay of human TSP1 and Properdin binding to *N*-acetylgalactosamine GalNAc of chondroitin sulphate (CS). Both bars were dealt in the same manner of targeting human Properdin using 1:10,000 rabbit polyclonal antibody against human Properdin (kindly provided by Dr. M.A, Youssef, University of Leicester, England). 10µg/ml chondroitin sulphate was coated in 96 well microtitration plates (Maxisorb, Nunc). 10µg/ml human TSP1 was added into physiological buffer (Tris-hydrochloride containing Ca^{2+} and Mg^{2+}) already contain 10µg/ml human Properdin. However, 10µg/ml human Properdin alone was used for purpose of comparison as a positive control on chondroitin sulphate and also as negative control on BSA. Both experiments were dealt in the same time of 1 hour incubation at 37°C. The observation of the competition was the ability of TSP1 to prevent Properdin binding mostly to chondroitin sulphate. That would indicate involvement of TSP1in complement regulation via competing Properdin on the residual binding sites either on bacterial pathogens or activated platelets. The development colour substrate was absorbed at 405nm and all reading values were recorded at same point of time for comparisons. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

Two Way ANOVA (Bonferroni's comparisons test)		
Binding to Chondroitin sulphate (CS)	**Significant? P < 0.05?**	**Mean Diff.**
Properdin/Chondroitin sulfate vs Properdin/BSA	Yes (***)	0.483
Properdin/Chondroitin sulfate vs Properdin+TSP1/ Chondroitin sulfate	Yes (***)	0.409
Properdin+TSP1/ Chondroitin sulfate vs Properdin/BSA	No (NS)	0.074

Table 5.6. The statistical significance results of (Figure 5.15) human Properdin binding to Chondroitin sulfate in the presence or absence of human TSP1 by using Two Way ANOVA (Bonferroni's comparisons test). Significant values = P < 0.05. Key words; (*); significant. NS; Not significant.

According to the statistical analysis (Figure 5.15) (Table 5.6) revealed that the binding of Properdin to CS was significantly observed comparing with binding to BSA (-ve control). Suggesting, Properdin could also participate on binding to activated platelet through not only platelet receptors, previously stated, but also through CS resulting and increasing the augmentation of C3b deposition of alternative pathway on activated platelet surface. However, the binding of Properdin to CS in the presence of TSP1, statistically, was not significant in comparison with BSA. Statistically, by subtraction the Mean Diff of binding Properdin to CS from Mean Diff in the presence of TSP1, it was remarkably resulted almost 80% of preventing properdin binding.

The observation would explain the role of TSP1 in competing Properdin, as a pattern-recognition molecule of AP, resulting down-regulation of complement activation on such activator patterns where expressed on activated platelets such as CS, or on bacterial pathogens like polysaccharides. Suggesting, TSP1 manifests to passively regulate AP through binding to the repeating units of sugar represented either in *N*-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc) by competing Properdin in the same manner as competing MBL on mannan and CS (Figure 5.9A,B).

5.2.4.4. The role of TSP1 in binding to factor B

Factor B is a serine protease cleaved by factor D resulting Bb that stick to C3b on the target surface where to form C3 convertase (C3bBb) which is stabilised by Properdin in order to form C3 convertase (C3bBbP) of AP.
It has been shown that the TSR4 and TSR5 in Properdin are responsible for the C3bBb complex stabilisation (Higgins et al., 1995). That would suggest the capability of other TSR proteins such as TSP1, which are identical or similar to TSR4, TSR5 repeats of properdin, to bind to factor B or stabilise the complex.

Human factor B (fB) (Kindly provided by Professor Robert B Sim, University of Oxford, England) was tested in binding ELISA to TSP1 and Properdin in order to determine the highest binding affinity comparing to each other. 10µg/ml TSP1 and Properdin were coated in 96-well microtitration plate (Maxisorb, Nunc) while serial dilutions of 10µg/ml fB were incubated into the coated molecules at 37ºC for 1 hour in the presence of Ca^{+2} and Mg^{+2}. The binding affinity was determined by the use of 1:1,000 rabbit polyclonal antibody (H-95) (Santa Cruz Biotechnology). The observed results were astonishing by manifesting the ability of fB to bind to TSP1 with significant binding affinity higher than the binding toward Properdin (Figure 5.16,A). That binding affinity was confirmed in competition assay of ELISA by targeting human Properdin using 1:10,000 rabbit polyclonal antibody against human Properdin (kindly provided by Dr. M.A, Youssef, University of Leicester, England). The binding of 10µg/ml human Properdin to 10µg/ml immobilised fB was in comparison with binding two molecules; 10µg/ml TSP1 and Properdin, to immobilised fB. The results observed the discovery of a competitor (TSP1) which is able to bind to immobilised fB and prevent fB from Properdin interaction that nearly led to make Properdin as a miss-functioned molecule in the presence of TSP1. Both the results are in comparison with binding to BSA served as negative control, and also to positive control (Figure 5.16,B).

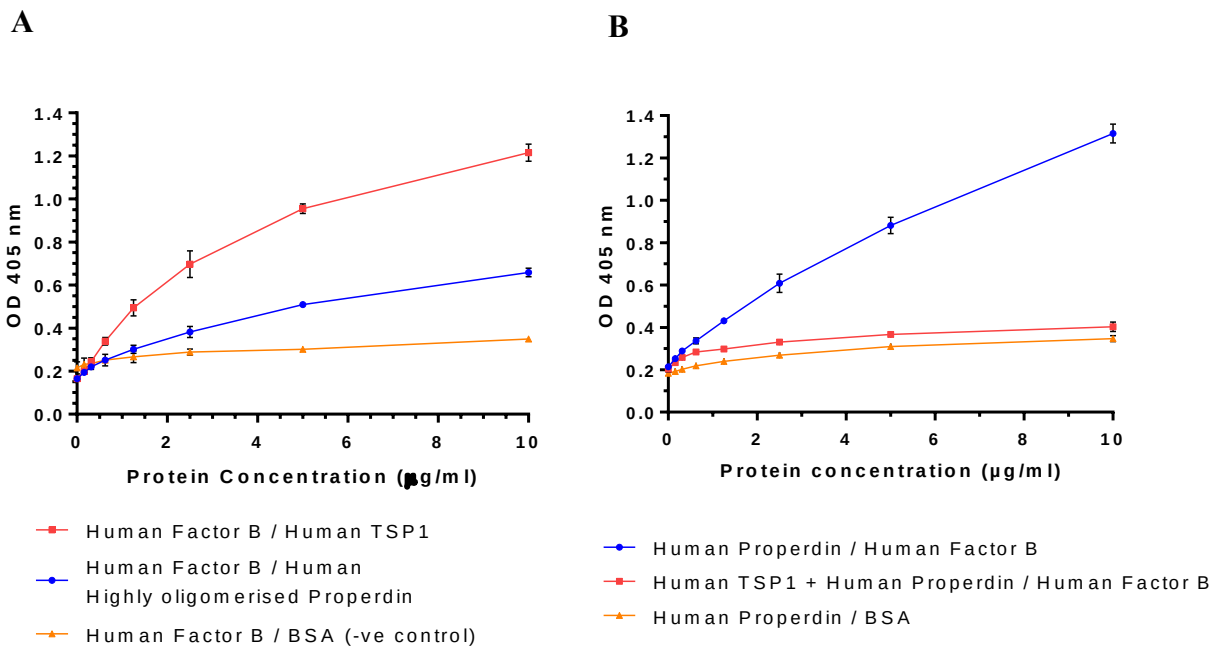

Figure 5.16. Direct and competition ELISA assay for Human factor B (fB) binding to human TSP1, Properdin. (A) 10µg/ml TSP1 and Properdin and also (B) fB were coated in 96 well microtitration plates (Maxisorb, Nunc). (A) 10µg/ml fB was applied onto the coated molecules using physiological buffer (Tris-hydrochloride containing Ca^{2+} and Mg^{2+}). (B) 10µg/ml Properdin with or without TSP1 was applied onto the coated fB. The binding was targeted by (A) 1:1,000 rabbit polyclonal antibody (H-95) (Santa Cruz Biotechnology) while (B) 1:10,000 rabbit polyclonal antibody against human Properdin (kindly provided by Dr. M.A, Youssef, University of Leicester, England). Both experiments were dealt in the same time of 1 hour incubation at 37°C. The observation of the binding affinity of (A) soluble fB was much higher toward immobilised TSP1 than Properdin, whereas, (B) soluble TSP1 was able to prevent soluble Properdin binding to immobilised fB. That would imply an indication of the capability of soluble TSP1 to bind immobilised fB leading to block Properdin's binding sites. Moreover, that would result down-regulation of complement activation. The development colour substrate was absorbed at 405nm and all reading values were recorded at same point of time for comparisons. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

Two Way ANOVA (Bonferroni's multiple comparisons test)			
fB/TSP1 vs fB/BSA	**Mean Diff.**	**Significant? P < 0.05?**	**P value Summary**
10µg/ml	0.866	Yes P< 0.0001	****
5µg/ml	0.653	Yes P< 0.0001	****
2.5µg/ml	0.408	Yes P< 0.0001	****
1.25µg/ml	0.227	Yes P< 0.0001	****
fB/Properdin vs fB/BSA			
10µg/ml	0.309	Yes P< 0.0001	****
5µg/ml	0.207	Yes P< 0.0001	****
2.5µg/ml	0.092	No P> 0.05	NS
1.25µg/ml	0.035	No P> 0.05	NS

Table 5.7. The statistical significance results of (Figure 5.16,A) human factor B binding affinity to either human Properdin and human TSP1 using Two Way ANOVA (Bonferroni's multiple comparisons test). Significant values = P < 0.05. Key words; (*); significant. NS; Not significant.

According to the statistical analysis of binding fB to TSP1 (Table 5.7) revealed that the binding affinity was significantly remarkable by higher up to 50% than binding to Properdin. The latest dilution of fB in the table 5.7 showed no significant binding affinity to Properdin, and vice versa for showing significant differences in binding to TSP1.

Two Way ANOVA		
	Significant? P < 0.05?	**P value Summary**
Properdin/fB vs Properdin/BSA	Yes P= <0.0001	****
Properdin/fB vs Properdin+TSP1/fB	Yes P= <0.0001	****
Properdin+TSP1/fB vs Properdin/BSA	No P= 0.622	NS

Table 5.8 The statistical significance results of (Figure 5.16,B) Properdin binding inhibition assay to factor B with or without an exogenous human TSP1 using Two Way ANOVA (Ordinary). Significant values = P < 0.05. Key words; (*); significant. NS; Not significant.

According to the statistical analysis of binding Properdin to fB with or without TSP1 (Table 5.8), the P value of binding Properdin P<0.0001 was significantly dropped up to P=0.622 to non-significant binding in the presence of equal concentration of TSP1. This result would reveal to confirm the previous results (Figure 5.16,A) which may illustrate the targeted molecules of the complement system by TSP1. Suggesting, TSP1 might prevent the initiation of C3 convertase formation by preventing fB to bind iC3 or C3b. These results would confirm the ability of TSP1 to bind factor B by which the activation of AP is likely targeted through fB. That would indicate the way of platelet to protect itself in case of thrombosis or haemostasis events where TSP1 is perhaps released for protection from complement activation cascade on their surfaces.

5.2.4.5. The role of TSP1 in binding to Complement C3

It has been shown that the TSR5 and TSR4 in Properdin are vital motifs for binding C3b and also for C3 convertase stabilisation of alternative pathway (Higgins et al., 1995). As same as the previous suggestion of possibility the TSP1 to bind to factor B, it also might capable to bind C3(H_2O) by which the C3 convertase formation may be disturbed or even the stabilisation of the complex may be prevented by TSP1. Methylamine Hydrochloride (Sigma) was used with 200mM to convert the human C3 intact molecule into C3(H_2O) through C3 α-chains (Gadjeva et al., 1998, Tack, 1983) of the N-terminal peptide (Osterberg et al., 1989) which is identical to the cleaved C3b active molecule that exposed active glutamyl carboxylate group that is able to covalently binds with hydroxyl or amino groups (Law et al., 1979, Tack et al., 1980, Hostetter et al., 1982, Janatova and Tack, 1981).

The purpose of the experiments is to investigate the binding ability of C3(H_2O) to TSP1 and also to find out whether the binding of TSP1 to C3(H_2O) could disturb or prevent the binding of C3(H_2O) to Properdin using Direct and Indirect (Inhibition assay) ELISA. Therefore, after treating Human C3 molecule (kindly provided by Dr. Hany Kenawy, University of Leicester) with 200mM Methylamine Hydrochloride for overnight at 4°C, the reactive molecule was incubated with the immobilised TSP1 and oligomerised Properdin (Figure 5.17,A). On the other hand, the reactive molecule of C3(H_2O) was incubated with immobilised oligomerised Properdin in the presence of different serial dilutions of soluble TSP1 (Figure 5.17,B). That binding affinity of C3(H_2O) was targeted using 1:5000 rabbit polyclonal anti-human C3c antibody (Dako)

The current results of the binding C3(H_2O) has observed the ability of the soluble TSP1 to bind to soluble C3(H_2O) and prevent the binding of C3(H_2O) to the immobilised Properdin (Figure 5.17,B). Meanwhile, soluble C3(H_2O) was able to bind immobilised TSP1 but with less binding affinity comparing to Properdin (Figure 5.17,A). That would indicate the involvement of TSP1 in complement regulation via C3(H_2O) by which the formation or stabilisation of C3 convertase complex of AP through Properdin is likely prevented which lead to a reduction of C3b deposition.

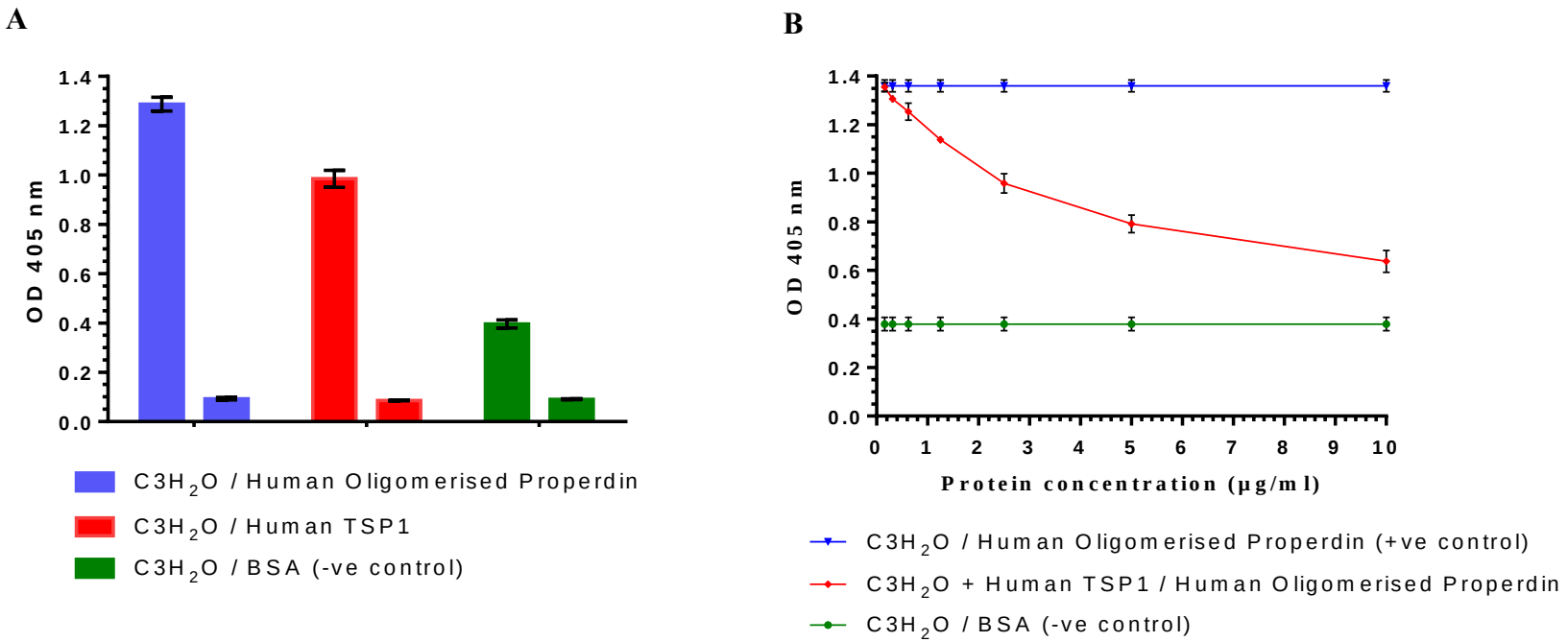

Figure 5.17. (A) Direct binding assay of human C3(H_2O) to oligomerised Properdin and TSP1. (B) Inhibition binding assay of C3(H_2O) to immobilised oligomerised Properdin by TSP1. (A) Purified Human intact C3 (kindly provided by Dr. Hany Kenawy, University of Leicester) was treated by 200mM Methylanmin Hydrochloride (Sigma) for overnight at 4°C to produce C3(H_2O). 10µg/ml TSP1 and oligomerised Properdin were coated in 96 well microtitration plates (Maxisorb, Nunc). After blocking, 10µg/ml of the treated C3(H_2O) was incubated using physiological buffer (Tris-hydrochloride containing Ca^{2+} and Mg^{2+}) on both coated materials including BSA as negative control. (B) 10µg/ml oligomerised Properdin was coated in 96 well microtitration plates (Maxisorb, Nunc). After blocking, different serial dilutions of TSP1 starting from 10µg/ml was incubated in the presence 10µg/ml C3(H_2O) for each dilution. All were incubated on the coated Properdin while only C3(H_2O) used to bind Properdin as positive control and also to bind BSA as negative control. Both experiments were dealt in the same time of 1 hour incubation at 37°C. C3(H_2O) was detected using 1:5000 polyclonal anti-human C3c antibody (Dako). The observation of the binding C3(H_2O) was the ability of soluble TSP1 to bind to soluble C3(H_2O) and prevent the binding of C3(H_2O) to the immobilised Properdin. Meanwhile, soluble C3(H_2O) was able to bind immobilised TSP1 but with less binding affinity comparing to Properdin. That would indicate involvement of TSP1in complement regulation via C3(H_2O) and prevent the formation of C3 convertase complex of AP. The development colour substrate was absorbed at 405nm and all reading values were recorded at same point of time for comparisons. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

Two Way ANOVA (Bonferroni's multiple comparisons test)		
$C3H_2O$ + Human TSP1 / Human Oligomerised Properdin vs $C3H_2O$ / Human Oligomerised Properdin (+ve control)	**Significant? P < 0.05?**	**Mean Diff.**
(10µg/ml) TSP1 concentration	Yes (****)	0.722
(5µg/ml) TSP1 concentration	Yes (****)	0.568
(2.5µg/ml) TSP1 concentration	Yes (****)	0.401
(1.25µg/ml) TSP1 concentration	Yes (***)	0.221
(0.625µg/ml) TSP1 concentration	Yes (*)	0.106
(0.3125µg/ml) TSP1 concentration	No (NS)	0.054

Table 5.9. The statistical significance results of Inhibition binding assay of $C3(H_2O)$ to oligomerised Properdin by TSP1 using Two Way ANOVA (Bonferroni's multiple comparisons test) of comparing between different concentrations of soluble exogenous 10µg/ml TSP1 in single concentration of soluble 10µg/ml human $C3(H_2O)$ and between the only 10µg/ml human $C3(H_2O)$, following incubation on binding to immobilised oligomerised Properdin. P value of all the significant results = P 0.05. Key words; (*); significant. NS; Not significant.

According to the statistical analysis of inhibiting $C3(H_2O)$ to bind to Properdin by serial dilutions of TSP1 (Table 5.9) revealed that the binding affinity of TSP1 to $C3(H_2O)$ was significantly able to prevent the binding of $C3(H_2O)$ to Properdin by comparing each dilution of TSP1 to a single concentration of $C3(H_2O)$. The significant inhibition was downward upon the serial dilutions of TSP1 while about 70% of the binding of $C3(H_2O)$ to Properdin was significantly dropped down comparing to the positive control.

5.2.5 Killing (Bactericidal) assay

The activation of complement system subsequently leads to C3 convertase formation of the central complement cascade resulting in the formation of membrane attack complex (MAC). The potential of bactericidal or killing role on pathogenic invaders is considered to be the ultimate result of the role of the cascade, by forming pillars of C9 supported by the terminal complement complex that anchored into the lipid bilayer of the pathogenic cell wall leading to holes or pores resulting in water influx from outside to inside leading to a certain bacterial lysis (Podack et al., 1982).

20% Human serum was used to evaluate the bactericidal activity of complement system in the presence of two different concentrations; 10µg/ml and 20µg/ml, of human recombinant TSP1 against *S.pneumoniae* strain D39. The bactericidal activity on D39 using normal human serum (NHS) (+ve control) was able to show gradual impact of killing starting from 30 min up to 120 min time point incubation using HBSS containing Ca^{+2},Mg^{+2}, whereas, the impact of NHS on D39 in the presence of 10µg and 20µg/ml TSP1 was not able to show any lytic activity in comparison with positive and negative controls; NHS, human in-activated serum (HIS) with or without TSP1. NHS was heated (HIS) at 60°C for 1-hour in order to destroy the complement system activation so considering as negative control showing no killing activity for D39. The viable pneumococcal count representing as colony formation unit (CFU) was incredibly increased with gradual time point in the presence of 20µg/ml TSP1 incubated with 20% NHS, while, 10µg/ml TSP1 was slightly enhancing pneumococcal growth located between the positive control (NHS) and 20µg/ml. All of which were compared with the negative controls which were neither enhancing pneumococcal growth nor decreasing CFU (Figure 5.18).

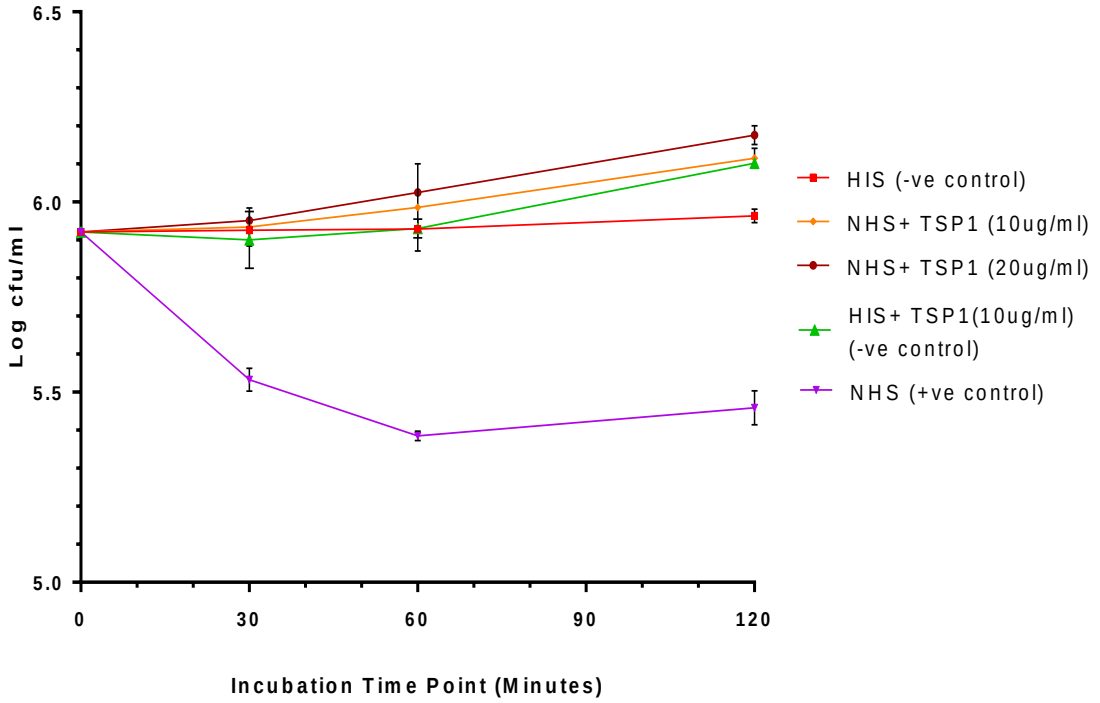

Figure 5.18. Determination the viable count of *S.pneumoniae* D39 strain facing complement activation cascade using 20% Normal Human Serum (NHS) with or without exogenous TSP1 (10µg/ml, 20µg/ml) at 37°C under orbital rotation. Colony Formation Unit (CFU) represent the recovered bacterial cells during the incubation with 20% NHS under certain period time points beginning from 0min, 30min, 60min and 120min. In each time, bacteria were plated into Blood Agar plate and then the plates were incubated at 37°C for overnight in candle jar to provide 5% CO_2. The viable count of bactericidal assay was calculated. Heat Inactivated Human Serum (HIS) with or without 10µg/ml TSP1 used as negative control. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

Two Way ANOVA (Bonferroni's multiple comparisons test)		
NHS + TSP1 (10µg/ml) vs NHS (+ve control)	**Significant? P < 0.05?**	**Mean Diff. CFU**
(Zero minute) Time Point	No (NS)	0.042
(30 minute) Time Point	Yes (****)	0.401
(60 minute) Time Point	Yes (****)	0.600
(120 minute) Time Point	Yes (****)	0.655

Table 5.10. The statistical significance results of Bactericidal assay using Two Way ANOVA (Bonferroni's multiple comparisons test) of comparing between different time points of 20% normal human serum (NHS) (+ve control) and between exogenous 10µg/ml+NHS, following incubation with *S.pneumoniae* (D39). All the significant results=P<0.0001. Key words; (*); significant. NS; Not significant. P value start <0.05

Two Way ANOVA (Bonferroni's multiple comparisons test)		
NHS + TSP1 (10µg/ml) vs HIS (-ve control)	**Significant? P < 0.05?**	**Mean Diff. CFU**
(Zero minute) Time Point	No (NS)	0.007
(30 minute) Time Point	No (NS)	0.008
(60 minute) Time Point	No (NS)	0.057
(120 minute) Time Point	Yes (**)	0.151

Table 5.11. The statistical significance results of Bactericidal assay using Two Way ANOVA (Bonferroni's multiple comparisons test) of comparing between different time points of 20% human inactivated serum (HIS) (-ve control) and between exogenous 10µg/ml+NHS, following incubation with *S.pneumoniae* (D39). All the non-significant results=P 0.4129. Key words; (*); significant. NS; Not significant.

Two Way ANOVA (Bonferroni's multiple comparisons test)		
NHS + TSP1 (20µg/ml) vs NHS (+ve control)	**Significant? P < 0.05?**	**Mean Diff. CFU**
(Zero minute) Time Point	No (NS)	0.035
(30 minute) Time Point	Yes (***)	0.417
(60 minute) Time Point	Yes (****)	0.639
(120 minute) Time Point	Yes (****)	0.717

Table 5.12. The statistical significance results of Bactericidal assay using Two Way ANOVA (Bonferroni's multiple comparisons test) of comparing between different time points of 20% normal human serum (NHS) (+ve control) and between exogenous 20µg/ml+NHS, following incubation with *S.pneumoniae* (D39). All the significant results = P < 0.0001. Key words; (*); significant. NS; Not significant.

According to the statistical analysis of the significance pneumococcal killing result using the bactericidal assay, the P value was calculated with no significant difference P=0.4129 between the use of NHS+10µg/ml TSP1 in comparison with HIS (-ve control) which would reveal the complete inhibition of complement killing assay as the same as human inactivation serum (Table 5.11). Whereas, the P value of the viable count of CFU was significantly increased P<0.0001 starting from 30min until 120min of using NHS (+ve control) in comparison with NHS+10µg/ml TSP1 (Table 5.10). The significance difference in mean of using 20µg/ml TSP1 comparing with 10µg/ml was significantly increased by increasing the viable count of CFU (Table 5.12). Suggesting, the increase of TSP1 concentration would increase the risk of bacterial infection through inhibiting the activation of complement cascade and then create such suitable growth situation by enhancing bacterial colonisation and dissemination to organs through peripheral blood circulation.

However, it has been recently suggested by group of Teizo Fjita that the alternative pathway activation dependent on lectin pathway serine proteases complexes consist of MASP-3 by which the alternative pathway is possibly induced resulting in the formation of C3 convertase through initiating the proteolytic cleavage of C3b bound factor B on the target surface (Iwaki et al., 2011). This hypothesis has been recently manifest-

ed through suggesting the driving of the alternative pathway activation on the surface of *N.meningitidis* has been likely to be through MBL and the complex of serine proteases; MASP-1 and MASP-2 of lectin pathway (Ali et al., 2014). In addition to some contributions of complement pathways for killing bacterial pathogens, Properdin has been demonstrated to enhance the killing of pneumococci and meningococci of pathogens at time point incubation from 60min and 90min through using two different concentrations; 5μg and 10μg/ml, of Properdin (Ali et al., 2014).

Despite the fact that the enhancement manner of AP activation by, either, the positive regulator Properdin of AP or the contribution of the pattern recognition molecules of lectin pathway initially represented by MBL, TSP1 has been found to be initially a remarkable competitor for both MBL and Properdin on target surfaces resulting in a decrease in C3b and C4b deposition levels. Here, in the current study, TSP1 prove the hypothesis of being cross linker from the haemostasis system, where process of platelet activation included, to complement system by functioning as competitor molecule in order to down regulate the activation of both lectin and alternative pathways.

It is strongly suggested through these findings the important role of TSP1 in protecting platelet from the waves of activation of complement cascade which is likelihood inhibited on the surface of certain pathogens associated with platelet activation at site of vessel injury where the pathogens take the opportunity of releasing the most abundant stored protein from α-granules of platelets. Moreover, certain organs like lungs where loads of TSP1 released as part of extracellular matrix layer of tissues which can be infected by certain respiratory pathogens like *S.pneumoniae*.

5.3 Discussion

During the haemostasis process of platelet activation in vascular injury, the complement system plays a crucial role in increasing the activation of platelets, besides the crucial protection role towards any invader microbial pathogens by opsonisation. Therefore, pathogenic microbes are likely and possibly take advantage in such circumstances of haemostasis activation process in vascular injury by camouflaging their cell walls with natural inhibitors of the complement system, being secreted and used by activated platelets. The association of such microorganisms like *S.pneumoniae* with activated platelets or thrombotic events at the site of vessel injury has been classified for microbial pathogenesis as an essential part of colonisation and dissemination (Rennemeier et al., 2007, Sjobring et al., 2002), while the complement system plays an essential role as a first-line defence of innate immunity for pneumococcal infection clearance (Tu et al., 1999, Brown et al., 2002, Yuste et al., 2005). It has been reported that the colonisation of *S.pneumoniae* is associated with both platelets and coagulation activation through TSP1 (Niemann et al., 2009) in vascular injury sites. This suggests other bacterial pathogens might also be supported by TSP1 in thrombotic events. As it is known that TSP1 is an essential initiator/agonist for platelet activation through the TSR, N-, C-terminal domains leading to platelet accumulation by adhering platelets onto ECMs in vascular injury, however, the conserved TSR domain has been found in different proteins that function in different attitudes in different systems. For instance, the TSR domain has been found in properdin, TSP1, TSP2 and parts of the membrane attack complex (MAC) of the complement system (Schwaeble and Reid, 1999b). Moreover, TSP1 was found identical to properdin of the complement system within the TSR repeat (Goundis and Reid, 1988, Robson et al., 1988) covering about 47% of identity (Goundis and Reid, 1988). The function of positive regulator properdin is to serve on stabilising C3b binding on the target surface, besides, enhancing the elevation of C3b deposition via stabilising the C3 convertase (C3bBb) of alternative pathway, so that increasing the half-life of C3 convertase of AP 18-40min (Schwaeble and Reid, 1999a). From the similarity in TSR domain and the function of properdin, it would brought questions about TSP1 regarding the impact of the similarity on complement activation on platelets, and also how to understand the ability of such pathogenic bacteria to take the opportunity of binding TSP1 on their surfaces for colonisation and dissemination, by linking the role of TSP1 in the complement system.

Therefore, the prospective advantages of this study provide and define the evidence for TSP1, where the TSR repeat domains are conserved in similar to properdin, and could play a crucial role in the complement system, being activated on the activated platelet surfaces.

The discussion will consider the following points; first of all, the role of TSP1 on the activation of the LP through screening C4 and C3 deposition levels using human serum, and also the relationship with mannose-binding lectin (MBL) on targeted surfaces in the presence of calcium ions. Secondly, the role of TSP1 in the AP activation will be discussed through screening C3 deposition levels in the absence of calcium ions, and also the relationship with properdin on the targeted surfaces. Lastly but certainly not least, defining the role of TSP1 on pathogenic surfaces like *S.pneumoniae* with respect to resisting complement activation. The discussion will illustrate the activity of human TSP1 regarding complement activation on the surface of activated washed platelets and also pathogenic surfaces.

5.3.1 TSP1 Plays an Important Role of Down-regulating Lectin Pathway (LP) and Alternative Pathway (AP) Activity of the Complement System through Competing Mannose-binding lectin (MBL) and Properdin on their Ligands

The activation of the complement system is classified to be initiated through three distinct pathways; classical pathway (CP), lectin pathway (LP) and alternative pathway (AP). Both CP and AP have been characterised regarding their components and the way of activation while LP still requires configuration due to new components which have recently been involved to the pathway (Thiel, 2007, Degn et al., 2009, Frauenknecht et al., 2013). The cascade of the complement pathways requires pattern recognition molecules for triggering complement activation through binding to microbial surfaces or to apoptotic cell surfaces (Frauenknecht et al., 2013) including activated platelets (Alonzo et al., 2012). These pattern recognition molecules are involved only into CP and LP, while AP is still unknown to have recognition molecules regardless the controversial topic of some researchers, who point out that properdin of AP is considered to be a pattern recognition molecule due to the ability to bind to necrotic, apoptotic cells and pathogenic surfaces (Kemper et al., 2010) while other scientific researchers exclude it from the pattern recognition molecules list of complement system (Ali et al., 2014). This is because recently it has been noticed that the properdin is not involved in initiating AP activation, but rather positively regulating and accelerating the activation cascade called amplification of AP, whereas the activation of AP is hypothesised to occur by supplying the C3b cleavage from activated CP and LP either dependently (Hourcade, 2006) or independently through the spontaneous hydrolysis cleavage of C3 to obtain C3(H_2O) (Schwaeble and Reid, 1999b, Ali et al., 2014). However, mannose-binding lectin (MBL) is one of the pattern recognition molecules of LP that binds through carbohydrates, for instance; the polysaccharides contents of bacterial surfaces (Kemper et al., 2010, Frauenknecht et al., 2013) or even activated platelets, leading to cascade activation. Through the activation cascade of LP, C2 and C4 of complement components are enzymatically cleaved by the serine protease MASP-2 initially to form C3 convertase (C4b2a) that cleaves the C3 intact molecule to form C5 convertase (C4b2aC3b) (Kidmose et al., 2012, Heja et al., 2012). Moreover, the opsonisation or phagocytosis process is initiated through triggering polymorphonuclear cells to engulf the target sur-

faces during complement cascade activation (Ali et al., 2014), and also leading to form MAC ultimately for lysis (McQuillen et al., 1994).

In the events of thrombus formation such as ischaemic stroke and myocardial infraction of cardiovascular disease, platelets adhere to the thrombotic molecules leading to platelets activation by which a coagulation cascade is possibly activated (Verschoor and Langer, 2013). It has been pointed out that in the events of coagulation or thrombosis, the complement system is found to synergise with the thrombotic event, leading to propagation of the coagulation cascade or platelet activation (Li et al., 2012, Verschoor and Langer, 2013). There are some scientific papers interested in screening the involvement of complement components on the surface of activated platelets. It has been found that C4 and C3, as native (inactive) or cleaved molecules (active), attach to the activated platelets while all have not been detected on the intact platelet surfaces (Hamad et al., 2010). In addition, the attachment of CP recognition molecule C1q to the surface of activated platelets has been identified with significant levels in comparison with binding to the intact platelet that showed only background (Hamad et al., 2010). That would refer to the hypothesis that chondroitin sulfate (CS), which is basically a glycosaminoglycan is released from the activated platelets to the fluid phase, and could initiate complement activation (Hamad et al., 2008). Besides, the activated platelet has been shown to expose CS on its surface (Ward and Packham, 1979). However, the natural role of TSP1 basically acts to bind to activated platelets essentially through binding to heparin sulfate or CS (Vischer et al., 1997). On the other hand, the complement components, C1q, C4 and C3 or their derivatives, bind to CS (Hamad et al., 2010) which may trigger complement activation. The current results of determining the role of TSP1 with regard to the complement activation on platelets manifested a new role in the functional activity of exogenous TSP1 in 5% human serum whereby screening the levels of C3b deposition has been significantly reduced on activated platelets in the presence of Ca^{+2} and Mg^{+2} compared with human serum, which served as a positive control using ELISA. Moreover, the impact of the functional activity of TSP1 toward screening C4b deposition on mannan was almost the same as C3b deposition on the activated platelet. That would refer to the CS expression on the platelet surface by which mannan, representing microbial polysaccharide surfaces' structural contents, which hardly differ from CS, representing platelets polysaccharide surfaces' structural contents, that is possibly recognised by MBL and other pattern recognition molecules of LP. The current result of binding human MBL to CS would suggest that the MBL might be responsible for LP

activation on the surface of the activated platelet by which TSP1 competed MBL on residual binding sites, either on CS or mannan. Basically, the binding results of TSP1 may indicate an explanation and observation that TSP1 likely mimics the binding interaction of the pattern recognition molecules; MBL and ficolins, of LP toward polysaccharide ligand residues like mannose and glucose, represented in mannan and zymozan, respectively, and also to the acetylated ligands represented in monosaccharide. That would just confirm the suggested role mentioned previously about competing MBL binding on its residual ligands through strong binding affinity toward the ligands, which possibly lead to blocking the binding sites in the residual polysaccharides, and also ficolins might be involved in the competition by TSP1. That would explain why the screening levels of C4b deposition using ELISA on mannan were incredibly down-regulated by the exogenous TSP1 in 5% human serum, 1:20 (5%) in tris-hydrochloride buffer containing Ca^{+2}, Mg^{+2}. Unlike the C4b deposition assay that depends on the exogenous C4b and the use of MBL binding buffer containing high salt concentration to dissociate and prevent the activity of serine proteases of the CP by introducing only MBL of lectin pathway to bind to mannan, the quantification of C4b deposition assay was assessed by using tris-hydrochloride buffer containing 140mM NaCl, due to the high salt concentration which possibly prevents the interaction of TSP1 as the interaction with heparin dissociated by high salt concentration. Therefore, the regulation of C4b deposition possibly occurred due to the competition of TSP1 with MBL on binding to polysaccharide like mannan, and also with the other pattern recognition molecules like ficolins and collectins of LP. That suggested role of TSP1 in the complement system might down-regulate the complement system *in vivo*, while it has been observed *in vitro* in reducing the formation of C3 convertase complexes and subsequently may lead to less lysis by MAC formation.

This result would point out a meaningful role of TSP1 that might be involved in the protection of activated platelets from opsonisation and phagocytosis through reducing the consumption amounts of C3b deposition as a result of complement activation on activated platelet surface in thrombotic events.

Indeed, activated platelets really do protect themselves from destruction of the complement system as it has been noticed in murine platelets (Barata et al., 2013) by a variety of such complement regulatory molecules, for example, binding factor H (fH) to their surfaces, or even expressing decay-accelerating factor (DAF) or CD55 which disrupted C3 convertase and MAC, respectively (Nicholson-Weller et al., 1985, Morgan, 1992). Basically, it has been observed that some of the complement regulatory molecules such

as fH eventually bind washed activated platelets specifically through the GPIIb/IIIα complex (integrin αIIb/β3) (Mnjoyan et al., 2008), and also bind through TSP1 leading to prevention of the formation of C3 convertase via binding to C3b in order to prevent the binding of factor B (fB) of AP (Vaziri-Sani et al., 2005, Verschoor and Langer, 2013). As a result of binding fH to C3b bound activated platelet surface with or without fB, it performs as a cofactor for factor-I (FI) to accelerate the breakdown of C3 convertase (C3bB) of AP and C3 convertase (C4ba2) of CP and LP into their inactive forms leading to the production of inactive convertases (Weiler et al., 1976, Whaley and Ruddy, 1976, Pangburn et al., 1977, Rodriguez de Cordoba et al., 2004). The current results hypothesised that the TSP1 may function as the same as the role of fH with respect to binding to C3b, through three binding sites (Zipfel, 2001, Hellwage et al., 2002), especially after noticing the capability of the soluble C3(H_2O) to bind to immobilised TSP1, but with affinity less than immobilised properdin, whereas the interaction between soluble C3(H_2O) and immobilised properdin was interrupted and inhibited up to 70% by soluble TSP1 (Figure 5.17) (Table 5.9). Moreover, the soluble fB was observed to bind to immobilised TSP1 in affinity with approximately 2-folds greater and more than the interaction with immobilised properdin, while the binding of soluble properdin to immobilised fB was prevented by the 10μg/ml soluble TSP1 (Figure 5.16). That hypothesis of TSP1 regarding binding to complement C3 convertase of AP may be supported by the fact that the co-factor fH molecule was found to bind to heparin, due to having three heparin binding domains (Zipfel, 2001, Vaziri-Sani et al., 2005) as the same as TSP1.

On the other hand, the function of properdin in the AP of the complement system is to bind and stabilise the C3 and C5 convertase complexes (C3bBb) ($C3_nBb$) on the target surface (Schwaeble and Reid, 1999a). The result of oligomerised properdin in augmenting C3b deposition of AP, *in vitro*, has agreed with Ali et al.'s results by which exogenous properdin has been reported to augment and restore the functional activity of the AP via C3 deposition in serum of properdin knockout mice (Ali et al., 2014). The TSR domain, which is represented in properdin, does not require calcium in order to function in AP, and vice versa regarding binding TSP1 to targets. It is essential to use EGTA for preventing CP and LP in order to distinguish the function of the AP (Fine et al., 1972, Fine, 1974) amongst the other pathways *in vitro*. As the role of EGTA is to consume calcium, it is impossible to determine the role of TSP1 in the AP without the need for

calcium ions. In 1997, it was observed by Scott et al. that the purified TSP1 was contaminated with heparin that was considered to be eluted with TSP1 using 40ml of heparin sepharose affinity chromatography column. Thereby, it has been revealed that the heparin contamination was up to 90% by which the AP was inhibited using a haemolytic assay conditioning by 10mM EGTA (Scott et al., 1997). The role of heparin in complement regulation has been mostly identified before discovering the LP. Complement activation was found to be regulated by heparin via interaction with the C1 complex (C1q, C1r and C1s) (Allan et al., 1979, Fiedel and Gewurz, 1980). Moreover, the regulation of the complement system by heparin found to be contributed with C7, C8 and C9 leading to preventing MAC formation using haemolytic assay (Tschopp and Masson, 1987). In addition to binding heparin to complement components, it has been found that the consumption of C3 has been decreased, preventing the deposition of C3b on the target surface and was also found to prevent the consumption of fB to be cleaved by factor D (fD) (Edens et al., 1993). Therefore, it has been suggested that the way for heparin to regulate the consumption of C3 is through binding to C3b on fB binding sites leading to down-regulating the C3 convertase of AP (Maillet et al., 1983). As a result of using the purified TSP1 by heparin sepharose affinity column containing 1ml of heparin sepharose beads, the AP was not inhibited through screening C3b deposition levels on mannan. The screening was in comparison with human serum w/out exogenous TSP1 which was observed in parallel with human serum while using heparin alone obviously showed a dramatic decrease of C3b deposition levels on mannan in a dose-dependent manner. That would confirm the functional activity of heparin while eventually indicating pure purification of TSP1 from heparin contamination in a level which could not sufficiently effect C3b deposition of AP conditioning by EGTA using ELISA. This suggests that using 1ml heparin sepharose affinity column could be useful to purify TSP1, regardless of the non-effective eluted levels of heparin on the complement system; if so, and then the C3b inhibition levels shall be detected. That would also confirm the necessity for calcium ion for the functional effectivity of TSP1 toward complement regulation through competing pattern recognition molecules of LP.

On the other hand, the current results of determining the role of TSP1 in the AP within the presence of oligomerised properdin, and also conditioning by Ca^{+2},Mg^{+2}, has surprisingly observed the significant ability of TSP1 to reduce C3b deposition on the target surface even in the presence of both equivalent amount concentrations of exogenous molecules; TSP1 and highly active oligomerised properdin, in the use of 1.25% human

serum while properdin was highly active in augmenting C3b deposition in the use of 5% human serum conditioning with EGTA. It would reveal that a postulated function of TSP1 is likely to interfere Properdin interaction with at least one of the AP components like C3b or $C3H_2O$ and fB. fB is a serine protease stick to C3b or $C3H_2O$ on the target surface, by which forming C3 convertase (C3bB) ($C3H_2OB$) requires factor D to cleave fB, resulting in Bb that is stabilised by properdin in order to form C3 convertase (C3bBbP) of AP. It has been identified that the TSR4 and TSR5 in properdin are responsible for the C3bBb complex stabilisation (Higgins et al., 1995, Schwaeble and Reid, 1999b). That would suggest the capability of other TSR proteins such as TSP1, by which the TSR4 and TSR5 located in TSR matched in identical positions to properdin repeats (Goundis et al., 1989), to bind to fB or to the complex.

The current results of identifying the role of TSP1 with regard to binding affinity to fB revealed that TSP1 has the ability to compete or inhibit properdin to react with fB using polyclonal anti-human properdin. In addition, fB has a greater significant level of binding affinity toward TSP1 by twofold more than properdin using monoclonal anti-human fB using ELISA (Figure 5.16). The cross-reaction is possibly processed to be in greater binding affinity than the capability of oligomerised properdin, and also the properdin binding site of fB is likely targeted by TSP1. Moreover, the results have shown the capability of soluble $C3(H_2O)$ to bind directly and significantly to immobilised TSP1, but in less affinity than immobilised properdin, whereas the interaction between soluble $C3(H_2O)$ and immobilised properdin was interrupted and inhibited up to 70% by the 10μg/ml soluble TSP1 (Figure 5.17) (Table 5.9).

That might be because of the fact is around 47% similar identity amongst the TSR repeats of properdin and TSP1 (Goundis and Reid, 1988), and also the interaction might be involved in other domains of TSP1, which require further investigations. However, the augmentation of C3b deposition levels of AP in target surfaces depends on binding affinity toward the active properdin that is also in dimer, trimer and tetramer cyclic forms, reacting to bind with C3bB, but the binding affinity and activity increase with regard to the higher active cyclic forms (Pangburn, 1989, Schwaeble and Reid, 1999b). It has been observed that the interaction of properdin to C3b was 1:1 while in the presence of fB, and the interaction was increased up to fourfold (DiScipio, 1981). This has been explained by observing the affinity of properdin to C3bBb cell bound by showing the enhancement interaction to be greater than C3b alone (Farries et al., 1988b). It has

been demonstrated that the association of C3b to fB was accelerated in the presence of properdin (Hourcade, 2006). That would explain the importance of fB for properdin interaction with C3b in order to initiate C3 convertase of the AP by which the interaction might be disrupted by TSP1 the same as the indication of binding TSP1 toward C3b. The current result of binding TSP1 to fB and C3b would suggest that TSP1 might prevent the initiation of C3 convertase formation by preventing fB to bind iC3 or C3b or preventing properdin to bind the C3 convertase complex of AP which might lead to dissociation and no longer being stabilised into the target surface and leading to decreasing the half-life of C3 convertase of AP. These results would confirm the ability of TSP1 to bind fB and C3(H_2O) by which the activation of the AP is likely targeted through the complex or even for C3b deposition of LP and CP.

Nevertheless, as properdin was considered to be one of the glycoconjugates proteins that bind to carbohydrates through binding to sulphated glycoconjugates through the motif (CPVTCG), which is similar to the amino acid sequence (CSVTCG) of TSR proteins (Holt et al., 1990, Cerami et al., 1992, Tuszynski et al., 1992, Higgins et al., 1995) including properdin and TSP1 (Ginsburg and Roberts, 1988). It has been found that TSP1 binds to glycosaminoglycan (GAG) ligands like where presented in bacterial cell wall as peptidoglycan layer and also presented in the activated platelets by secreting chondroitin sulphate (CS) which contains chains of sulphatide galactose sugar *N*-acetylgalactosamine (GalNAc) (Figure 5.3). Moreover, it has been shown that TSP1 associated with bacterial pathogens through binding host cell and peptidoglycan leads to bacterial adhesion (Rennemeier et al., 2007). Similarly, TSP1 is one of the adhesion molecules that processed platelets for the tethering step with the exposed sub-endothelial layer in haemostasis process. Therefore, the adhesion process of platelets to the sub-endothelial layer probably occurs on through either platelets adhesion receptors or probably through the exposed CS on activated platelet surface. The current study binding properdin to CS has observed a significant binding, while the significance is mostly competed by 80% via equal amounts of TSP1 using ELISA (Figure 5.15). Despite the fact that properdin consists of three repeat motifs having similar amino acid sequences where localised in TSR1 and TSR2 (CSVTCG), and the amino acid sequence (CPVTCG) presented in TSR4 that are namely responsible for interactions with carbohydrates like CS. Also TSR5 has been proposed to have another motif that could be responsible to bind carbohydrates after using polyclonal antibody targeting TSR5, pre-

venting properdin binding to C3 and sulphatides (Perdikoulis et al., 2001) and also the properdin lack of TSR5 was unable to both stabilise C3 convertase and bind to both C3 and sulphatides (Higgins et al., 1995) like CS, although TSP1 consists of two motifs of CSVTCG localised in TSR2 and TSR3. The result of the competition would suggest other domain motifs involved in the interaction to CS beside TSR repeats in TSP1.

The observation would explain the role of TSP1 in competing properdin as a pattern recognition molecule of AP resulting in down-regulation of the complement activation on such activator patterns where represented on activated platelets such as CS, or on bacterial pathogens like polysaccharides. This suggests TSP1 might be a passive regulator for AP through manifesting the binding to either *N*-acetylglucosamine (GlcNAc) or *N*-acetylgalactosamine (GalNAc) by competing properdin in the same manner as competing MBL on mannan or CS, respectively (Figure 5.9A,B).

That would indicate an illustration in the process of platelets manner about manipulating the complement activation in the event of thrombotic lesion, by releasing TSP1 for platelet adhesion and maybe for protection from pattern recognition molecules that trigger complement activation.

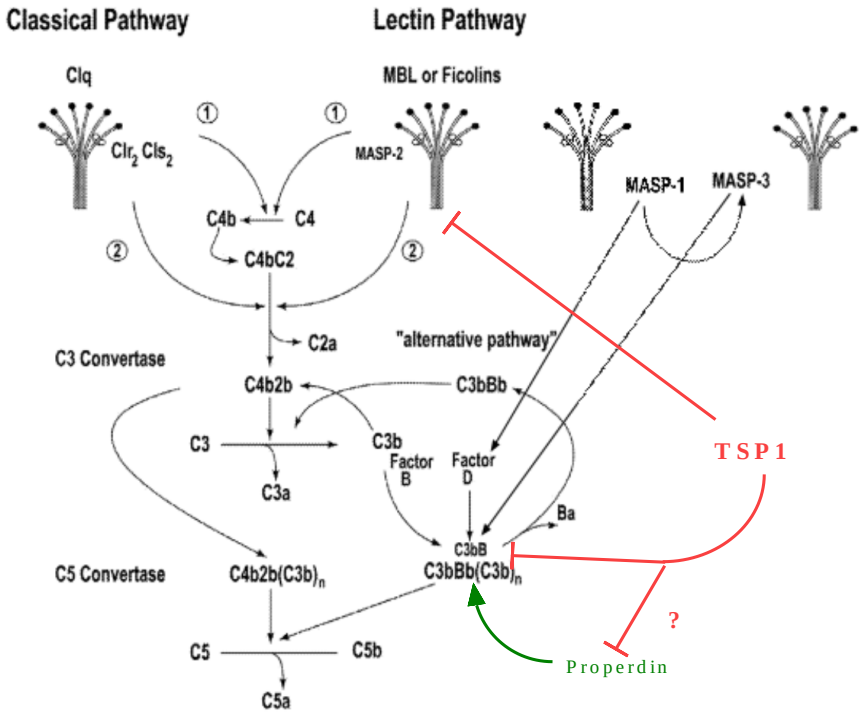

Figure 5.19 A simplified diagram shows the regulation roles of TSP1 in the activation of Lectin pathway and Alternative pathway. Adapted from (Kindly provided by Professor W Schwaeble, University of Leicester, UK.) (Demopulos et al., 2015).

5.3.2 TSP1 Plays an Important Key Role for Bacterial Pathogens by Offering Cover from the Complement System

As mentioned previously, the interaction of TSP1 has been reported to be involved in some microbial mechanical pathogenicity of a variety of microorganisms; gram-positive and gram-negative, depending on the cell wall content of peptidoglycan (PG) (Rennemeier et al., 2007). It has been reported that an indication of TSP1 may play a role of making a bridge between the invader pathogen and the host cell, which can lead to microbial adherence and colonisation (Rennemeier et al., 2007). It has been observed that the exogenous TSP1 enhances and stabilises the association between activated platelets and encapsulated *S.pneumoniae,* which based on a theory that stated by Niemann that is an enhancer molecule might be involved in the microbial adhesion mechanism for colonisation (Niemann et al., 2009). However, PG has been found to be recognised by pattern recognition receptors (PRR) like toll-like receptor 2 (TLR2) and CD41 of polymorphonuclear cells (PMNs) (Yoshimura et al., 1999, Dziarski and Gupta, 2006). Therefore, TSP1 has been found using flow cytometry to interfere with that interaction between PRR of PMNs and PG which is presented in staphylococcus and streptococcus (G+ve bacteria) which may cause impairment interaction leading to protection from phagocytosis by PMNs to the gram-positive bacteria *in vitro* (Rennemeier et al., 2007).

The current results confirmed the binding ability of TSP1 to the residual polysaccharide surfaces such as mannan and zymozan (Figure 5.3), represented on the cell wall of various bacterial pathogens classified under either Gram-positive or Gram-negative, but the capsulation is likely the essential key factor for binding TSP1, while Rennemeier's results point out the interaction as toward the PG (Rennemeier et al., 2007), by ignoring that some G-ve bacteria forming capsulation like *Neisseria meningitidis*. The Gram-positive and Gram-negative bacteria like *S.pneumoniae* D39 strains, *S.aureus* and *N.meningitidis* group A, B, have been observed to bind to TSP1 using ELISA (Figure 5.1).

The pattern recognition molecules; MBL or ficolins, of LP attach to the residual carbohydrate containing *N*-acetyl-D-glucosamine GlcNAc exposed on the cell wall surface of microbes (Roos et al., 2003, Thiel, 2007), besides properdin of AP has been observed to bind to sulphatides of carbohydrates (Higgins et al., 1995) like chondroitin sulphate (CS).

On the other hand, there are many examples of how the invader pathogens could manipulate the complement inhibitors in order to provide a protection for themselves from innate immunity consequences but because of the study focus on certain objectives of how such a protein like TSP1 involved in thrombosis and platelet activation events cross-talk to react in the complement system would be manipulated by pathogenic invaders, rather than studying the role of certain microorganisms of escaping the activation of complement system. However, this would also give an advantage for certain microorganisms to illustrate their pathogenicity for those who interest in working for this field.

Although host cells protect themselves from the self-damage of the complement activation by complement regulatory proteins, e.g. MCP, DAF and factor H etc., (Wallis et al., 2007), fH was found to bind to *N.meningitidis* through factor H-binding protein (fHbp) (Pangburn et al., 2000, Schneider et al., 2006) similar to platelets in the principle of binding (Nicholson-Weller et al., 1985, Morgan, 1992) by eliminating C3b deposition leading, to decay the C3 convertase through binding to C3b, by allowing the binding of factor I to cleave C3b into intact derivatives (iC3b, C3c and C3dg) (Pangburn et al., 2000, Turnberg and Botto, 2003), or binding to fB already bound to C3b, and preventing C3 convertase formation of AP (van Deuren et al., 2000). In addition to such examples of microbes manipulating regulatory inhibitors against the complement system, *S.pneumoniae* binds fH and C3 through the virulence factor pneumococcal surface protein (PspC) which own the defence to accelerate the proteolysis of C3 on its surface, resulting in protection from phagocytosis (Jarva et al., 2002). Moreover, it is known that *S.aureus* recruits factor I through its cell wall protein surface called clumping factor A (ClfA) (Hair et al., 2008, Hair et al., 2010). FH has been recently found to bind the cell wall of *S.aureus* leading to disturb the complex of AP (Sharp and Cunnion, 2011, Sharp et al., 2012).

However, upon the competition results and complement screening functional assays, it is obvious that TSP1 has competed the essential pattern recognition molecules of LP and AP; MBL (Figure 5.9) and properdin (Figure 5.15), respectively, on their binding sites either on the microbial polysaccharide residues or on other molecules like fB (Figure 5.16) and C3(H_2O) (Figure 5.17) resulting in the reduction of C3 convertase formation or disturbing the stabilisation of the complex on the microbial surfaces by which MAC formation may be reduced or prevented. Therefore, it would be revealed that TSP1 is likely to be manipulated by microorganisms through the binding to PG, which

is associated with platelet activation in vascular injury (Niemann et al., 2009), for not only disturbing the phagocytosis process through PG receptors of leukocytes (Rennemeier et al., 2007) but also may be used against complement activation. The complement system has ultimate function defence of lysis microbial pathogens called bactericidal process by MAC formation on the pathogenic invaders. The MAC formation is combined with about 10-16 molecules of polymerised C9 that are only polymerised when the C5bC6C7C8 complex is formed on the pathogenic surface by which C9 forms pore structure causing bacterial lysis (Podack et al., 1982). Thus, the functional activity of TSP1 on reducing the functional activity of the complement system through either C4b or C3b deposition has been set for the test for bactericidal assay using *S.pneumoniae* D39 strain (Figure 5.18). The current result of TSP1 on the bactericidal assay has astonishing impact by which the treated *S.pneumoniae* D39 with exogenous TSP1 in 20% human serum was alive after several incubation points even until 120min of incubation in parallel with the negative control of using the same bacterial strain that is also incubated with 20% heat inactivated serum (HIS), whereas the colony formation unit (CFU) of the bacteria was gradually decreased by time incubation using 20% human serum (positive control) (Figure 5.18). This result has indicated a new unknown role for TSP1 on the surface of capsulated *S.pneumoniae* or bacterial pathogenic invaders on the activation of the complement system.

However, the complement pathways contribute and activate each other for eliminating the pathogens. It has been recently suggested by the group of Teizo Fjita that the AP activation is dependent on lectin pathway serine protease complexes consisting of MASP-3 by which the AP is possibly induced, resulting in the formation of C3 convertase through initiating the proteolytic cleavage of C3b bound factor B on the target surface (Iwaki et al., 2011). This hypothesis has been recently manifested through suggesting the driving of the AP activation on the surface of *N. meningitidis* has likely to be through MBL and the complex of serine proteases; MASP-1 and MASP-2 of the lectin pathway (Ali et al., 2014). In addition to some contributions of complement pathways for killing bacterial pathogens, properdin has been demonstrated to enhance the killing of pneumococcal and meningococcal pathogens at different points of incubation from 60min and 90min through using two different concentrations; 5µg and 10µg/ml, of properdin (Ali et al., 2014).

Despite the fact that the enhancement manner of AP activation by either the positive regulator properdin of AP, or by the contribution of the pattern recognition molecules of LP initially represented by MBL, TSP1 has been found to be a remarkable competitor or inhibitor initially for both MBL and properdin on the target surfaces, resulting in a decrease in C3b and C4b deposition levels and, then leading to pneumococcal survival amongst complement activation. Here, in the current study, TSP1 is likely to prove the hypothesis of being a cross-linker between the haemostasis system, where the process of platelet activation is involved, and between the complement system, by functioning as competitor or inhibitor regulatory protein in order to down-regulate the activation of both lectin and alternative pathways. Therefore, TSP1 might have the advantages of both fluid-phase and membrane-bound inhibitors of controlling complement activation (Walport, 2001) by binding fB or C3-like fH, and also when secreted to bind in return into the target surfaces covered by residual carbohydrates to prevent MBL and properdin attachment, and might also prevent other pattern recognition molecules of L. TSP1 is likely to be a unique soluble and non-soluble competitor of controlling complement activation. That would suggest further investigations in future for studying the relationship of TSP1 with C3b and fI.

There are several questions pointed out during the discovery which require answers. Is TSP1 exposed for all bacterial pathogens or just for those associated with platelet activation in vascular injury sites? Also, would the respiratory tract pathogens like *S. pneumoniae* require TSP1, released as part of the extracellular matrix layer of tissues with loads concentration, to cause colonisation and dissemination from the respiratory tract to the bloodstream, causing bacteraemia under TSP1 protection from innate immunity; the complement system and polymorphonuclear cells (phagocytosis)? Would this unknown role of TSP1 be used for platelet protection in the same manner as protecting bacterial pathogens from bactericidal activity and phagocytosis?

It is strongly suggested through these findings that the importance role of TSP1 is likely to assess in platelet protection from the initiators of the complement cascade activation in the same manner as the inhibition occurring on the pathogenic surface associated with platelet activation, especially when TSP1 increased the association with activated platelets (Niemann et al., 2009) at the site of vessel injuries, where the pathogens take the opportunity of releasing the most abundant stored protein from α-granules of platelets.

As the supposed role for TSP1 is to be a competitor or inhibitor, TSP1 may function to create a deficient condition for some complement components of activating complement cascade, but locally at the site of vessel injuries where an abundance of TSP1 is secreted locally. That supposed deficient condition which may be created by the local TSP1 may increase the susceptible risk of infections like pneumococcal and meningococcal, etc. For instance, it has been noticed that there is an increased risk of pneumococcal infections related to MBL-deficiency (van Kessel et al., 2014). Moreover, MBL deficiency in children has raised the susceptibility of pneumococcal infection; thereby, the carriage of *S. pneumoniae* via nasopharynges was twofold higher for children suffering from MBL-deficiency compared to MBL-sufficiency (Valles et al., 2010, Vuononvirta et al., 2011), whereas, the deficiency of MBL in adults has been reported as being responsible for an increase in pneumococcal disease with more severity in infection (Eisen et al., 2008, Brouwer et al., 2013). Moreover, C2 deficiency in children is also associated with pneumococcal infections (Jonsson et al., 2005). A significant high risk of *S.pneumoniae* has been reported with fB and C3 deficiencies (Brown et al., 2002, Mueller-Ortiz et al., 2004). Polymicrobial peritonitis was also noticed to be associated with properdin-deficient mice models compared with litter-mates of wild-type mice (Stover et al., 2008). However, there is an association tendency with a high risk of meningococcal infection in the deficiency of one of the following; in properdin of AP or the components of the terminal pathway (C5, C6, C7, C8, or C9) (Morgan and Walport, 1991, Fijen et al., 1995, Spath et al., 1999). Therefore, as a result of binding and the competition and the functional assays of bactericidal activity or C3 and C4 deposition assays, TSP1 might prevent the consequences of complement activation, possibly leading to create a suitable condition for pathogenic invaders to survive in such conditions.

However, the current findings observed that TSP1 binds to both Gram-negative and Gram-positive bacteria, and to either capsulated or non-capsulated strains compared with the negative control BSA using ELISA. The current results conflict with Rennemeier's results probably did not show negative control, and also showed the binding of TSP1 to variety of capsulated and non-capsulated G+ve bacteria but not to capsulated G-ve bacteria, with an indication that the peptidoglycan of gram positive bacteria is the targeted ligand for TSP1 (Rennemeier et al., 2007) while the current findings indicated the capsulation of either G+ve and G-ve bacteria in consideration of bacterial colonisation and dissemination.

As a result of binding TSP1 to several pathogens like *S.aureus* (DSM20233) strain represented G+ve bacteria, and *S.pneumoniae* ($_{\text{wild type}}$ D39) represented capsulated G+ve bacteria, while *N.meningitides* (group $_{\text{wild type}}$ A-Z2491, $_{\text{wild type}}$ B-MC58) represented two different serogroups of capsulated G-ve bacteria (Figure 5.1), the current results of TSP1 to these microorganisms (Figure 5.1) agreed and matched the interaction results of Rennemeier et al. (2007).

However, the binding was followed by different strains of *N.meningitides* that the capsulation has been knocked out to represent non-capsulated G-ve bacteria (Figure 5.2). *N.meningitides* group B was genetically modified in the capsulation gene of sialylated lipooligosaccharide (LOS) called siaD $MC58_{\Delta}antS_{\Delta}SiaD$ and $MC58_{\Delta}SiaDcay$, which are unable to express polysaccharide capsule (Unkmeir et al., 2002) in order to illustrate and differentiate the interaction of TSP1 with and without capsule for the same species of the pathogenic microbe. Other strains of *N.meningitides*, e.g. N176.1 and N199.1 were also targeted in the capsulation gene (Oldfield et al., 2013) (Figure 5.2). All of which have observed the binding capability of TSP1 to either G-ve or G+ve bacteria (Figure 5.1) and also to both capsulated and non-capsulated strains of *N.meningitidis* (Figure 5.2). The result would indicate that the capsule might not only the target ligand for TSP1 on *N.meningitidis* and also would suggest other factors like virulence factors which may be involved in or interfere with the interaction of TSP1 on pathogenic surface. However, the binding to the polysaccharide materials represented in mannan, zymozan, chondroitin sulphate (CS) and the *N*-acetylated-BSA would also explain the binding affinity of TSP1 toward its ligands where involved in capsule contents and also on the activated platelet wall (Figure 5.3). Moreover, the binding affinity of TSP1 has been observed to favour the binding toward GAG containing chains of galactose *N*-acetylgalactosamine (GalNAc) that is represented in CS, more than mannan and zymozan which contains polysaccharide chains of sugar mannose and glucose, respectively. Following binding affinity to mannan, binding to *N*-acetylated-BSA was downward and differentiated from other polysaccharides due to maybe representing the monosaccharide ligands of *N*-acetylmuramic acid (MurNAc) (Figure 5.3).

The binding would illustrate how such bacterial pathogens obtain a benefit from the interaction of TSP1 to defeat the first-line defence represented on the complement system of innate immunity, besides providing bacterial colonisation and dissemination, and also might lead to internalisation in endothelial cells, especially when the RGD sequence of fibronectin is found to bind to the integrin receptor $\alpha_5\beta1$ of endothelial cells causing in-

ternalisation for *N.meningitidis* through the Opc protein surface (Unkmeir et al., 2002). This suggests the RGD sequence of TSP1 would require investigation to determine the involvement of TSP1 in the process of meningitis disease.

All in all, the interaction of TSP1 is likely to imply the importance of the molecule to its role as a cross-talker beyond the ECMs grid or the haemostasis/thrombosis process toward the complement system. TSP1 might be exploited by different bacterial pathogens through attaching not only through microbial PG surfaces contents, but also through composition of the capsule which would increase the binding opportunity even if the bacteria are classified as a Gram-negative species. However, that would lead to other considerations about the ability of such virulence factors on the surface of bacterial pathogens to bind to TSP1 against complement activation, suggesting the pathogenic invaders may take advantage of exploiting TSP1 to function against the host line defence as a way of escaping from the innate immune system, and disseminating to organs through the bloodstream.

Chapter 6
(Final Summary)

6. Final Summary

Previous studies have reported the effect of complement components on activated and intact platelets. The interaction of Properdin forms, dimer, trimer, and tetramer, with intact washed platelets has been shown but with no impact was reported. The previous study used native properdin forms purified from human plasma. While, the current study showed the activity of properdin especially the higher oligomerised form on resting platelets. The aim of this thesis was to find out the role of active properdin in platelet activation and interaction with adhesive molecules of ECMs like collagen and the atherogenic particles like LDL in haemostasis/thrombosis process from the point of structural homology in the TSR repeats with TSP1. Furthermore, this thesis aims to investigate the role of TSP1 in complement system from the point of structural homology in the TSR repeats with properdin. The thesis used *in vitro* experimental approaches. The experimental results of this thesis revealed the following:

6.1 Active higher oligomerised properdin cross-talk on triggering platelet activation

In the events of rupture veins or some diseases like atherosclerosis and myocardial infraction and etc., that activate coagulation cascade including platelet activation, and complement system. That activation is as a result of synergism of each other in the inflammatory process by which some platelet secretions like CS, α-thrombin, and some platelet receptors, possibly trigger complement activation. Once complement cascade sufficiently activated, then, the anaphlatoxin shreds like C5a, C3a, C4a, trigger the activation of the inflammatory cells like PMNCs that releases active properdin (but with unknown cyclic form, which has not been shown before) into the inflammatory sites.

It is now noticed that the cross-talk and the interference between complement system and platelet activation through the activity of oligomerised properdin which might be released from inflammatory cells around the vascular injury sites, moreover, and through the released TSP1 from activated platelets.

None of the following properdin forms; dimer, trimer and tetramer, was able to bind to human resting washed platelets but only bind to activated washed platelets. However, oligomerised properdin shows the ability to bind either to intact or activated washed platelets.

The binding activity of properdin to intact platelets would possibly occur through CD42 (GPIb/V/IX); CD42α (GPIbα) and CD42d (GPV), CD41α (integrin-αIIb), CD36 (GPIV, GPIIIb) and CD47/IAP in similar with TSP1. The binding effectivity on resting platelets could trigger platelet activation by exposing the adhesive molecules P-selectin (CD62p) on their membrane surfaces. That activation is likely propagated by enforcing the induction through releasing the stored active α-thrombin from α-granules, even with little concentrations but, that are able to bind in return into PAR-2, PAR-4 receptors of activated platelets. Moreover, the released TSP1 from α-granules stimulates circulating intact platelets or binds in return on activated platelet providing a sufficient adhesion and activation support for preliminary stages on platelets or even for aggregation process in association with Fg.

The activation potency either by TSP1 or oligomerised active properdin seems to sufficiently play a key role in releasing PMPs, which expos TF and most of platelet adhesive receptors. The procoagulant PMPs cause more platelet adhesion and trigger the activation of both extrinsic and intrinsic pathways of the coagulation cascade by forming firm fibrin clot.

The result of human highly oligomerised properdin in the haemostasis/thrombosis system through activating platelets implies the importance function in preventing blood loss. Therefore, the role of highly oligomerised properdin likely goes beyond AP of the complement system through the association with thrombotic events by binding to platelet membrane receptors and transforming platelets to burst their essential thrombotic contents into the vascular injury site.

6.2 Properdin forms cross-talk on interacting with extracellular matrix (ECMs) proteins and the atherogenic particles like LDL, cholesterol and triglycerides

In addition, ECMs proteins are expressed by sub-endothelial cells layer which is an essential part in manipulating platelet adhesion process either by direct contact between platelet receptors and ECMs, or indirectly by engaging the adhesion interaction between the receptors and ECMs via TSP1. The oligomerised active properdin seems to bind to ECMs proteins that exposed to blood stream after injury. The interaction appeared to the most effective and adhesive matrix glycoproteins proteins; Collagen Type-I, vWF and Fn. While, the other forms of properdin like dimer, trimer and tetramer, appeared to

bind to the ECMs but with less gradual binding affinity in serial order upon the size of cyclic forms. That would suggest in future work is to screen the active properdin released from activated neutrophils and other inflammatory cells, to bind to ECMs. It is also suggested that properdin is likely either to serve as an adhesive molecule or manipulated to make a bridge between the exposed matrix and the adhered platelet to strengthen the interaction in the thrombotic events.

Likewise, the binding affinity of properdin forms to Fg gradually increased in serial order upon the size of cyclic forms. That may imply the association of properdin with Fg interaction either to platelets or supporting fibrin polymerisation for strengthen clot formation as the same as TSP1.

The oligomerised form of properdin has been also observed to bind to the atherogenic particles like LDL, triglycerides and cholesterol, by which the high levels in plasma are noticed and implied to cause many diseases like cardiovascular disease (CVD) and atherosclerosis disease after proceeding platelet adhesion.

6.3 Application of recombinant TSP1 down-regulates the complement system through alternative pathway (AP) and lectin pathway (LP)

The activation of complement system by, for example; CS, plays a crucial role of increasing the activation of platelets by such as, C9 which is found to bind to activated platelets and stimulate the production of PMPs. That activation cascade of complement system requires inhibitor regulatory proteins either membrane-bound or fluid phase regulators to control the cascade and prevent attacking self-platelets from opsonisation. The new findings summarised the crucial function of TSP1 to negatively regulate complement cascade activation by binding to C3 and fB of AP component, leading to prevent the stabilisation of the C3-convertase by properdin which then may slow-down the downstream of complement activation cascade. Besides, TSP1 showed competition utility on binding to *N*-acetylgalactosamine (GalNAc) of CS. That competition was manifested only toward the following recognition molecules of complement system; MBL and properdin. The competition resulted in reducing C3 and C4 deposition on the exposed surfaces (activated platelets and residual polysaccharides). Therefore, releasing the abundance amount of TSP1 from α-granules may participate in regulating complement system activation.

That implies the understanding of platelet behaviour in such thrombotic events by regulating complement activation and, in meanwhile, utilising some of complement components for supporting the activation in injury sites.

The released TSP1 has been noticed over many previous reports that could be used as an adhesive protein for microbial pathogens like *S.aureus, S.pneumoniae and N.meningitidis* by binding to the polysaccharide capsule or to peptidoglycan layer in G+ve bacteria. That could help microorganisms in colonisation and dissemination. Besides, with the new findings of TSP1 in complement regulation, the pathogenic bacteria that associated with platelet activation may take the opportunity of releasing abundance amounts of TSP1 to use it against complement system in injury sites. The protection of TSP1 for microbial pathogens from the consequences of complement cascade resulting MAC formation may be occurred especially on *S.pneumoniae* (D39 wild-type) using killing assay. Therefore, the cross-talk is likely exploited by a variety of microbial pathogens for colonisation, dissemination and also protection from the complement system consequences like opsonisation, phagocytosis.

6.4 Final conclusion

In conclusion, the thesis shows for the first time the significance of both TSP1 and active properdin in complement down-regulation and platelet activation process, respectively. That would indicate the co-operation between the two systems in order to stop bleeding and in meanwhile down-regulate the complement activation.

Moreover, the two components may involve into synergism and antagonism process between haemostasis and complement system. That may promote wound healing, prevent blood loss and, therefore, may cooperate in such uncontrolled events as atherosclerosis disease, resulting in thrombus formation and leading to myocardial infarction. Our understanding of the interaction between platelet and complement system is expanding.

Chapter 7
(Appendices)

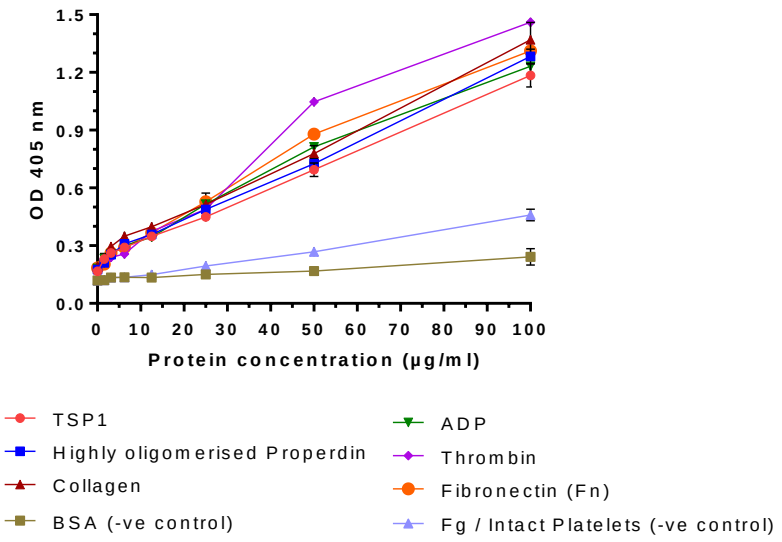

Figure 7.1. Under using different agonists, activated platelets bind to Fg. TSP1, oligomerised Properdin, Collagen, Thrombin, Fn, and ADP were used to maintain platelet activation under static condition while washed platelet used as negative control without adding agonist into it. Platelet was then coated in 96-well plate as previously described. Serial dilutions of Fg starting from 100µg/ml in TBS buffer. Mouse mAb for human-Fg (Santa cruze Biotechnology) was used in 1:5000. This result would indicate involvement of oligomerised Properdin in platelet activation. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

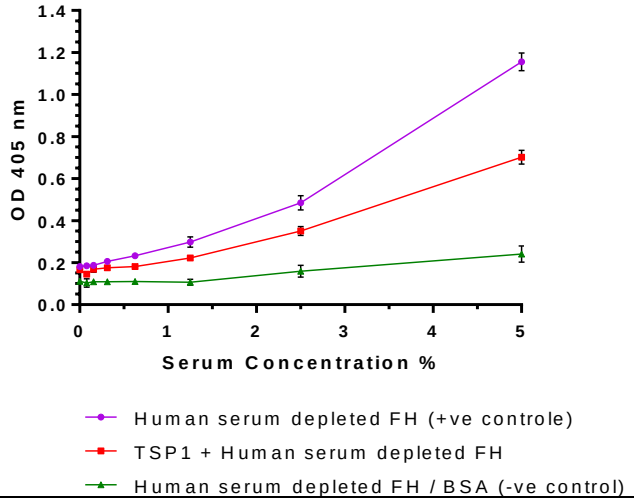

Figure 7.2. Quantifying C3b deposition levels of complement activation on Mannan in the presence of TSP1 using ELISA based assay. Mannan coated in 96-well micro-titration plate. Barbital buffer saline (BBS) containing (Ca^{2+},Mg^{2+}) was used to serially dilute human serum depleted factor-H 1:20(5%) (kindly provided by Dr. Robert B. Sim, Oxford University) pre-incubated with or without TSP1 at range of concentrations from 10-0µg/ml. The C3 deposition was detected using anti-human C3c antibody. The inhibition of C3b deposition was certainly observed in the use of exogenous TSP1 in comparison with human serum depleted factor H served as positive control while the negative control served to observe the amount of exposing C3b deposition on BSA. This result would indicate involvement of TSP1 in the complement system regulation. The experiment was repeated three times and the result was represented in duplicate value. The relative values of all results are represented as mean±SEM.

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