The role of mannose binding lectin associated serine protease MASP-3 in complement mediated haemolysis and the utility of recombinant properdin in fighting *Streptococcus pneumoniae* infection

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Statement of originality

This accompanying thesis submitted for the degree of PhD entitled (the role of mannose binding lectin associated serine protease MASP-3 in complement mediated haemolysis and the utility of recombinant properdin in fighting *Streptococcus pneumoniae* infection) is based on work conducted by the author at the University of Leicester mainly during the period between April 2011 and April 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

Signed: **Date:**

Abstract

The role of mannose binding lectin associated serine protease MASP-3 in complement mediated haemolysis and the utility of recombinant properdin in fighting *Streptococcus pneumoniae* infection

Bayad Mawlood Saeed

Complement is a part of our innate immune defence system and plays an important role in fighting infection and maintaining the integrity of our body. Complement activation is tightly regulated through complement regulators, which allow their activation on foreign cells, like pathogens and stop their activation on self-cells. In states that complement activation causes damage to the self-cells (unwanted complement activation on self-cell) that arises due to deficiency of complement regulators, down regulating complement system is essential. In this condition, targeting specific pathway of complement system that mediates the pathology and allowing other pathways functioning is important. For example, results in this thesis show complement mediated haemolytic activity in human, mouse and chicken is mediated by the alternative pathway and, MASP-3 deficiency in human and mouse abolishes their haemolytic activity, thus targeting MASP-3 by monoclonal antibody may be of therapeutic value in the treatment of the AP mediated diseases e.g. Paroxysmal Nocturnal Haemoglobinuria. By this, the functions of both classical and lectin pathways of the complement system in these patients will be preserved. On the other hand, it is of therapeutic value to enhance complement activation in a state that complement activation is necessary for promoting their immune functions like on pathogen surfaces. For example, results of this thesis demonstrate that enhancing complement functional activity through the addition of small quantities of highly active recombinant properdin (Pn) provides significant therapeutic benefits in models of *S. pneumoniae* infection.

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Table of contents

List of tables

List of figures

List of abbreviations

aHUS	atypical Haemolytic Uraemic Syndrome
AP	Alternative Pathway
BBS	Barbital Buffered Saline
BHI	Brain Heart Infusion
BSA	Bovine serum Albumin
C1-INH	C1-inhibitor
C4bp	C4 Binding Protein
CCP	Complement Control Protein
CFU	Colony Forming Unit
CL-11	Collectin 11
CL-K1	Collectin Kidney 1
CP	Classical Pathway
CR	Complement Receptor
CRD	Carbohydrate Recognition Domain
DAF	Decay Accelerating Factor
DDD	Dense Deposit Disease
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside Triphosphate
EGTA	Ethyleneglycoltetraacetic acid
fB	Factor B
fD	Factor D

fH	Factor H
FHL-1	Factor H Like Protein 1
fI	Factor I
FL MASP-3	Full-length MASP-3
GPI	Glycosylphosphatidylinositol
GPI-AP	glycosylphosphatidylinositol Anchored Protein
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IP	Intraperitoneally
kDa	Kilodalton
LDH	lactate dehydrogenase
LEA-1	Lectin pathway Effector Arm 1
LEA-2	Lectin pathway Effector Arm 2
LP	Lectin Pathway
MAC	Membrane Attack Complex
MAp19	19 kDa Mannose Binding Lectin Associated Protein
MAp44	44 kDa Mannose Binding Lectin Associated Protein
MASP	Mannose binding lectin associated serine protease
MBL	Mannose Binding Lectin
mProperdin	Murine Properdin
NHS	Normal Human Serum

NO	Nitric Oxide
OD	Optical Density
OMS721	Anti-human MASP-1 antibody
PBS	Phosphate Buffered Saline
Pn	Recombinant Properdin
PNH	Paroxysmal Nocturnal Haemoglobinuria
PNH-sc	Subclinical Paroxysmal Nocturnal Haemoglobinuria
PspA	Pneumococcal Surface Protein A
PspC	Pneumococcal Surface Protein C
RBCs	Red Blood Cells
SGMI-1	Anti-human MASP-1 antibody
SGMI-2	Anti-human MASP-2 antibody
TBS	Tris Buffered Saline
TCC	Terminal Complement Complex
tr MASP-3	Truncated MASP-3
v/v	Volume/volume
w/v	Weight/volume
WTS	Wild type serum

Chapter one: Introduction

1.1 Immune system

Immunity is a state in which a multicellular organism has sufficient biological defences to avoid infection, disease, and can discard apoptotic, malignant or necrotic cells from its body. The immune system is composed of two interconnecting networks, the innate immune system and the adaptive immune system.

The innate immune system, which is phylogenetically older, provides immediate immune actions (Hoffman *et al*., 1999), leading to direct destruction or isolation of invading pathogens. This immunity is composed of anatomical barriers (skin, nasal mucosa, and urogenital and intestinal epithelium); antimicrobial peptides and enzymes in body fluids and a large number of germ-lined encoded receptors and fluid phase recognition molecules binding to various components of pathogen associated molecular patterns (PAMPs) (St John and Abraham, 2013; Kawai and Akira, 2010). What discriminates the innate immune system from the adaptive immune system is the lack of memory functions for specific antigens, which means that the innate immune response does not increase its specificity in subsequent and repeated infections with the same pathogen. Central components of innate immunity are neutrophils, macrophages and the complement system (Silva, 2010). Phagocytosis of cellular debris, dead cells and opsonised bacteria is mediated through neutrophils and macrophages. In addition to enhancing this phagocytosis, the complement system can also directly destroy pathogens.

In contrast to the innate immune response, the adaptive immunity mounts antigen-specific immune responses. This adaptive immune response involves a complex network of highly specialised immune cells, which are often triggered

to respond to pathogens by alarm signals or danger signals provided by the innate immune system. This acquired immunity adapts to specifically recognise novel pathogen associated antigens through either cell surface resident adaptive receptors or through specific immunoglobulins, i.e. antibodies secreted into plasma or the lymphatic system by antibody producing cells. This adaptive response is made possible through a complex selection process involving a complex network of highly specialised immune cells equipped with receptors that allow them to communicate with each other. These immune cells included antigen presenting cells that take up the antigen through phagocytosis, split it into fragments and present these antigen fragments in conjunction with their own histocompatibility complex to receptors on T cells, i.e. white cells of the haematopoietic system and trigger activation on those T cells which are recognising the antigen in context with the presented histocompatibility complex. Another highly specialised type of immune cells, the B cell is later responsible for the production of antibodies and is characterised through the presentation of specific B cell receptors on its surface. These B cell receptors are transmembrane anchored-immunoglobulins which differ from cell to cell in their antigen binding interphase, a process made possible through a genetic process based on hypermutations to encode highly variable regions within the immunoglobulin genes (Schatz *et al.,* 1992). The same genetic mechanism of hypermutation has been described in genes encoding T cell receptors.

During ontogenesis, the adaptive immune system learns to tolerate self-antigens in order to prevent autoimmune disease. This is achieved by driving all those T cell or B cell clones that recognise self-antigens during the embryonic development up until the early post-natal phase into apoptosis (i.e. cell death) to prevent auto-reactivity against self-antigens. After the first post natal phase,

the phase where the immune system induces tolerance against self-antigens is over and instead of driving reactive T or B cells into apoptosis, antigen recognising cell clones are driven to divide and multiply. The better the surface receptors of these cells bind either the antigen alone or the antigen presented within the histocompatibility complex, the stronger the signal of their T cell or B cell receptors trigger clonal expansion. This leads to a positive selection process, which favours expansion of those immunocytes that most effectively recognise the antigen.

This highly simplified description of the principle organisation of adaptive immune response involves highly complex regulatory mechanisms to maintain the homeostatic balance between immune responsiveness to protect the body against infection and the prevention of hyper-responsiveness leading to autoimmune disease. Since the primary adaptive immune response requires the involvement of so many different effector cells and the time to allow clonal expansion processes for reactive immunocytes to multiply and differentiate from dormant precursor cells into effector cells, the development of an effective adaptive immune response is not fast and can take up to a week, the main work to fight of infections and kill pathogens in the naïve host is carried by the innate immune system. Since cells of the adaptive immune response, once gone through clonal expansion, maintain in the body for decades, the adaptive immune response is much faster responding to the same or a similar pathogen upon secondary infection, i.e. the immune memory of the cell based adaptive immune response provides a highly effective and highly specific responsiveness. Again, activation products generated by the innate immune system as a primary line of the immune defence promote and synergistically support the development and maintenance of adaptive immunity.

The production and release of antigen specific immunoglobulins by the B cell system of adaptive immunity generates a highly effective humoral immune response where antibodies specifically bind to pathogens or debris to mediate phagocytosis and depletion by the reticuloendothelial system, or lysis and opsonisation through classical pathway or lectin pathway mediated complement activation or preventing toxicity by binding and masking toxins released. The cellular effector system of the adaptive immune response generates cytotoxic cell that specifically respond to antigen, such as viral peptides or aberrant surface structures to kill and eliminate virally infected cells or cells that are transformed or malignant in order to fight the development of cancers (Goldsby *et al.*, 2003; Medzhitov, 2007; Oshiumi *et al.*, 2003).

Like the innate immune system, also the adaptive immune system is tightly regulated through arrays of immune regulators in order to limit its functions towards pathogens and abnormal cells. Deficiency of any of these regulators usually leads to arising autoimmune disease.

1.1.1 Complement system

Complement system is a far-reaching chief component of the humoral and cellular immune system and represents one of the main effector mechanisms of innate immunity (Reid, 1983). It evokes highly efficient inflammatory responses to pathogens including viruses, bacteria and parasites, neoplastic insults, damaged tissues and other non-self surfaces. In 1896, Jules Bordet described complement for the first time as a component of normal plasma which possesses bactericidal activity (Bordet, 1898; Ehrnthaller *et al.*, 2011). He described that this bactericidal activity is heat liable and can be destroyed by incubating serum for 30 minutes at 56°C. From the beginning of its discovery,

scientist thought the role of complement system is confined to innate immunity with no role in adaptive immune responses. With the advance in science, the role of the complement system within the adaptive immune response has been redefined demonstrating that it forms the bridge between the two internetworking systems of immunity (Carroll, 2004a; Dunkelberger and Song, 2010**).**

The complement system consists of more than 35 components including circulating proteins, receptors and arrays of positive and negative regulatory proteins. Approximately 5-15% of the total protein content in human plasma is made up of soluble complement proteins (Brouwer *et al.,* 2006; Dunkelberger and Song, 2010). Under normal conditions, complement proteins are present in their inactive zymogen form (proenzymatic state). These proteins are arranged into the order of proteolytic cascades, and activation of these cascades is initiated by recognition of pathogen associated surface structures through specific recognition subcomponents.

Complement activation leads to the release of potent anaphylatoxins, the coating of the surface of pathogens (opsonisation) with different complement activation products (e.g. C3b) and finally to the lysis of some pathogens and foreign cells through membrane attack complex (Walport, 2001; Dunkelberger and Song, 2010). Thus, the most prominent physiological functions of complement are opsonisation, chemotaxis, lysis (osmolysis of the membrane of foreign cells), agglutination of antigen–bearing agents and facilitation of the uptake of dead or modified host cells (such as apoptotic cells and cellular debris) by phagocytes.

Most but by far not all complement components are synthesized in the liver. There are three activation pathways that drive complement activation: the classical pathway, the alternative pathway, and the lectin pathway (see figure 1.1) (Fujita, 2002; Schwaeble *et al*., 2002). All three pathways participate in the generation of C3 convertase complexes, which cleave the abundant plasma component C3. Cleavage of C3 by C3 convertases releases a small 9kDs fragment of the alpha-chain of C3 (i.e. the anaphylatoxin C3a) and the major activation product C3b which contains a reactive thioester bond that is activated during cleavage and allows C3b to covalently bind to hydroxyl groups or nucleophilic surface structures present on activator surfaces. The ability of C3b to covalently bind to surfaces upon C3 cleavage is a central characteristic feature of complement activation. The binding of C3b to the surface of invading microorganism initiates the formation of alternative pathway convertase complexes on the pathogen or activator surface leads to further complement activation which finally results in the release of potent proinflammatory components and the formation of membrane attack complex through terminal cascade activation (Walport, 2001).

Activation of the classical pathway is mainly antibody driven initiated through the binding of the globular heads of C1q with the Fc regions of immune complex-bound antibodies, while initiation of the lectin pathway activation depends on carbohydrate recognition processes mediated by lectin pathway specific carbohydrate recognition molecules. In contrast and until now, the initiation events of alternative pathway activation were thought to be independent of specific pattern recognition molecules or antibodies. The prevailing past and present view is that the alternative pathway activation route is initiated through spontaneous hydrolysis of C3 which can then bind to the alternative pathway zymogen factor B for factor B to be subsequently

cleaved by factor D to form the alternative pathway C3 convertase, C3bBb, to cleave more C3 in a self-feeding cycle of amplification. Activation is held in check by an array of regulatory fluid phase components, which controls a continuous balance between spontaneous low grade activation and negative regulation through potent down-regulatory components. In addition to fluid phase resident complement control proteins in blood plasma and other body fluids, all host cell surfaces are equipped with surface bound negative regulators and complement receptors to avoid cytotoxicity through autologous complement attack. Deficiencies of complement regulatory components on the surface of host cells can render these cells susceptible to autologous complement attack and lysis leading to serious pathologies.

It is widely accepted that the alternative pathway provides a physiologically relevant amplification loop to also enhance complement activation initiated by either the classical or the lectin pathway (Lachmann, 2009).

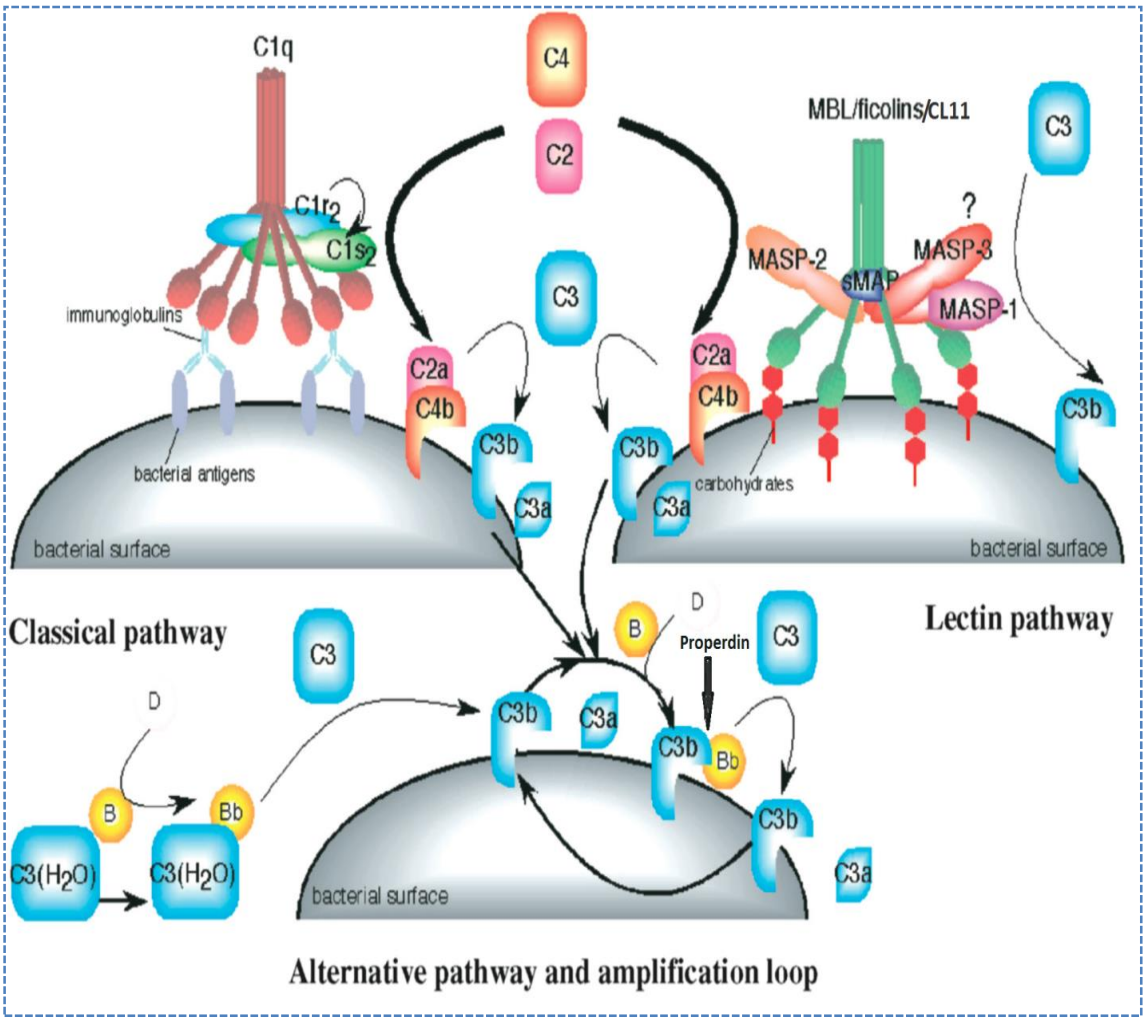

Figure 1.1 : An overview of the complement activation
A schematic presentation of different complement pathways activation (the classical pathway, the lectin pathway and the alternative pathway) (modified from Fujita, 2002).

1.1.1.1 The classical pathway

The classical pathway initiates complement activation mainly through the binding of the classical pathway recognition subcomponent C1q to antigen-antibody complexes. This is very much in line with Paul Ehrlich's historical statement that complement activation provides cytotoxic activity to antibodies (which Paul Ehrlich called Amboceptors) (Ehrlich *et al.*, 1899). The C1 complex has a total molecular weight of approximately 790 kDa and is composed of the

18 chain C1q recognition subcomponents and the C1r/C1s heterotetramer C1s:C1r:C1r:C1s (Gaboriaud, 2004) (see figure 1.2). The recognition subcomponent, C1q, binds to Fc regions of the antibodies of the IgM and IgG class (IgG1, IgG2, IgG3, but not IgG4) within immune complexes on microbial, necrotic, infected and apoptotic cells (Phillips *et al.,* 2009; Schwaeble *et al.,* 2002; Thiel *et al.,* 2000). C1q has also been reported to activate the classical pathway through binding directly to bacterial lipopolysaccharides, apoptotic cells, C-reactive protein, viral envelop and poly anionic compounds (Navratil *et al.,* 2001).

C1q binding facilitates the auto-activation of zymogen C1r in the centre of the C1s:C1r:C1r:C1s heterotetramer placed in the inside of the C1 complex. Activated C1r in turn activates the C1s proenzyme and C1s subsequently cleaves C4 into C4a and C4b (Kusumoto *et al.,* 1998; Arlaud *et al.,* 2002). The smaller particle (C4a) will be released into the plasma and act as a mild anaphylatoxin, while the larger particle (C4b) will bind to the microbial or targeted cell surface (Wallis *et al.,* 2007). Subsequently, the complement component C2 will bind to C4b on activating surfaces and it will become substrate of a second C1s mediated activation event cleaving C4b-bound C2 into a smaller cleavage product (C2b) and the larger cleavage fragment C2a which remains bound to the C4b fragment to form the classical pathway C3 convertase, C4bC2a. This convertase complex subsequently cleaves the abundant complement component C3 into the small (9kDa) cleavage fragment C3a and the large fragment C3b. C3a is a complement anaphylatoxin which recruits inflammatory cells to the location of complement activation, while C3b covalently binds to the activator surface via its reactive thioester and acts as an opsonin for C3b coated pathogen surfaced rendering the C3b coated cells. C3b coated bacteria or microbial debris are susceptible to phagocytosis and

elimination through the reticuloendothelial system. Several molecules of C3b will also bind in close proximity of the formed C3 convertase. The association of several C3b fragments in close proximity of the C3 convertase complex, C4bC2a, changes the substrate specificity of the convertase from cleaving C3 to cleaving C5 to generate the classical pathway C5-convertase, (C4b2a(C3b)n, (Gros *et al.,* 2008; Pangburn and Rrawal, 2002). This C5 convertase cleaves the complement component C5 to the small fragment (C5a), a very potent anaphylatoxin and the large fragment (C5b). The larger fragment (C5b) then recruits and assembles C6, C7, C8 and multiple C9 molecules to form the membrane attack complex (MAC) (Müller-Eberhard, 1986).

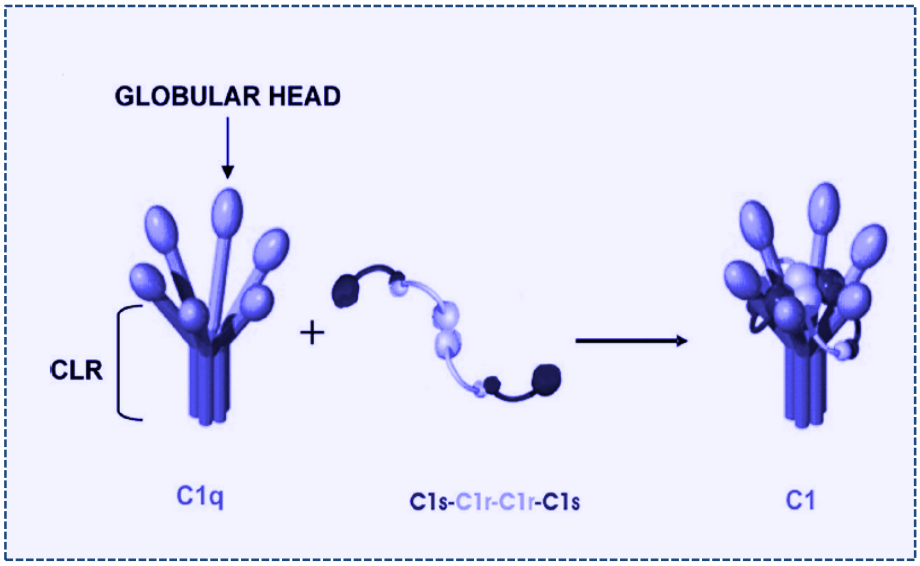

Figure 1.2: Structure of the classical pathway C1 component
The figure shows the structure of the collagen-like region (CLR) and globular heads of C1q and heterotetramers of C1r and C1s (Pflier *et al.,* 2010).

1.1.1.2 Membrane attack complex (MAC)

Complement pathway mediates its lytic function through the formation of the MAC, which is composed of five complement components, C5b, C6, C7, C8 and C9 (see figure 1.3) (Podack *et al.,* 1976). Formation of the MAC is initiated by C5b, which subsequently binds a single molecule of the complement component C6. Then a single molecule of C7 binds to the C5b6 complex and this binding leads to conformational changes that enable binding of the C7 and forms C5b67. Complement component C8 which is composed of C8β and C8*α-y* binds to the formed C5b67 complex. C8β chain binds to the C5b67 complex while the C8*α-y* chain, which is hydrophobic, inserts into the lipid bilayers of the cell wall and leads to the formation of C9 polymers which composed of 10-16 subunits (Bubeck, 2014; Tschopp, 1984). Like C8*y*, the C9 polymers will also insert into the cell wall and form a pore through the lipid bilayers that may lead to lysis of the MAC-targeted cell. Insertion of the C9 polymers through which the cell wall permits excessive intracellular Ca^{+2} influx of water to inside the cell which consequently disturbs electrolyte balance causes osmotic instability resulting in cell lysis and death (Podack *et al.,* 1982). This terminal activation sequence from C5b to C9 does not involve any enzymatic activation steps as long as any of the three activation pathways provide the C5b activation product (DiScipio *et al.,* 1983).

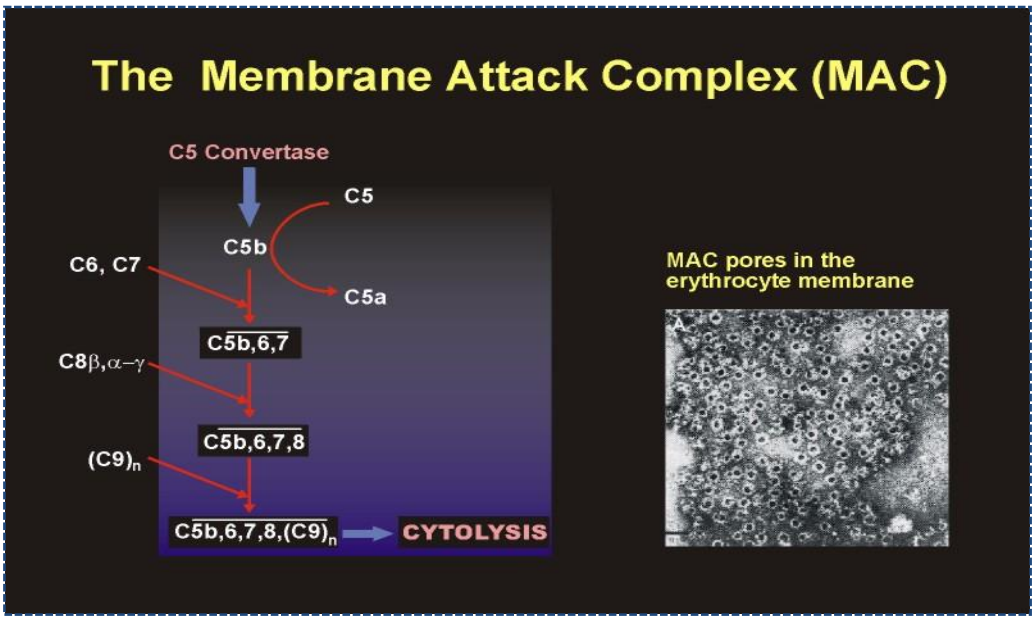

Figure 1.3: The terminal pathway of complement
Diagram of the terminal activation sequence leading to the formation of the membrane attack complex (left), holes in the erythrocyte membrane formed by membrane attack complex (right). (*http://www.complement-genetics.uni-mainz.de*).

1.1.1.3 The lectin pathway

The lectin pathway (LP) and the alternative activation pathway of complement are phylogenetically represented at the roots of the evolutionary tree, while the classical pathway activation root co-evolved with the emergence of immunoglobulins at the evolutionary stage of cartilaginous fish (Dodds, 2002). The lectin pathway initiation complexes somehow show close resemblance to the architecture of C1 complex of the classical pathway. Since constantly improving images of the classical pathway initiation complex C1 with the recognition subcomponent C1q and the associated serine proteases C1r and C1s have been depicted in textbooks for decades, there has been a trend to module the architecture of the lectin pathway activation complexes on the known structure of the C1 complex, especially since the lectin pathway activation complexes are also composed of multimolecular recognition subcomponents

forming dimeric, trimeric, tetrameric, pentameric and hexameric complexes of homotrimeric subunits and associated lectin pathway specific seine proteases (Wallis, 2007).

The recognition molecules of the LP are Mannan-binding lectin (MBL), ficolins and collectin 11 (CL-11) (Ali *et al.*, 2012; Hansen *et al.*, 2010; Schwaeble *et al.*, 2002). Interactions of these recognition molecules with microbial surface structures or altered self-components initiate the activation of the LP through three different MBL-associated serine proteases (MASPs, i.e. MASP-1, MASP-2 and MASP-3) which form complexes with them (Schwaeble *et al.*, 2002). In addition, both the *MASP2* and the *MASP1 gen*e encode truncated non enzymatic gene products, termed MAp19 and MAp44, (MAp19 is a truncated gene of the *MASP2* gene of approximately 19kDa, MAp44 a gene product of the *MASP1* gene of approximately 44kDa). These gene products are encoded by mRNAs generated by alternative splicing of the heterologous nuclear RNA transcripts of these genes and both MAp19 and MAp44 are binding to LP complexes and considered to down regulate the LP activation by competing with MASPs` for the binding sites to the LP recognition subcomponents (Degn *et al.*, 2009; Schwaeble *et al.*, 2002; Skjoedt *et al.*, 2010; Wallis, 2007).

MASP-2 is a key component of the LP as the deficiency of MASP-2 makes it impossible for the lectin pathway C3 convertase, C4bC2a, or the C5 convertase C4bC2a(C3b)n to form (Schwaeble *et al.*, 2011). Similar to activated C1s, activated MASP-2 acts on C4 and cleaves it into two segments, large segment C4b and small segment C4a. It also cleaves C2 to large segment C2a and small segment C2b. C2a binds to C4b to form C3 convertase (C4b2a) which in turn attaches to microbial surface. C3 convertase cleaves C3 to large fragment (C3b) and small fragment (C3a). C3b binds to formed C3 convertase and lead to formation of a C5 convertase, that cleaves C5 to large fragment C5b and small

fragment C5a. C5b will initiate the formation of membrane attack complex (MAC) which can mediate cell lysis (Schwaeble *et al.,* 2011).

1.1.1.3.1 Recognition molecules of the lectin pathway

The initiation of lectin pathway activation appears to be even more complex than that of the activation of the classical pathway and involves more components. The recognition molecules of the LP are MBL, collectin 11 and ficolins.

1.1.1.3.1.1 Mannose Binding Lectin

The first ever described recognition subcomponent of the lectin pathway is mannose binding lectin (MBL). MBL, which is a member of collectin family, is mainly synthesised in the liver (Brouwer *et al.,* 2008). It is formed from oligomers of structural subunits (see figure 1.4). Each MBL structural subunit is composed of homotrimers of identical polypeptide chains which are approximately 32 kDa in size (Garred *et al.,* 2006; Sastry *et al*., 1984). The polypeptide chain consists of an N-terminal cysteine-rich domain, a collagen like domain, neck region and carbohydrate recognition domain (CRD) (White *et al.,* 1994). Through its collagen-like domain, MBL binds to MBL-associated serine proteases in calcium dependent manner. The CRD will bind to specific carbohydrate ligands on the surface of pathogens (Wallis *et al*., 2005).

The genomic organisation of MBL is different between human and rodents. In humans, MBL is expressed by one single structural *MBL* gene which is located on chromosome 10, while in rodents two different MBL proteins (MBL-A and

MBL-C) are expressed by two functional MBL genes, namely *MBL-A* and *MBL-C* which are located on chromosomes 14 and 19 respectively (Garred *et al.,* 2006; van der Bol *et al.,* 2010; White *et al.,* 1994). In plasma MBL is found as polymers of homotrimers, mainly dimer, trimmers and tetramers (Kilpatrick, 2002; Lipscombe *et al.,* 1995). MBL has high affinity to mannose and N-acetyl-glucosamine oligosaccharides which are surface components of different bacteria, yeast and fungi (Brouwer *et al.,* 2008). In addition to this, MBL is able to recognise patterns on the surface of apoptotic cells and activate complement system (Stuart *et al.,* 2006). Furthermore, MBL can bind to IgM and activate the lectin pathway on immune complex (McMullen *et al.,* 2006). MBL deficiency, which is the most common human complement deficiency, increases the risk of meningococcal diseases (Bax *et al.,* 1999).

1.1.1.3.1.2 Collectin 11

Collectin 11 (CL-11) or Collectin kidney (CL-K1) belongs to the collectin family. It is first described about 10 years ago by Keshi and his colleagues. This recognition molecule is encoded by *COLLEC11* gene which is localized at human chromosome 2 (2p25.3) (Keshi *et al.,* 2006). CL-11 mRNA has been found in all organ tissues with a very high level in the kidney, adrenal gland and the liver. It also expressed in monocytes, T cells, B cells and vascular epithelium (Hansen *et al.,* 2010; Keshi *et al.,* 2006). Its plasma concentration is 2.1 μg/ml. It possesses four domains; N-terminal domain, collagen like domain, neck domain and carbohydrate recognition domain (CRD). CL-11 has potent binding activity via its CRD to D-mannose and L-fucose in the Ca^{+2} dependent manners (Hansen *et al.,* 2010). CL-11 was co-purified with the MASP-1 and it can interact with the MASP-1 and/or MASP-3 in the plasma. Furthermore, it has ability to

bind to bacteria, viruses and fungi and forms complexes with the influenza A virus DNA. So, the ability to interact with MASPs for different pathogens indicates that CL-11 plays a role in the initiation of the complement system activation (Hansen *et al.*, 2010).

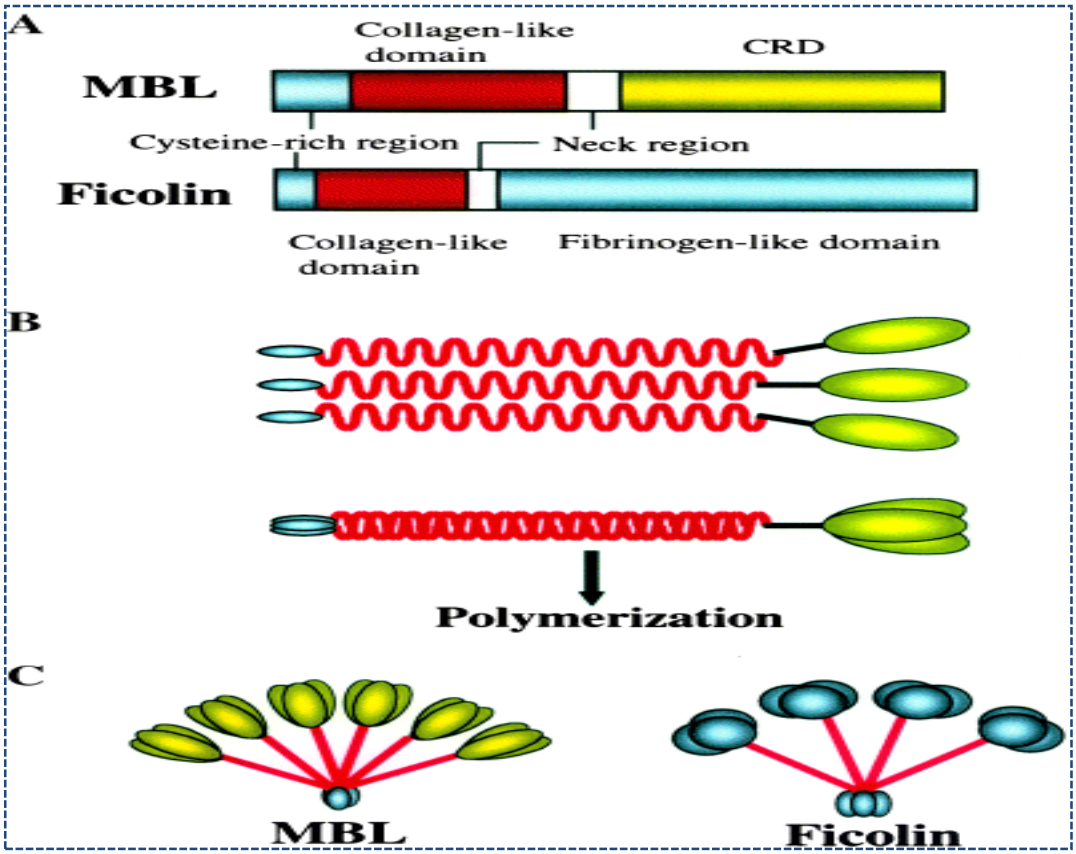

Figure 1.4: Mannan binding lectin (MBL) and ficolin structures
(A) MBL and ficolin structures. (B) Each MBL subunit has three identical polypeptides. (C) MBL present in several sizes of oligomers, hexametric form of MBL and tetrameric form of L-ficolin are shown in this picture (Fujita *et al.*, 2004).

1.1.1.3.1.3 Ficolins

Ficolins form a family of proteins that are, like collectin, composed of collagen-like domains and a C-terminal carbohydrate binding domain. While the carbohydrate binding domain of collectin is a typical lectin domain, the

carbohydrate binding domain of ficolins is formed from a fibrinogen-like domain that preferentially binds to acetylated molecules. Other than that, the structure of ficolins very much resembles that of MBL and CL-11. Ficolin complexes are composed of homotrimers of single ficolin chains which themselves are composed of N-terminal sequence, followed by a collagen-like domain, followed by a neck region and a C-terminal fibrinogen-like domain which acts as an analogue of the carbohydrate recognition domain in MBL and CL-11 (Yongqing *et al.,* 2012).

In man, three types of ficolins have been prescribed which are M-Ficolin (Ficolin-1), L-Ficolin (Ficolin-2 or Ficolin/P35) and H-Ficolin (Ficolin-3 or Hakata antigen) (Boyajyan and Mkrtchyan, 2013; Endo *et al.,* 1996). The degree of similarity between the primary structure of L-ficolin and M-ficolin is approximately 80%, while H ficolin shares only 45% of similarity with L- and M- ficolin (Yongqing *et al.,* 2012). The length of the collagen-like region differs among these proteins. Carbohydrate recognition domain of all ficolins recognise a wide range of carbohydrate moieties on different pathogen surfaces particularly *N*-acetylglucosamine, and acetylated structures on apoptotic cells and DNA (Jensen *et al.,* 2007; Endo *et al.,* 2011). Despite their function as the initiator of the lectin pathway activation, ficolins can act as opsonin, enhancing the phagocytosis of apoptotic cells by phagocytes, thus clearing body from dead cells (Boyajyan and Mkrtchyan, 2013). Ficolins circulate in the plasma in a complex with the Mannose binding lectin associate serine proteases.

The gene encoding L-ficolin, *FCN2*, is located on chromosome 9 (9q34) (Endo et al., 1996). It is mainly expressed in hepatocytes with the serum concentration of about 5.15 μg/ml (Endo *et al.,* 1996; Le *et al.,* 1998) while H-ficolin (which is present at a serum concentration of about 18.4 μg/ml) is mainly expressed in the liver and the lung (Endo et *al.,* 1996; Le *et al.,* 1998). M-ficolin has the lowest

plasma concentration among ficolins, with an average plasma concentration of 1.07 μg/ml. The gene that encodes M-ficolin is the *FCN1* gene located on chromosome 9 (9q34). In human, M-ficolin is expressed in bone marrow, spleen, alveolar epithelial cells and peripheral monocytes (Wittenborn *et al.,* 2010).

Ficolins have also been identified in the secretory granules of neutrophils, in type II alveolar epithelial cells in the lung and in monocytes (Liu *et al.,* 2005). In rodents, there are only two types of ficolins; ficolin-A and ficolin-B, since the rodent orthologue of H-ficolin gene is not expressed, i.e. a pseudogene (Endo et al., 2004). Ficolin-A is the mouse orthologue of human L-ficolin. It is highly expressed in the liver and the spleen (Fujimori *et al.,* 1998). It is often considered to be the only serum ficolin in mouse and rat, since Ficolin-B is somehow associated with membrane structures. Ficolin-A is encoded by the *FcnA* gene located on chromosome 2 (Endo *et al.,* 2004). Ficoln-A forms complexes with MASP-2 in the plasma and initiates the activation of the lectin pathway (Endo *et al.,* 2005). Ficolin-B, which is expressed highly in bone marrow, is encoded by the *FcnB* gene located on chromosome 2. Because of a sequence mutation in the binding motif responsible for the binding of MASPs, mouse ficolin-B cannot bind to MASP-2 (Girija *et al.,* 2011).

1.1.1.3.2 Mannose binding lectin associated serine proteases

Mannose binding lectin associated serine proteases (MASPs) are members of a serine protease family and share a high degree of homology with the classical pathway specific serine proteases C1r and C1s (Schwaeble *et al.,* 2002). There are three types of MASPs present in mammals, which are MASP-1, MASP-2, and MASP-3 (in addition to MAp19, a non-enzymatic, truncated product of *MASP2* gene and MAp44 a non-enzymatic, truncated product of *MASP1* gene)

(Schwaeble et al., 2002). C1r and C1s as well as MASP-1, MASP-2 and MASP-3 share the same modular organisation being composed of an N-terminal CUB1 domain, followed by an epidermal growth factor-like domain (EGF), a second CUB domain(CUB1), followed by two complement control protein domains (i.e. CCP1 and CCP2) and the C-terminal serine protease domain. The truncated alternative gene product of the *MASP2* gene, MAp19, is only composed of the two N-terminal domains of MASP-2, i.e. the CUB1 and EGF domains followed by a unique C-terminal sequence of four amino acids encoded by the alternative splice exon that also represents the translation termination codon and the unique 3'-untranslated region of the MAp19 mRNA (Schwaeble *et al.*, 2002). While truncated alternative gene product of the *MASP1* gene, MAp44, is composed of four N-terminal domains of MASP-1 and MASP-3, i.e. the CUB1, EGF, CUB2 and CCP1 domains followed by a unique C-terminal sequence which consists of seventeen amino acids (see figure 1.5) (Deng *et al.*, 2009). Both MAp19 and MAp44 are considered to act as competitive inhibitors of lectin pathway activation since they may compete with MASP-1, MASP-2 and MASP-3 for the binding to lectin pathway recognition subcomponents by binding to the MASPs-binding site within the collagen-like domains of ficolins, MBL and CL-11 via their CUB1 and epidermal growth factor-like domains in a Ca^{+2} dependant manner (Wallis, 2007).

MASPs are present in the serum in a zymogen state. When the lectin–MASPs complexes bind to target carbohydrate structures on microbes, MASP-1 and MASP-2 are cleaved and activated via autolysis at a cleavage single site in the short linker region between the CCP2 domain and serine protease domain. The activated serine protease domain stays bound to the N-terminal heavy chain via a single disulphide bond. In contrast, MASP-3 does not undergo autolysis and needs to be cleaved by either MASP-1 or MASP-2 (Wallis, 2007). Activation of

MASP-1, MASP-2 and MASP-3 leads to the conversion of the single chain zymogens into the N-terminal A-chain (heavy chain) and the C-terminal B-chain (or light chain) which comprises the entire C-terminal serine protease domain (Schwaeble *et al.,* 2002). MASP-1, MASP-3 are alternative splicing product of a single structural gene, *MASP1*, located on chromosome 3q27-28 in human (Takada *et al.,* 1995; Schwaeble *et al.,* 2002), while MAp19 and MASP-2, are alternative splicing products of the *MASP2* gene located on human chromosome 1p36.3-2 (Stover *et al.,* 1999; Schwaeble *et al.,* 2002; Stover *et al.,* 1990). The molecular weights of MASPs by Western blotting are approximately 90 kDa, 74 kDa and 94 kDa for MASP-1, MASP-2, and MASP-3 respectively (Schwaeble *et al.,* 2002).

MASP-2 is a key enzyme for lectin pathway of complement activation since only MASP-2 can cleave both C2 and C4 to form the lectin pathway specific C3 convertase C4bC2a (Rossi *et al.,* 2001). A mouse strain with a targeted disruption of the *MASP2* gene is therefore deficient of this C3 convertase (Schwaeble *et al.*, 2011). Neither MASP-1 nor MASP-3 is able to activate complement system to mediate the formation of this convertase in the absence of MASP-2. In contrast, a mouse strain deficient in both MASP-1 and MASP-3 present with a residual, by still fully functional lectin pathway since they can form (although with some delay) C4bC2a convertase complex.

The exact function of MASP-1 is still a mystery and has not yet been convincingly defined: On one hand, it is generally accepted that MASP-1 is quite efficient in cleaving C2, however, its lacking cleavage activity towards C4 shows that without MASP-2 functional activity no C4bC2a complexes can be formed (Fujita, 2002, Schwaeble *et al.* 2011, Megyeri *et al.,* 2013). Other recent work claims that MASP-1 is an exclusive and obligate activator of MASP-2 (Héja *et al.*, 2012). Fully ignoring the facts that the lectin pathway complexes

formed in plasma and serum of MASP-1/3 deficient mice showing that complexes exclusively loaded with MASP-2 can activate the lectin pathway in absence of MASP-1 (Schwaeble *et al.*, 2011) and birds and reptiles are all MASP-1 deficient, but have a fully operational lectin pathway running exclusively on MASP-2 (Lynch *et al.,* 2005). Likewise, recent work claims that MASP-1 has a critical role in converting pro factor D to mature factor D based on the observation that MASP-1/3 deficient mice are deficient in alternative pathway functional activity caused by low to undetectable levels of active Factor D (Takahashi *et al.,* 2010, Iwaki *et al.,* 2010). However, reconstitution of alternative pathway functional activity in MASP-1/3 deficient mice with recombinant MASP-1 failed to restore this defect (Takahashi *et al.,* 2010). MASP-1 was also shown to MASP-3 (which is a non-autoactivating enzyme).

A similar degree of mystery is surrounding the quest for the natural substrates of MASP-3: While the early papers on MASP-3 failed to identify a physiological substrate for MASP-3, a subsequent study showed that MASP-3 can cleave the insulin like growth factor binding protein 5 and a derived synthetic peptide (Cortesio and Jiany, 2006). MASP-3 is predominantly expressed in the liver, but biosynthesis was also shown in extra-hepatic sites showing that a substantial amount of MASP-3 is also locally produced in spleen, small intestine, tonsils, lung, thymus and brain tissue (Lynch *et al.,* 2005).

The serum concentrations of human MASPs are as follows: Median concentration of MASP-1 is 11 μg/ml, median concentration of MASP-2 is 0.4 μg/ml while the median concentration of MASP-3 is 5 μg/ml (Degn *et al.,* 2010; Thiel *et al.*, 2012). These huge differences in the median plasma concentrations between the three different lectin pathway specific enzymes have a significant impact on the way these enzymes contribute to the molecular events leading to lectin pathway mediated complement activation. MASP-2 alone was shown to

be sufficient to translate binding of the lectin pathway recognition complexes into complement activation, since it is the only lectin pathway specific serine protease that efficiently cleaves and activates C4 and C2 to form the LP C3 convertase C4bC2a. Since MASP-1 lacks the ability to cleave C4, MASP-1 cannot compensate for the absence of MASP-2 rendering the LP deficient since neither the LP C3 convertase nor C5 convertase (i.e. C4bC2a or C4bC2a$(C3b)_n$) can form. Undoubtedly, MASP-1 plays an essential and physiologically meaningful supportive role by accelerating the rate of LP activation through its ability to cleave C2 (which facilitates the formation of the LP C3 convertase C4bC2a), and as an essential component of the activation network where MASP-1 cleaves and activates either MASP-1 or MASP-2, or MASP-2 cleaves MASP-2 or MASP-1. To appreciate this critical role of MASP-1, it is important to accept that there are fundamental differences in the mode of activation between the classical pathway activation mechanism and that of the lectin pathway. While the serine proteases of the CP, C1r and C1s, form a heterotetramer within the C1 complex, where C1r initiates activation when the CP recognition subcomponent, C1q, binds to CP activating surfaces (such as immune complexes) and cleaves its only substrate C1s. The strictly heterotetrameric conformation C1s:C1r:C1r:C1s embedded within the hexameric C1q recognition subcomponent allows that all initiation steps of CP activation steps can occur within a single C1 complex. This allows each individual C1 complex to initiate complement activation independently from other C1 complexes. In contrast, the LP specific serine proteases MASP-1 and MASP-2 and MASP-3 form sturdy rod-like homodimers or heterodimers which dimerise through antiparallel binding interactions of their N-terminal structural domains (i.e. CUB1, EGF, CUB2) to expose the C-terminal serine protease domains diametrically opposite of each other on either end of the dimer. Binding of these serine protease dimers to lectin pathway recognition complexes will expose either end of their serine protease domains

far apart from each other, making it stochiometrically impossible that serine protease domains within each dimer can interact or activate each other.

Binding of LP complexes to their ligands can initiate low grade autoactivation of MASPs zymogens, but to amplify this autoactivation event to a physiologically meaningful event, many LP activation complexes will have to bind in close proximity to each other to allow enzymatically active serine protease domains to convert MASPs zymogen dimers of neighbouring complexes into their enzymatically active form. Such chain reaction events of activation are only possible when many LP complexes bind in close proximity to each other on activator surfaces. Activated MASP-1 can cleave zymogen MASP-1 or zymogen MASP-2, like MASP-2 can cleave zymogen MASP-1 or zymogen MASP-2, as both MASP-1 and MASP-2 can cleave MASP-3, while MASP-3 cleaves neither MASP-1, nor MASP-2. In order to understand the relation between MASP-1 and MASP-2, it has to be understood that MASP-1 is more than 27-times as abundant than MASP-2 in serum and plasma (i.e. 11 microgram/mL MASP-1 compared to 0.4 microgram/mL MASP-2). In absence of MASP-1, the availability of sufficient quantities of MASP-2 is a limiting factor, which leads to a reduced rate of the LP activation.

The function of MASP-3 is still obscure; recent studies have suggested that it may play a role in the alternative pathway). In 2011, Iwaki *et al.* hypothesised that activated MASP-3 can cleave C3(H_2O) bound factor B leading to the formation of alternative pathway C3 convertase (Iwaki *et al.,* 2011). Thus MASP-1 and MASP-3 (alternative splice products of the *MASP1* gene) might cross-link the lectin and alternative pathways.

In 2010, Takahashi *et al.* showed that MASP1/3 deficient mouse serum lacks AP functional activity (Takahashi *et al.,* 2010). Furthermore, they found these mice

also are deficient in active factor D and only profactor D (factor D in zymogen state) is present in their sera. Therefore, addition of active factor D to this serum restores AP functional activity (Takahashi *et al.,* 2010). One year later, Iwaki and his colleagues demonstrated that MASP-1, MASP-2 and MASP-3 triple knockout mouse serum has lower complement activity in comparison to wild type mouse serum and this functional activity is restored by reconstitution of this triple knockout serum with recombinant MASP-3 and also they showed that the physiological activator of factor D is MASP-3 as it directly activates factor D *in vitro* (Iwaki *et al.,* 2011).

Deng *et al*. claimed that neither MASP-1 nor MAP-3 has role in the AP activation. Assessing AP haemolytic activity of 3MC serum of a Turkish patient with a frame shift mutation within the *MASP1* gene in the sequence encoding the leader peptide shared by MASP-1, MASP-3 and MAp44 (or MAP-1), they claimed to see little no difference between this 3MC patient serum and normal human serum. They also claimed that addition of recombinant factor D restored AP functional activity even in the absence of MASP-1 and MASP-3 (Degn *et al.,* 2012). These statements have not been accepted by the community of experts in the field. Takahashi *et al.* replied in a letter to the Editor of the Journal of Immunology directly as a comment to this publication providing evidence that the restoration of AP activity by adding factor D in the absence of MASP-1 and MASP-3 to serum is most likely due to the fact that the factor D used by Degn *et al.* contained activated factor D and not pro-factor D (Takahashi *et al.,* 2013).

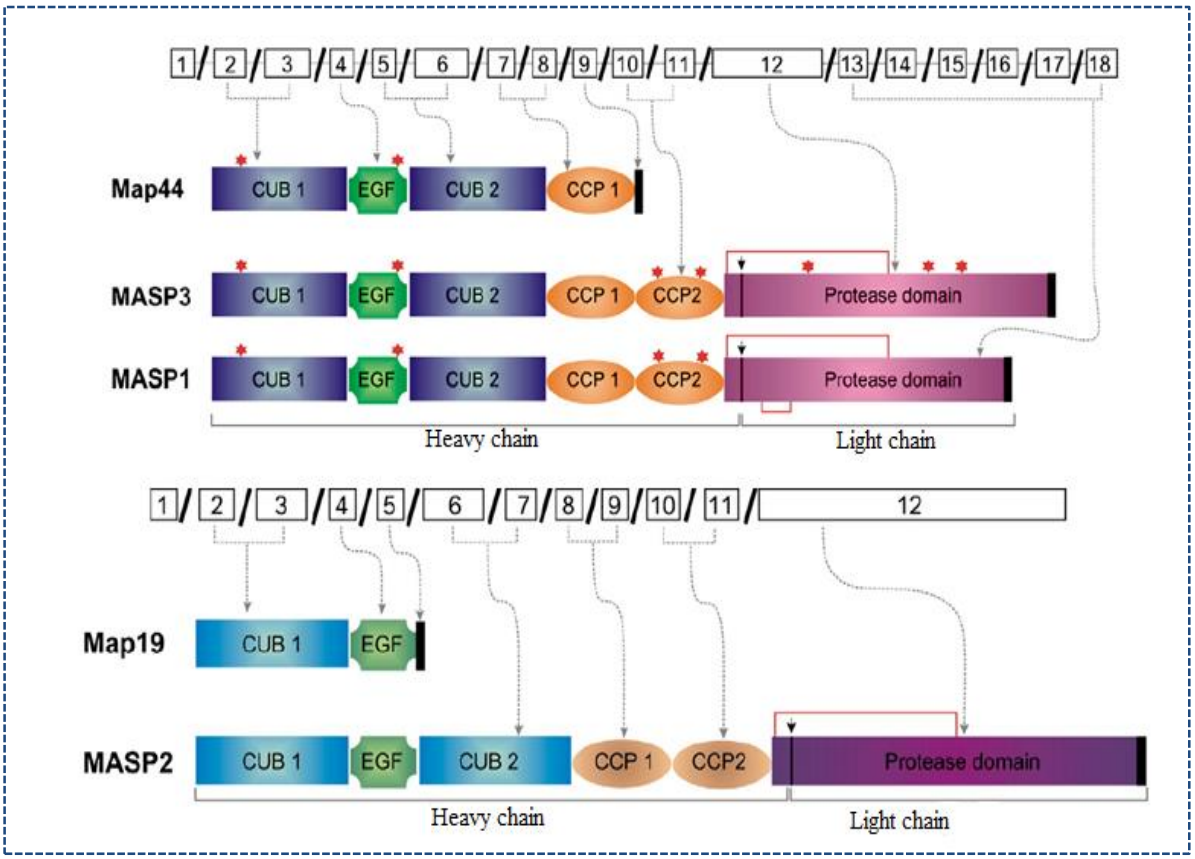

Figure 1.5: Genomic organisation of *MASP1/3* and *MASP-2* genes and their resulting proteins structures (modified from Yongqing *et al.*, 2012)

1.1.1.4 The alternative pathway

The alternative pathway of complement activation provides an innate and non-adaptive first line of defence against invading microorganisms. The components of the alternative pathway include factor B, factor D and properdin (Thurman and Holers, 2006). In contrast to the other two activation pathways of complement, this activation pathway is not driven by specific recognition molecules and it is believed to sustain a constant low state of activation that can be quickly switched towards fulminant complement activation due to the intrinsic amplification loop that the alternative pathway provides if the activation promoting signals outbalance the activity of the negative alternative

pathway regulators, a phenomenon widely known as "tick-over mechanism" (Lachmann, 2009).

Recent work hypothesised that properdin, the so far only know positive regulator of the alternative pathway could also act as a recognition molecule of the alternative pathway by binding to pathogen-associated pattern on microbes and apoptotic cells and initiating the formation of the alternative pathway convertase C3bBb on the surface of pathogens, an appealing suggestion (Kemper *et al.,* 2010), however, subsequent work has cast a shadow over this hypothesis, since properdin binding to microbial surfaces was shown to be dependent of the presence of complement C3 (Harboe *et al.,* 2012). The loss of properdin binding activity to the surface of pathogens in absence of C3 makes it evident that properdin cannot bind on its own to pathogen surfaces, but requires that C3 convertase complexes, i.e. C3bB and C3bBb, to which properdin binds with high affinity and which are stabilised through properdin binding, have to be present first to allow properdin to bind.

Turnover of this pathway begins either via the supply of initial C3b through the lectin pathway, the classical pathway or through the spontaneous C3 hydrolysis, which is occurs in blood, to form C3(H_2O) (Ali *et al.,* 2014). C3(H_2O) has an average half-life of approximately 60 μS (Sim *et al.,* 1981). Formed C3(H_2O) has an altered conformation (Pangburn *et al*., 1981), allowing the binding of plasma factor B in a Mg^{+2}-dependant manner which in turn leads to the formation of the pro-convertase C3(H_2O)B. In the same manner C3b binds to factor B to form C3bB. The binding of factor B to C3(H_2O) or C3b allows factor D to cleave factor B into Ba and Bb. Ba is released from the C3bB or C3(H_2O)B complex and the major cleavage fragment Bb remains part of the complex to form the alternative pathway C3 convertase C3(H_2O)Bb or C3bBb. The alternative pathway C3 convertase converts more C3 to C3a and C3b and this

leads to the generation of more C3b.

C3 convertase of the alternative pathway is a relatively unstable enzyme complex, because the dominant serum regulatory component factor H has high affinity to bind to complex bound C3b. By this, factor H decays this complex and acts as a cofactor to factor I in the factor I mediated conversion of C3b to iC3b (which is no longer serving as an active component of this convertase) and therefore the factor I mediated conversion to iC3b renders this convertase complex inactive. Properdin, a 53 kDa plasma protein, is the only positive regulator of the complement system that stabilise alternative pathway C3 convertase and the alternative and lectin/classical pathway C5 convertases (i.e. $C3bBb(C3b)_n$ and $C4bC2a(C3b)_n$, respectively).

The switch of substrate specificities of the alternative pathway C3 convertase, C3bBb, and the classical/lectin pathway C3 convertase, C4bC2a, to convert C5 into C5b and C5a is achieved when several molecules of C3b bind to or in close proximity of the existing C3Bb or C4bC2a complexes to form the alternative pathway ($C3bBb(C3b)_n$) or classical/lectin pathway ($C4bC2a(C3b)_n$) C5 convertases. These C5 convertase binds and activates C5, releasing the potent complement anaphylatoxin C5a and initiating the terminal complement activation cascade leading to the formation of the membrane attack complex (C5-9).

Most recent work indicated that Mannan- binding lectin associated serine proteases-1 and -3 (i.e. MASP-1 and MASP-3) have important roles in the maturation of the alternative pathway activation as MASP-1/3-deficient mice have no ability to maintain the alternative pathway activation (Takahashi *et al*., 2010). The alternative pathway augments the physiological function of the other complement activation pathways by providing a positive amplification

feedback loop of complement activation (Lachmann, 2009). As this alternative pathway amplification loop augments all pathways of complement activation targeting the alternative pathway therapeutically may be a highly effective way to target complement system mediated inflammatory disorders as long as the alternative pathway amplification loop is critically involved in the pathophysiology of this particular condition (Thurman and Holers, 2006).

1.1.1.4.1 Complement components of the alternative pathway

Complement components of the alternative pathway are factor B, factor D and properdin (Thurman and Holers, 2006). Recently role of MASP-1 and MASP-3 in the alternative pathway also been described.

1.1.1.4.1.1 Factor B

The proenzyme factor B, which is a primary alternative pathway zymogen component, is composed of three N-terminal complement control protein (CCP) domains, that are connected to a von Willebrand factor A (vWF) domain and a C-terminal serine protease (SP) domain by a 45-residue long linker. The vWF and SP domains form Bb, while the Ba fragment is composed of the three CCP domains and the linker. The C3b binding to factor B depends on the metal ion dependant adhesion site (MIDAS) motif in the von Willebrand factor A domain of the Bb fragment. This process is Mg^{+2}-dependant (Pryzdial and Isenman, 1987).

1.1.1.4.1.2 Factor D

Factor D (fD), also known as a dipsin, is a member of serine protease family and a critical component of the alternative pathway of complement system (Volanakis and Narayana, 1996). It is expressed in different tissues, but its major source for biosynthesis is adipose tissue (Stanton *et al.,* 2011). Its plasma concentration is in the range of approximately 2 μg/ml (Barnum *et al.,* 1984). In plasma, it was previously claimed that more than 99% of factor D is mature factor D and just about 1% is profactor D, indicating that profactor D is converted to factor D directly after its biosynthesis (Lesavre and Muller-Eberhard, 1978; Yamauchi *et al.,* 1994;). My results put this statement in questions.

1.1.1.4.1.3 Properdin

Properdin is a glycoprotein which is present in the blood in a soluble state. Up to now, it is the only known positive regulator of complement activation. Louis Pillemer described properdin in 1954 as a plasma component that can activate the complement system in the presence of Mg^{+2} without involvement of immune complexes (Pillemer *et al.,* 1954). The gene that is responsible for the expression of properdin in human, *Complement Factor Properdin* (*CFP)*, is located on short arm of chromosome X (Xp11.3-Xp11.23) (Coleman *et al.,* 1991). Normal plasma concentration of properdin ranges between 5-15 μg/ml (Schwaeble and Reid, 1999).

Properdin presents in serum as a cyclic dimer (P2), trimer (P3) and tetramer (P4), which formed by head to tail association of monomers in a stable ratio of 20:54:26 respectively (Smith *et al.,* 1984; Schwaeble and Reid, 1999). Oligomerisation of properdin is an early intracellular event. Therefore, no

monomer can be found in the serum (Farries and Atkinson, 1989). The monomer has an approximate molecular weight of 53 kDa. Each properdin monomer is composed of six homologous structural units which are called thrombospondin structural homology repeats (TSRs) or thrombospondin type I repeats, and an N terminal domain (see figure 1.6). Each unit consists of approximately 60 amino acids and are named from TSR1 to TSR6.

The function of different units are varied, TSR5 is important for C3b and sulphatide binding, TSR4 is important for C3bBb stabilization and TSR6 is important for oligomerisation (Perdikoulis *et al.,* 2001; Schwaeble and Reid 1999). The functional activity is variable among polymers and it increases with the increasing size of the polymers. The tetramer is approximately ten times more potent than the dimer (Pangburn, 1989). Unlike other complement components, properdin is not synthesized in hepatocytes. There are different cellulous sources of properdin production: properdin mRNA is expressed in monocytes and macrophages (Schwaeble *et al*., 1994), mast cells (Stover *et al*., 2008), T cells (Schwaeble *et al*., 1993), and peripheral blood neutrophils (Wirthmueller *et al.,* 1997).

Properdin can bind to C3b and C3bBb complexes. Properdin`s binding affinity to cell bound C3 convertases greater than cell bound C3b. Moreover, the binding affinity of properdin to bind to cell bound C3b is higher than soluble C3b (Farries *et al.,* 1989). Properdin binds to and stabilizes the C3bBb complex (alternative pathway C3 convertase) and increases its half-life 5-10 folds (Fearon and Austen, 1975). By this stabilisation, properdin counteracts the function of Factor H which enhances inactivation of the alternative pathway C3 convertase through binding to C3b bound complex and by acting as a cofactor for factor I which mediates conversion of C3b to iC3b, inactive form of C3b (Schwaeble *et al.,* 1987). In addition to this, properdin can bind to the C3b or iC3b bound

surface and resemble as a receptor for C3b or preformed C3bBb complex through its unoccupied C3b binding sites (Hourcade, 2006).

Properdin has an essential role in alternative pathway activation as properdin deficiency in serum leads to abolishment of serum ability to activate the AP and addition of purified properdin to properdin deficient serum can restore alternative pathway activation. This restoration is dose-dependent and complete restoration can be achieved by approximately 50% of normal concentration (Schwaeble and Reid, 1999). Despite of these functions, native properdin is thought to act as a pattern recognition complex and promote complement activation as it binds to zymosan (Spitzer *et al.* 2007), *Chlamydia pneumoniae* (Cortes *et al*., 2010) and late apoptotic and necrotic cells (Ferreira *et al.,* 2010). Unfractionated properdin (Pn), (containing non physiological higher level polymers is formed during long-term storage and freeze-thawing), binds to *Neisseria meningitis* and *Neisseria gonorrhoeae* (Agarwal *et al.,* 2010).

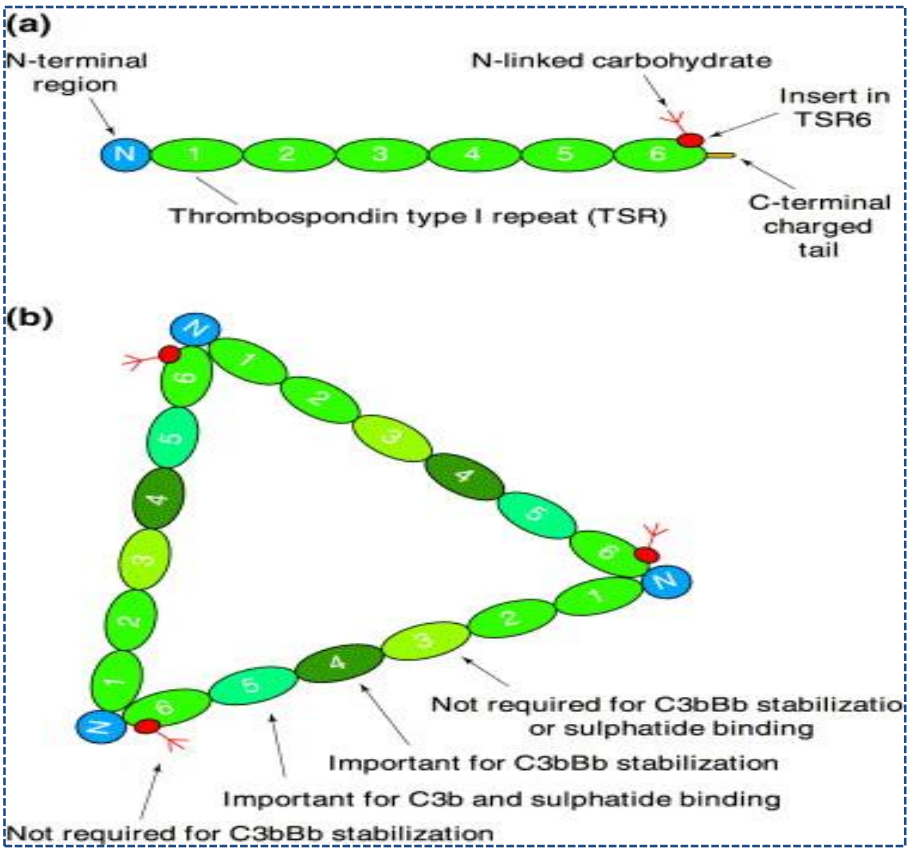

Figure 1.6: Structure of properdin
The monomer structure and (b) the trimer structure and functional regions of TSR. Adapted from (Schwaeble& Reid, 1999).

1.1.1.5 Biological effects of complement activation

Complement has crucial role in innate immunity as it eliminates invading pathogens, apoptotic cells and dead cells; also it is important for guiding adaptive immunity (Dunkelberger and Song, 2010). Activation of the complement system mediates many biological functions including opsonisation (leading to phagocytosis of pathogen and cellular debris), production of anaphylatoxins (leading to chemoattraction of leukocytes), lytic action (membrane attack complex mediated lysis) and activation of the adaptive immune response.

Pathogen opsonisation is one of the main activities of the complement system and thus enhances uptake of invading pathogens by phagocytes. C3 is a core to opsonisation, after cleavage of C3 by the C3 convertases, the early product of C3 is C3b which remains covalently bound to the surface pathogen. C3b cleavage products iC3b, C3c and C3dg act as ligands for specific receptors on leucocytes (Kemper and Köhl, 2013). C4b, C3b, iC3b and C3dg on the surface of the pathogen serve as ligands to the complement receptor 1 (CR1, CD35), complement receptor 3 (CD11b/CD18) and complement receptor 4 (CD11c/CD18) which are expressed on macrophages, polymorphonuclear (PMN) cells and monocytes (van Lookeren *et al.,* 2007). In addition to this, C3b and C4b can bind to complement receptor 1 (CR1) on the erythrocytes and this allows erythrocytes to bind to complement opsonised molecules and antigen-antibody complexes, enhancing their clearance by taking them to the monocyte phagocytic system and reticuloendothelial system, thus preventing deposition of immune complex in vulnerable tissues (Miyaike *et al.,* 2002). The complement system also initiates clearance of apoptotic cells through the CR3 and CR4 on macrophages which bind to iC3b bound apoptotic cells and hence enhances phagocytosis of apoptotic cells (Mevorach *et al.,* 1998). The role of some complement components on the clearance of apoptotic cells have been described like MBL (Stuart *et al.,* 2005), L-ficolin and H-ficolin (Kuraya *et al.,* 2005). Some other components of the complement system also act as opsonin such as MBL and ficolin on their own upon finding their target surfaces (Teh *et al.,* 2000; Kuhlman *et al.,* 1989).

Another important function of complement system is to direct killing of pathogens by the MAC. Some Gram negative bacteria are liable to be killed directly by the MAC. This lytic immune structure disrupts the cell membrane of pathogens through forming pores in the cell membranes, which permit excess

intracellular Ca^{+2} influx and water to inside the cell, which consequently disturb electrolyte balance and causes osmotic instability resulting cell lysis and death (Tschopp, 1984; Podack *et al.*, 1982).

Complement system mediates chemotaxis function as activation of this system leads to formation of small peptides (C4a, C3a and C5a), which are potent anaphylatoxin especially C3a and C5a. They induce local inflammation by driving different types of immune cells to the site of infection. Furthermore, these small peptides can activate a variety of different cells through intracellular signalling pathways. They stimulate mast cells leading to mast cell degranulation and can activate T cells (Ember *et al.*, 1994; Morgan *et al.*, 1983; Nataf *et al.*, 1999; Shin *et al.*, 1968). Also, they induce physiological changes of the smooth muscle as they enhance vascular permeability and this leads to extravasations (Williams and Jose, 1981). They enhance expression of adhesion molecules by endothelial cells in a site of infection, by this; facilitate migration of inflammatory cells to the infection site. Also, they enhance cytokine release by different cell types (Ember *et al.*, 1994; Schraufstatter *et al.*, 2002). C5a also can activate monocyte and PMNs, upregulate surface expression of CR1 and CR3, to increase their ability to bind to endothelial cells and increase their phagocytic activity.

Another function of complement system is to guide adaptive immune system. C3b degradation products can also serve as ligands for CD35 and CD21 on B cells and by this share in the activation of B-cell which leads to production of antibodies to specific antigens and differentiate memory B-cell (Roozendaa and Carroll, 2007).

1.1.1.6 Complement system regulation

The complement system is regulated tightly by a sophisticated mechanism which mediated through a number of fluid-phase and cell-bound regulators (see figure 1.7). By this, it prevents self-tissue from damaging by the unwanted complement activation and allows activation of the complement system on hazardous surfaces including pathogen and apoptotic cells. Varieties of diseases arise as a result of deficiency of these regulators like paroxysmal nocturnal haemoglobinuria, atypical haemolytic uraemic syndrome (Markiewski and Lambris, 2007).

1.1.1.6.1 Fluid phase regulators

These regulators control complement activity in a fluid phase and include: C1 inhibitor, factor H, C4 binding protein (C4BP), Factor I (fI) and S protein. C1 inhibitor (C1INH) is the only inhibitor to complement component C1. It belongs to the family of serine protease inhibitors. It controls the classical pathway and lectin pathway activation through displacing the $C1r_2$-$C1s_2$ complex from the C1 complex (Sim *et al*. 1979) and by this, inhibits the initiation of the classical pathway (Sim et al., 1979) and inactivating MASP-1 and MASP-3, so that regulating the lectin pathway (Wagner and Frank, 2010). It may also regulate the alternative pathway via binding to C3b and by this prevents cleavage of C3b by factor D (Jiang *et al.,* 2001).

C4 binding protein (C4BP) is another member of serine protease family that regulates both classical and lectin pathways (Blom *et al.,* 2004). C4BP binds to C4b and serves as a cofactor for fI, which is an active plasma serine protease, for cleaving C4b to inactivated fragments iC4b, C4c and C4d. Thus prevents C4b-C2a binding and inhibiting C3 convertase formation (Blom *et al.,* 2004; Seya *et*

al., 1995). Another complement regulator in this group is fI. This protease inactivates complement components C3b with the contribution of the cofactor (factor H) and C4b with the contribution of the cofactor C4BP (Seya *et al.,* 1995).

The potent fluid phase inhibitor of the alternative pathway is factor H (fH). Despite its function of binding to C3b on the cell surfaces and in fluid phase and serves as cofactor for fI, fH destabilises and accelerates the decay of alternative pathway C3 and C5 convertases (Pangburn, 2000; Wagner and Frank, 2010). fH has affinity to bind to host marker recognition sites like terminal sialic acid residues and this affinity makes fH act as a guide to prevent complement activation on the host cells and thus prevent autoimmunity (Pangburn, 2000). Like fH, factor H like protein 1 (FHL-1) mediates its function by acting as a co-factor for fI for C3b inactivation (Wagner and Frank, 2010). Clusterin and S-protein prevent host cell lysis by interfering with MAC formation (Wagner and Frank, 2010). Complement system anaphylatoxins, C3a and C5a, are inactivated by serum carboxypeptidase N (SCPN) (Bokisch and Muller-Eberhard, 1970).

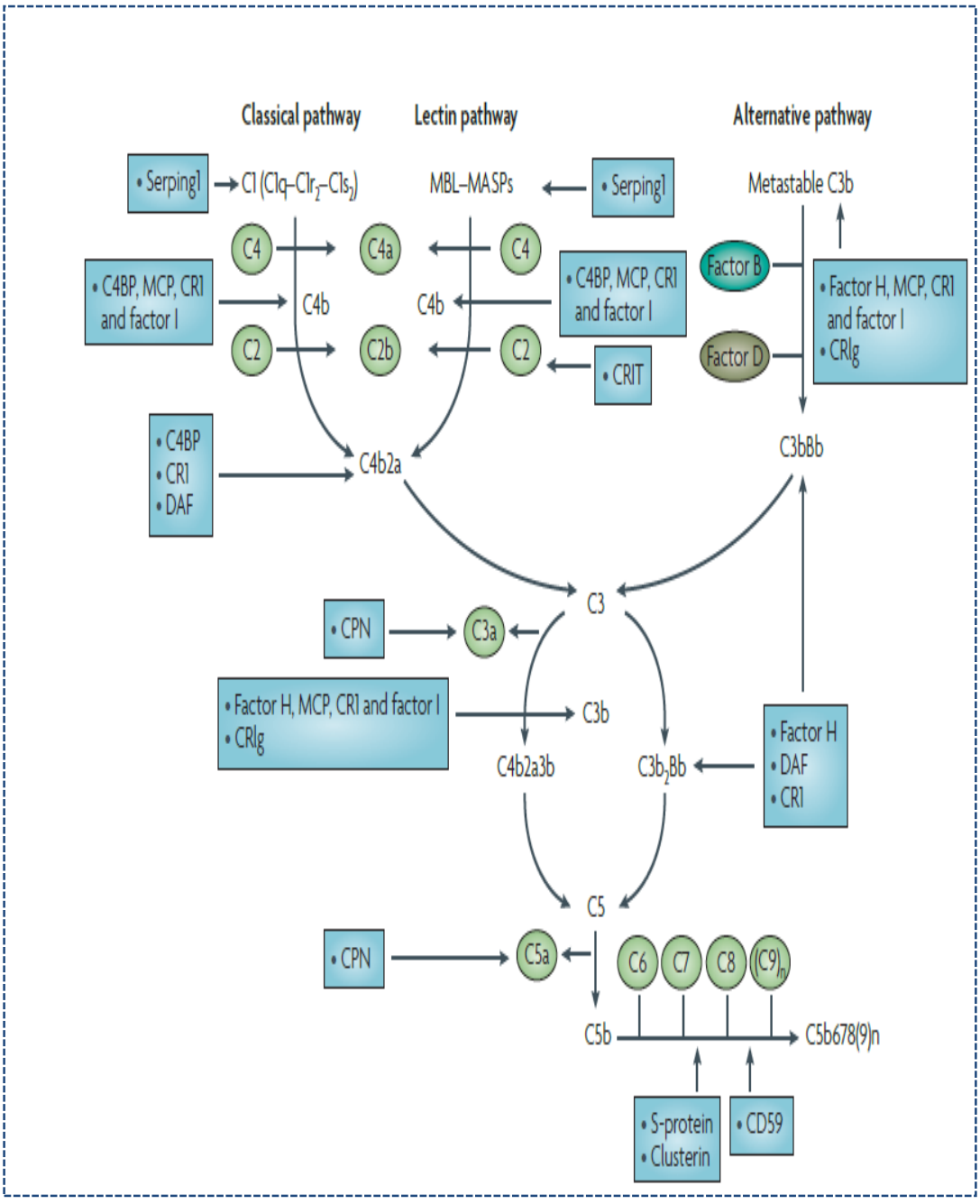

Figure 1.7: The schematic presentation of cell bound and fluid phase complement regulators (Wagner and Frank, 2010)

1.1.1.6.2 Cell surface complement regulatory proteins

These regulators are expressed on host cells surface to prevent host cells damage by unwanted complement activation (see figure 1.7). These are Complement receptor 1 (CR1), Membrane cofactor protein inhibitor (MCP, CD46), decay accelerating factor (DAF) and Protectin.

Complement receptor 1 (CR1) which also called CD35 serves as a receptor for C3b and C4b and by this destabilises and enhances the decay of all complement pathways C3 and C5 convertase complexes. It also acts as a co-factor for fI mediated cleavage of C3b and C4b into inactive smaller fragments (iC3b and iC4b), thus inhibiting self-cell damage by overshooting complement activation (Khera and Das, 2009). MCP is another membrane bound regulator that acts as a cofactor for fI to inactivate the C3b and C4b (Whaley and Schwaeble, 1997).

Another cell surface complement regulator is DAF (CD55). It is a glycophospholipid-anchored membrane protein, which is present on the surface of various host cells including erythrocytes. It decays the classical pathway and the alternative pathway C3 and C5 convertases (Lublin and Atkinson, 1989). Protectin (CD59) is another glycophospholipid-anchored membrane protein that inhibits membrane attack complex formation by preventing polymerization of C9 molecules on the formed C5b-8 membrane complex. Absences of Protectin and/or DAF on erythrocytes make these erythrocytes susceptible to lysis by complement activation and this leads to a disease which called paroxysmal nocturnal haemoglobinuria.

1.1.1.7 Complement deficiency

Complement system has essential role in immune system; plays pivotal roles against microbial agents, enhances clearance of the immune complex and apoptotic cells, and performs variety of functions in the process of tissue remodelling (Langer *et al.,* 2010). Uncompensated complement component deficiency of any complement pathway via complement bypass mechanism may lead to impairment in the function of the system, increasing susceptibility to certain pathogens and arising autoimmune diseases. In contrary, deficiency of uncompensated complement regulator causes uncontrolled complement activation and thus inducing host cell attack and self-tissue damage (Mayilyan, 2012). Complement deficiencies account for about 1-6% of primary immunodeficiency. They can be hereditary, usually autosomal recessive in nature or acquired secondary to excessive complement consumption, decreased synthesis, autoantibodies or excessive consumption of complement components (Grumach and Kirschfink, 2014).

1.1.1.7.1 Deficiencies of the classical pathway

Usually deficiency of components of the classical pathway is associated with immune complex autoimmune disease. Deficiency of C1q increases the risk of systemic lupus erythematosus (SLE) (Amano *et al.,* 2008). Almost all individuals with C1q deficiency develop SLE this indicates the important role of C1q in the immune complex clearances (Leffler *et al.*, 2014). Treating SLE patient who has C1q deficiency with fresh frozen plasma which restores the C1q level alleviates the pathology (Mehta *et al.,* 2010). C1q deficient individual with SLE has been cured after haematopoietic stem cell transplantation as a result of reconstituting C1q in this individual, which is excreted from bone marrow derived monocytes

(Arkwright *et al.,* 2014). Deficiency of other components of the classical pathway; C1s, C1r, C4 and C4 is also associated with increasing risk of developing SLE but with a lesser extent than C1q deficiency.

1.1.1.7.2 Deficiencies of the lectin pathway

The most common cause of complement deficiency is MBL deficiency which emerges mostly due to polymorphisms in MBL genes. About 10% of general population are MBL deficient. The deficiency of this carbohydrate recognition molecule is associated with increasing susceptibility to infection and autoimmunity (Sorensen *et al.,* 2005). MBL deficiency leads to impairment in opsonisation (Supper *et al.,* 1989) and increasing risk of rheumatoid arthritis (Jacobsen *et al.,* 2001). Also it has been found that it increases the risk of atherosclerosis and arterial thrombosis (Ohlenschlaeger *et al.,* 2004; Jacobsen *et al.,* 2001). However, low MBL level has been shown to be associated with variety of disorders, its high incidence in the general population reveals that MBL deficiency manifests only in specific circumstances when other parts of the immune system function improperly (Peterslund *et al.,* 2001; Sorensen *et al.,* 2005). In children, recurrent respiratory tract infections and allergic disorder are associated with L-ficolin deficiency (Atkinson *et al.,* 2004). MASP-2 deficiency, which is a rare condition, is found to be associated with recurrent severe infections and variety of autoimmune diseases (Stengaard-Pedersen *et al.,* 2003).

1.1.1.7.3 Deficiencies of the alternative pathway

Deficiencies of the alternative pathway are due to deficiency of either factor B (fB), factor D or properdin. Among them, the properdin deficiency (positive

regulator of complement system) is the most common cause of the alternative pathway deficiency. fD deficient serum has reduced bactericidal activity and associated with severe meningococcal infection (Sprong *et al.,* 2006). Recently a case with the history of recurrent pneumococcal and meningococcal infection was found to be factor B deficient (Slade *et al.,* 2013).

Properdin deficiency, which is an X-linked recessive inherited disorder, is associated with an increased risk of meningococcal diseases, recurrent otitis media and pneumonia (Fijen et *al.,* 1999; Schejbel *et al.,* 2009). Properdin deficient individuals have 250 fold increased risk to suffer from meningococcal infection than general population (Fijen *et al.,* 1999). Moreover, meningococcal infection has high mortality rate and more morbidity among properdin deficient patient compared to the general population (Fijen *et al.,* 1999). This indicates the important role of properdin in fighting infections. There are three types of properdin deficiency; type I (in which the properdin is absent in serum) type II (where properdin concentration in serum is less than 10% of normal concentration) and type III (with normal serum concentration of dysfunctional properdin) (Schejbel *et al.,* 2009).

1.1.1.7.4 Complement component C3 deficiency

C3 is a key complement component of all three pathways of complement system activation as it participates in the formation of C3 convertase of all complement pathways and C3a (potent anaphylatoxin). There are two types of C3 deficiency, inherited and acquired (Peleg *et al.,* 1992). C3 deficiency, which is a rare autosomal disease, is a serious complement defect (Botto *et al.,* 2009). It is associated with autoimmune diseases and increasing risk of recurrent fulminant infections caused by different bacteria as pneumococci *(*Singh and Rai, 2009*),*

meningococci (Peleg *et al.,* 1992) and *H. influenza* (Ross and Densen, 1984). C3 deficient individuals are defective in both innate and adaptive immune responses as impairment of the functions of B cell, T cell and dendritic cells were found in C3 deficient individuals (Botto *et al.,* 2009).

1.1.1.7.5 Deficiencies of terminal complement component

Terminal complement component (TCC) deficiency impairs the complement function against the bacteria that are susceptible to lysis by complement system. Genetic deficiencies of one or more of the terminal complement components (i.e.C5, C6, C7, C8 and C9) are associated with increasing susceptibility to recurrent meningitis (Figuera and Densen, 1991). The deficiency of complement component C5 differs from deficiency of other components of TCCs, in that C5a, which is a strong anaphylatoxin and chemotactic factor, is also not formed in C5 deficiency. Deficiency of C9 also increases risk of meningococcal infections, but to a lesser degree than other TCCs component deficiencies. For example deficiency of C7 increases risk of meningococcal infection by 10000 folds in comparison to general population while deficiency of C9 increases the risk by 1400 folds (Nagata *et al.,* 1989). This implies for some protective mechanisms of C5-8 complex alone in the absence of poly C9. No association has been identified between autoimmune diseases and TCCs deficiency.

1.1.1.7.6 Complement regulatory components deficiencies

Heterozygous C1 inhibitor deficiency, which is autosomal dominant, occurs with a prevalence rate of approximately 1/50000 (Gompels *et al.,* 2005; Grumach and Kirschfink, 2014). C1 inhibitor involves in the regulation of complement, fibrinolytic, kinin and clotting pathways. C1 inhibitor deficiency associates with hereditary angioedema (HAE). HAE is a condition characterized by increasing vascular permeability to plasma due to excessive production of vasodilator bradykinin in the absence of enough quantity of functional C1-inhibitor. This leads to collection of fluid (oedema) in the tissue of face, larynx, extremities and abdomen (Cancian, 2014; Cugno *et al.* 2009). Acquired C1 inhibitor deficiency, which is a rare condition, is associated with an excessive lymphoproliferation as seen in autoimmune disease and lymphoma. In these individuals, concentration of C1q is usually low and the presence of C1 inhibitor antibodies is common (Markovic *et al.,* 2000).

Deficiencies of the alternative pathways fluid phase regulators, fH and fI are associated with immune mediated diseases like SLE, glomerulonephritis and atypical haemolytic uraemic syndrome (Reis *et al.,* 2006; Thurman and Holers, 2006). Individuals with a deficiency of fH or fI are also more susceptible to infection due to secondary depletion of fB and C3 by continuous spontaneous activation of alternative pathway in vivo (Reis *et al.,* 2006). There are three types of deficiencies; complete deficiencies, subtotal deficiencies, and dysfunctional phenotypes. Even heterozygous mutations of fI and/or fH may initiate the development of atypical haemolytic uremic syndrome (Kavanagh *et al.,* 2008) which is a syndrome characterized by microangiopathic haemolytic anaemia, thrombocytopenia and glomerular thrombotic angiopathy that leads to renal failure.

Membrane bound regulator deficiencies such as the deficiency of DAF and/or CD59 are uncommon disorders. They are associated with an increased sensitivity of erythrocytes to lysis by the complement system, developing a syndrome known as paroxysmal nocturnal haemoglobinuria which will be discussed in detail later.

1.1.1.8 Alternative pathway and diseases

In the past two decades, because of advance in complement research, the role of AP in mediating disease has become more recognisable (Thurman and Holers, 2006). As mentioned earlier, AP is activated through spontaneous continuous turnover of the C3, so this pathway needs continuous regulation and active control. Thus, any defect in the AP regulation causes damage to the host cell and arising diseases. Furthermore, because the AP provides amplification loop for all other complement pathways, therapeutic targeting of the AP could result in amelioration of complement mediated diseases (Thurman and Holers, 2006). Membrane proliferative glomerulonephritis type II is one example of the AP mediated diseases. This disease, which also called Dense Deposit Disease (DDD) is characterised as a progressive renal disease, associated with decreased C3 plasma levels, and leads in most cases to renal failure with high morbidity and mortality. This DDD is often associated with autoantibodies, called C3 nephritic factors, which stabilises the AP C3 convertase. It is also associated with deficiency or dysfunction of the fH (Holers, 2008; Walker, 2007).

Another example is atypical haemolytic uraemic syndrome which mostly happens in children. It is characterised by thrombocytopenia, non-immune microangiopathic haemolytic anaemia and renal failure. It has poor prognosis with mortality rate about 25% and occurrence of end stage renal failure in about

50% of the cases (Constantinescu *et al.*, 2004; Noris and Remuzzi, 2009). Low C3 serum level and high level of C3b, C3c and C3d and C3 deposition in the glomeruli and arterioles in acute phase of the disease indicate the central role of complement system in this syndrome (Holers, 2008). Furthermore, AP complement components abnormalities like mutation in fH gene (Rougier *et al.*, 1998), autoantibodies against fH (Dragon-Durey *et al.*, 2005), and a mutation in the gene that encodes MCP (Richards *et al.*, 2003) have been described in these patients. Paroxysmal nocturnal haemoglobinuria is a disease, which is mediated by the inability of erythrocytes to prevent complement mediated lysis.

1.2 Paroxysmal nocturnal haemoglobinuria

PNH is an acquired haematological disorder, which is characterised by the clinical features of haemolytic anaemia, bone marrow failure, thrombosis and mostly a poor quality of life (Parker *et al.*, 2005). This disease emerges resulting from somatic mutation in the X-linked *phosphatidylinositol glycan class (A)* gene (Takeda *et al.*, 1993). This *phosphatidylinositol glycan class (A)* gene, which is located on the X chromosome in human, encodes proteins which are essential for the formation of a glycosylphosphatidylinositol (GPI) moiety which work like the membrane anchor for different cellular proteins (Parker, 2007). As a result of this mutation, the surface of affected haematopoietic stem cells and their progenies become deficient in the GPI-anchored proteins. Among these proteins, are decay accelerating factor (DAF or CD55) and membrane inhibitor of reactive lysis (CD59) (Holguin *et al.*, 1989; Holguin *et al.*, 1990; Nicholson-Weller *et al.*, 1982). As mentioned earlier, DAF decays the classical/lectin pathways and the alternative pathway C3 and C5 convertases (Lublin and Atkinson, 1989) and inhibits membrane attack complex formation by preventing polymerization of C9 molecules on the formed C5b-8 membrane complex. Thus, both CD55 and CD59 they prevent autologous lysis of host erythrocytes and host cells by the hosts complement system. Absence of these complement regulators on the surfaces of cells make them vulnerable to lysis through uncontrolled complement activation.

1.2.1 Classification of paroxysmal nocturnal haemoglobinuria

According to association with other diseases, presenting features and clinical manifestations, PNH is classified into three subcategories; classic PNH, PNH in

a setting of another defined bone marrow abnormality and subclinical PNH (PNH-sc). In patients with classic PNH, the evidence of intravascular haemolysis (reticulocytosis, high serum lactate dehydrogenase (LDH), low serum haptoglobin and high serum indirect bilirubin) are seen but there are no associations with another defined bone marrow abnormalities. Patients within the second subcategory, have evidence of intravascular haemolysis, both clinically and in laboratory tests, but simultaneously have a history of a myelodysplastic syndrome (MDS), aplastic anaemia or other myelopathies (e.g. myelofibrosis). Patients with PNH-sc have no evidence of vascular haemolysis (neither clinically nor in laboratory tests), but a small fraction of blood cells (either erythrocytes, granulocytes or both) are deficient in the GPI-anchored proteins and this subcategory is usually present with a concomitant bone marrow failure (Parker *et al.,* 2005).

1.2.2 Diagnosis of paroxysmal nocturnal haemoglobinuria

The current standard test for the diagnosis of PNH is flow cytometry (de Latour *et al.,* 2008). Previously, Hams tests were used to diagnose PNH. The basic principle of a Hams test is that when acidifying serum, the red blood cells deficient with GPI-anchored proteins becomes susceptible to lysis by complement (Rosse, 1991). Since the late 1980s flow cytometry has been used to diagnose PNH (de Latour *et al.,* 2008). Absence or decreased expression of GPI-antigens on the surface of haematopoietic cells is the principle for the diagnosis of PNH. This can be detected by using monoclonal antibody against CD55 or CD59 (Richard *et al.*, 2000). This test is more sensitive than Hams test as it has the ability to identify the quantity of deficient blood cells and can identify even a small clone size of PNH cells (de Latour *et al.,* 2008). By using this test, the

blood cells in PNH patients can be divided into 3 types; erythrocytes with complete deficiency of GPI-anchor proteins are named PNH III, while PNH II has about 10% of normal expression (subtotal deficiency of GPI-anchored protein) and PNH I has a normal GPI-anchored protein expression (Parkers *et al.,* 2005; Rosse, 1973). A considerable number of studies explore that measuring the fraction of neutrophils which are deficient in GPI-anchored proteins is more reliable than GPI-deficient red blood cells because the lifespan of neutrophils among these patients are normal as they are resistance to lysis by complement system even if they lack complement regulators, CD55 and CD59 (Hall and Rosse, 1996) while the lifespan of erythrocytes deficient GPI-anchored proteins are shorter than normal erythrocytes as they are susceptible to lysis by complement system.

1.2.3 Natural history of paroxysmal nocturnal haemoglobinuria

PNH is a chronic disease with clinical manifestations of anaemia, fatigue (80%) and thrombotic events (16%). Symptoms include dyspnea (64%), abdominal pain (44%), chest pain (33%), back pain, severe headache, excessive weakness, and recurrent infections. It affects all age groups and both sexes. A classic symptom of haemoglobinuria, which is bright red urine, occurs in 62% of patients. Mostly patients notice their urine has a dark tea-colour, especially in the morning, and clears as the day progresses (Parker *et al*., 2005; Schrezenmeier *et al*., 2014). Moreover, haemoglobinuria might be precipitated by some conditions like, alcohol, infections, exercise, or stress. In retrospective analysis, 10 year survival rate in these patients from 1940 to 1970 was 50%, but this number increased with the better medical care to 75% in more recent years even without use of eculizumab, which is an anti-C5 monoclonal antibody (de Latour

et al., 2008; Schrezenmeier *et al.,* 2014). A recent study by Schrezenmeier *et al.* which evaluates the data from 1610 patients, which enrolled in the International PNH Registry in 2012, shows the age at the disease starts ranged from 3 to 87 years (median age 32 years). Overall, median disease duration was 4.6 years (ranging from 1 to 47 years). The PNH granulocyte clone size ranged from 0.01% to 100% with the median of 68.1%. These patients suffered from poor quality of life and 14% of these patients had a history of renal function impairment (Schrezenmeier *et al.,* 2014). Spontaneous remission may happen, both clinically and laboratory, in 15% of the cases but this is not related to the severity of the disease or history of having complications (Hillmen *et al.,* 1995).

Table 1.1: Symptoms of paroxysmal nocturnal haemoglobinuria

Common symptoms	**Other symptoms**
- Fatigue	- Abdominal pain
- Dyspnea	- Sclera icterus
- Headache	- Dysphagia
- Haemoglobinuria	- Confusion
- Erectile dysfunction	- Chest pain

1.2.4 Pathophysiology of thrombosis in PNH disease

Thromboembolism is the main cause of death in these patients with knowing reasons, causing death in about 40% to 67% of these patients (Hillmen *et al.,* 2007; Schrezenmeier *et al.,* 2014). Even having a small proportion of PNH cells raises the risk of thrombosis considerably (up to approximately four times in comparison to normal individuals) and this risk increases linearly proportional to the PNH clone size (Hall *et al.*, 2003; Hoekstra *et al.*, 2009). Prevalence of

thrombosis among these patients is about 16% and it may happen in different places. Intra-abdominal and cerebral veins are the most popular sites. Moreover, more than one site are affected in one five of the cases (Hill *et al.*, 2013; Schrezenmeier *et al.*, 2014). Budd-Chiari syndrome which is an obstruction of the hepatic venous flow at the level of either hepatic veins or inferior vena cava is presented with abdominal pain, ascites and hepatomegaly. It has a wide range of complications and mortality. It happens in 7.5-15% of PNH patients (Hoekstra *et al.*, 2009).

Several mechanisms are involved in the thrombus formation in PNH patients including platelets activation, complement mediated haemolysis and depletion of nitric oxide. Moreover, in most of the cases more than one mechanism are involved. The main cause of increasing incidence of thrombosis in PNH individuals is platelet activation. Platelet activated as a result of uncontrolled complement activation on platelet-deficient GPI-anchored proteins. However platelet fH, which is secreted by platelet α granules, compensates CD55 deficiency, deficiency of CD59 ensures complement activation. Activated platelets, in turn, cause further activation of the alternative pathway (Del conde *et al.*, 2005; Martin *et al.*, 1995) and the classical pathway (Hamad *et al.*, 2008; Peerschke *et al.*, 2006). Forming MAC complex on the surface of platelets leads to expression of the considerable amount of microparticles on the surface of these platelets (Shattil *et al.*, 1992). These microparticles are pro-coagulant (Wiedmer *et al.*, 1993) and serve as ligands to tenase and prothrombinase complexes, thus enhancing coagulation (Pasquet *et al.*, 1996; Sims *et al.*, 1989).

A number of studies clearly clarify that increased haemolysis is directly associated with the rise in the incidence of thrombosis (Luzzato and Gianfaldoni, 2006; Moyo *et al.*, 2004). Haemolysis increases free haemoglobin and this causes diminishing of nitric oxide (NO), enhancing activation and

aggregation of platelets (Olson *et al.*, 2004). So, intravascular haemolysis in PNH patients is another promoter of thromboembolism (Lewis *et al.*, 2006; Nyska *et al.*, 1999). NO prevents platelets aggregation, adhesion molecules expression and procoagulants secretion (Arnold *et al.*, 1977; and Voetsch *et al.* 2004). Scavenging of NO by free plasma haemoglobins, as a result of haemolysis and the limited bioavailability of this free radical cause platelet activation and enhance binding of adhesion molecules to the vascular epithelium (Radomski *et al.*, 1987; Schafer *et al.*, 2004).

1.2.5 Treatment of paroxysmal nocturnal haemoglobinuria

Treatment of PNH patients are symptomatic and curative treatments. The curative treatment of PNH is bone marrow transplantation, but this treatment is associated with considerable morbidity and mortality. Because of these, this procedure is not suitable option for treating most patients (Schrezenmeier *et al.*, 2014). The symptomatic treatments are blood transfusion, iron and folate supplement, thrombolytic and anticoagulants.

Nowadays eculizumab, which is anti C5 monoclonal antibody, is the only complement system inhibitor drug and becomes a standard treatment for PNH patients with symptoms (Roth *et al.*, 2011). It decreases intravascular haemolysis, frequency of blood transfusion, thrombotic events, renal impairment, pulmonary hypertension and fatigue in these patients, and by these effects improves the quality of life of the PNH patients (Hillmen *et al.*, 2006; Roth and Duhrsen, 2011). In thrombotic event, thrombolytic therapy (McMullin *et al.*, 1994; Sholar and Bell, 1985), and eculizumab (Brodsky *et al.*, 2012) should be given immediately. Furthermore, radiologic intervention may be necessary in some patients with Budd-Chiari syndrome (Griffith *et al.*, 1996).

Thrombocytopenia should be corrected by transfusion. Low dose heparin enhances C3b binding to the PNH erythrocytes because it activates the AP of complement system, while high concentration of heparin inhibits complement activation (Logue, 1977). In contrary, even high concentration of heparin does not change the procoagulant tendency of PNH clone platelets (Ninomiya *et al.*, 2000).

Regarding secondary prevention of thrombosis, considerable studies indicate that anticoagulation does not show benefit in preventing the recurrence of thrombosis (Chenq *et al.*, 2010; de Latour *et al.*, 2008). So, anticoagulants alone are not a sufficient measure as a secondary prevention (Emadi and Brodsky, 2009). Moreover, heparin may enhance thrombocytopenia and raises the risk of thrombosis as thrombocytopenia by itself causes further activation of platelets. Increasing risk of haemolysis is another side effect of using heparin (Hall *et al.*, 2003), by this enhances complement activation and may lead to expansion of the PNH clone size because of coexistence of bone marrow failure (Emadi and Brodsky, 2009). Eculizumab decreases the incidence of thrombus recurrence by 85% in PNH patients (Hillmen *et al.*, 2007).

An interesting point that should be mentioned is that although using eculizumab reduces the risk of thrombus recurrence, the risk of thrombosis still remains high in these patients in comparison to normal population. Explanation for this may be the fact that it inhibits the complement pathway at the C5 level only and thus C3 convertase complexes have been formed and C3b has already been bound to the surface of PNH clones erythrocytes and platelets.

1.2.6 Incompleteness of the effect of eculizumab therapy

Despite using eculizumab, most of the patients remain anaemic and have evidence of extravascular haemolysis like reticulocytosis, increased bilirubin level and decreased haptoglobin level (Risitano *et al.,* 2009; Roth *et al.,* 2011). This is due to entrapment of the erythrocyte coated C3b in the hepatosplenic macrophages (Hill *et al.,* 2010; Risitano *et al.,* 2009). This extravascular haemolysis happens as eculizumab inhibits RBCs lysis and at the same time enhances C3b deposition on the PNH erythrocytes because it does not inhibit C3 activation as it works on the later stage of the complement cascade. Another point is that this drug is expensive and most countries cannot provide it to their patients (Risitano, 2012). Furthermore, eculizumab does not have effect on associated bone marrow abnormality (Roth *et al.,* 2011; Roth and Duhrsen, 2011). It also increases ferritin level and use of iron chelating agent may be necessary in some patients (Roth *et al.,* 2011).

Lastly, because it inhibits the formation of MAC from all complement pathways, the risk of meningococcal infection increases among these patients. Because of this, all patients prior to receiving eculizumab should receive vaccination against *Neisseria meningitides* serogroups (A,C,Y,W135) (Bouts *et al.,* 2011). Moreover, prophylactic low dose antibiotic still advisable as the vaccine not available for *Neisseria meningitis* serogroup B. Some studies show that using eculizumab increases risk of getting this infection even those patients who received vaccination especially in children and patients that receive immunosuppressive treatment (Bouts *et al.,* 2011; Struijk *et al.,* 2013).

1.3 3MC syndrome

The term 3MC refers to Carnevale syndrome, Mingarelli syndrome, Malpuech syndrome and Michels syndromes. The clinical signs of this syndrome are characterised by cleft lip and/or palate, ptosis, characteristic facial expression (blephorophismosis and hypertelorism, blepharoptosis, and highly arched eye brows), limb anomaly, spinal anomaly and craniocynostosis. All mentioned syndromes are autosomal recessive disorders (Titomanlio *et al.,* 2005).

The 3MC syndrome is caused by mutations in the genes *MASP1* and/or *COLLEC11 (*Rooryck *et al*., 2011*). MASP1* encodes two proteases of the lectin pathway of complement activation, while *COLLEC11* encodes an MBL-like pattern recognition molecule of the lectin pathway of complement system. Mutation in the *MASP1* gene can either make individuals deficient in MASP-3 or both MASP-1 and MASP-3 since the *MASP1* gene encodes both MASP-1 and MASP-3. These proteins play role in the embryonic development. These two genes act as guidance cues in the migration of the cranial neural crest cells into the frontonasal process and pharyngeal arches during embryonic development (Rooryck *et al.,* 2011). There is no significance difference in clinical presentation due to which gene of *MASP-1* or *COLLECT11* was mutated so most probably the proteins coded for have closely related functions in the embryonic development (Rooryck *et al.,* 2011).

1.4 *Streptococcus pneumoniae*

Streptococcus pneumoniae (pneumococcus) is a gram positive, diplococcic, ovoid (also spherical), capsular, aero-tolerant anaerobic, non-motile and non-spore forming bacterium. The favourite medium for growing *Streptococcus pneumoniae* is the media with a source of catalase like blood or serum in a presence of 5-10% of CO_2 at temperature 37°C. *S. pneumoniae* is Optochin sensitive, a property used to differentiate these bacteria from other streptococci. It is one of the most frequently occurring pathogens in children and adults with each year being the infectious agent believed to be responsible for more than 100 000 000 cases of ear infection, 5000 000 cases of pneumonia, 100 000 cases of meningitis, and 10 000 000 cases of bacteraemia worldwide (Varon *et al.,* 2010). Pneumococci produce a wide variety of virulence factors including capsular polysaccharides, pneumolysin, pneumococcal surface protein A (PspA) and pneumococcal surface protein C (PspC). Depending on their ability to produce different virulent factors, different strains of pneumococci cause different type of diseases (Kadioglu *et al.,* 2008; Mitchell and Mitchell, 2010).

1.4.1 *Impact of pneumococcal diseases on human health*

Streptococcus pneumoniae infections are the leading cause of community acquired pneumonia, bacteraemia and meningitis in children and adult and the leading cause of acute otitis media in children worldwide (Linch and Zhanel, 2009). It is a respiratory pathogen and can cause different types of invasive and non-invasive diseases. Invasive pneumococcal diseases (IPD) means isolation of the pathogen in sterile components of the body like blood (bacteraemia), cerebrospinal fluid, and pleural fluid causing variety of infectious pathologies as pneumonia, septicaemia, peritonitis, osteomyelitis, endocarditis, cellulitis,

brain abscesses and meningitis. IPD mostly affect children under the age of 2 years, individuals over the age of 65 years and immunocompromised individuals at any age (Lynch and Zhanel, 2010; Naheed *et al.*, 2009). *Streptococcus pneumoniae* also causes non-invasive disease such as otitis media (Lynch and Zhanel, 2010). Annually, 1.6 million people die due to IPD worldwide, children under the age of 5 years are counting for 1 million of this number (Lynch and Zhanel, 2010; Lynch and Zhanel, 2009). IPD mortality rate varies according to the age, is about 3% in children under the age of 5 years and 18-20% for those over the age of 5 years (Harboe *et al.*, 2009). The incidence of IPD has substantially decreased in children and adult following introduction of pneumococcal conjugate vaccine (PCV7) in children (Centres For Disease Control and Prevention, 2009). However, PID due to non–PCV7 serotypes increased significantly following introducing this vaccine (Lynch and Zhanel, 2010). Because of this reason, in 2010, PCV13, which is a new pneumococcal conjugate vaccine which consists of thirteen pneumococcal capsular polysaccharides replaced PCV7 in the UK and USA (Jefferies *et al.*, 2011).

1.4.2 Antimicrobial resistance pneumococci

Antimicrobial resistance pneumococci has increased substantially over the last 30 years, and this is mostly influenced by the ways of using antibiotics, density of the population and dissemination of some international clones due to globalisation. Using specific antibiotic classes not only increases the resistance to that specific class but also could influence emerging resistance to unconnected antibiotic classes (Lynch and Zhanel, 2010). Vancomycin resistance group B streptococcus has been reported in some cases (Park *et al.*, 2014). The incidence of penicillin non susceptible *S.pneumoniae* varies in

different countries, reaching 50% in some countries and remaining as low as 4% in some others countries. This variation is mostly associated with prior antibiotic use. The incidence of multidrug resistance *S.pneumoniae*, resistance of at least three antibiotic classes, is about 9-24% in the United States while in Europe is about 15.8% (Lynch and Zhanel, 2010).

1.4.3 Role of complement in S .pneumoniae infection

Complement system is a part of innate immune system which plays important role against different pathogens. Individual with deficiency of components of complement system are more susceptible to *S.pneumoniae* infection (Picard *et al.,* 2003). Complement system has a vital protective role against *Streptococcus pneumoniae* infection. All complement pathways play role in fighting pneumococcal infection (Ali *et al.,* 2012; Brown *et al.,* 2002). C3 deficient patients have increased susceptibility to pneumococcal infection especially invasive pneumococcal diseases. One out of eight of C2 deficient patients develop invasive *S. pneumonia* infection and 33% of C4 deficient patients have bacterial infection including pneumococcal diseases (Figueroa and Densen, 1991; Picard *et al.,* 2003; Ross and Densen, 1991).

Complement depleted mice are more prone to develop sepsis after nasopharyngeal colonisation of pneumococci in comparison to wild type mice (Bogaert *et al.,* 2010). Moreover, complement depleted rabbit have more bacteria in their brains after challenging with *S.pneumoniae* in comparison to controls (Tuomanen *et al.,* 1987). After introducing of *S.pneumoniae* intracisternally, C3 deficient mice have 20 folds higher bacterial titres in cerebrospinal fluid comparing to wild type mice (Rupprecht *et al.,* 2007).

Brown and his group showed the deficiency of the classical pathway in mice leads to increase bacteraemia, increase bacterial lung burden and decrease survival in comparison to wild type mice (Brown *et al.,* 2002). Moreover, leukocytes in cerebrospinal fluid are severely diminished in C1q deficient mice with pneumococcal meningitis in comparison to their wild type mice, which indicate the significant impairment of immune response to pneumococcus in experimental model of meningitis (Rupprecht *et al.,* 2007).

A lot of studies show the impact of the alternative pathway against the *S. pneumoniae* infection as factor B deficient mice have increased bacterial burden in lung and blood with higher mortality rate in comparison to their control group (Brown *et al.,* 2002; Tu *et al.,* 1999). Meanwhile, the survival of *S. pneumoniae* was significantly higher in the middle ear of factor B deficient mice than their control group (Tong *et al.,* 2010). *In-vitro* studies, the C3b deposition kinetic on the *S. pneumoniae* surface is slower in factor D deficient mice sera as compared to sera of wild type mice (Xu *et al.,* 2001). C1q/fB double knockout mice have shown less complement deposition onto *S. pneumonia*e surfaces (Brown *et al.,* 2002).

Recent studies have defined a central role for the lectin pathway of complement activation in the fight against *S. pneumoniae* infection. Mice with gene-targeted deficiency of MASP-2 showed a high bacterial burden in blood and lung tissues with higher mortality rates (Ali *et al.,* 2012). Ficolin A and CL-11 (the newly discovered recognition molecules for the lectin pathway) were found to be the specific recognition molecules for *S. pneumonia*e. Mice with genetic defects of Ficolin A or CL-11 have a higher susceptibility to *S. pneumonia*e infections with higher bacterial load in blood and lung tissues (Ali *et al.,* 2012). Human L ficolin can bind to pneumolysin, bacterial endotoxin, and activate lectin pathway of complement system (Ali *et al.,* 2013).

S. pneumoniae develops different strategies to escape from the complement system and facilitates its colonisation (Dieudonne-Vatran *et al.,* 2009; Jarva *et al.,* 2002). *S. pneumoniae* has developed different ways to decrease complement system mediated opsonisation and thereby minimise phagocytosis. Undergoing phase variation and becoming encapsulated is one of the methods that pneumococci used as polysaccharide capsule act as nonspecific barrier to reduce deposition of complement components on the bacterial surface, so minimising consequent interaction with phagocytes. A second way is by interaction of some surface proteins of pneumococci with specific complement components. For examples, PspA inhibits C1q and PspC has ability to bind to fH and by this inhibits the formation of AP C3 convertases (Adriani *et al.,* 2013; Dieudonne-Vatran *et al.,* 2009; Jarva *et al.,* 2002).

1.5 Aims and hypothesis

1.5.1 Aims of the study

The aims of this study are to:

a. Assess the role of each complement pathways (the classical, the lectin and the alternative pathway) individually in the complement mediated haemolysis by using different transgenic mice sera and human serum with the deficiency of MASP-3 (3MC) and compares their haemolytic activity to wild type mouse and normal human sera respectively.

b. Assess the utility of recombinant form of properdin (Pn) in fighting the *S. pneumoniae* infection in the experimental mouse module.

1.5.2 Hypothesis

These aims are based on two hypothetical assumptions:

a. Since a critical role of MASP-3 in supporting the alternative pathway functions has been established in my supervisor's laboratory at the beginning of my study, we hypothesized that inhibition or absence of MASP-3 may result in reduction of the alternative pathway mediated haemolysis in haemolytic pathology.

b. Recombinant properdin may enhance local complement activation and accelerate complement mediated clearance of pathogens.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Reagents and chemicals

- 1kb plus DNA ladder	Invitrogen or New England Biolabs
- Agarose, electrophoresis grade	Melford
- Barbital	Sigma-Aldrich
- Blood agar base	Oxoid
- Bovine serum albumin (BSA)	Sigma-Aldrich
- Brain heart infusion (BHI) medium	Oxoid
- C3d biotin	Quidel
- Calcium chloride	Sigma-Aldrich
- CD59 PE	Abnova
- CD235a PE: Cy5	BD Biosciences
- CD71 FITC	BD Biosciences
- Chicken blood	Harlan Laboratories
- Deoxynucleotides, PCR grade (dNTPs)	Promega
- Ethylenediaminetetraacetic acid	Sigma-Aldrich
- Ethylene glycol tetraacetic acid	Sigma-Aldrich
- Zymosan	Sigma-Aldrich
- Foetal bovine serum	Sigma-Aldrich
- Foetal calf serum	Harlan
- Formalin	Fisher Scientific
- Glacial acetic acid	Fisher Scientific
- Guinea pig blood	Harlan laboratories
- Heparin	Sigma-Aldrich
- Hoarse blood	Oxoid

- Low Molecular Weight DNA Ladder — New England Biolabs
- Magnesium chloride — Sigma-Aldrich
- Mannan — Sigma-Aldrich
- Optichin disks — Sigma-Aldrich
- Phosphate Buffered Saline (PBS) — Oxoid
- Proteinase K — Promega
- Rabbit blood — Harlan laboratories
- Sodium chloride — Fisher Scientific
- Taq DNA polymerase — Thermo
- Trizma base — Sigma-Aldrich
- TRIzol — Invitrogen
- Tris-HCl — Sigma-Aldrich
- Triton X-100 — BDH laboratories
- Tween 20 — Sigma-Aldrich

2.1.2 Antibodies and recombinant proteins

- Goat anti-rabbit IgG (whole molecule)Alkaline phosphatase antibody
    - Sigma Aldrich
- Rabbit anti-human C3c polyclonal antibody
    - Dako UK LTD
- Recombinant human and murine properdin
    - Dr Youssif Mohammed Ali, Department of infection, Immunity and inflammation, University of Leicester (UK)
- Recombinant MAp44
    - Omeros Corporation Seatle (USA)

- Recombinant human full length MASP-3
    - Omeros Corporation Seatle (USA)
- Recombinant human truncated MASP-3
    - Omeros Corporation Seatle (USA)
- Recombinant mouse MASP-3
    - Dr. Sadam Yaseen Department of infection, Immunity and Inflammation, University of Leicester (UK
- Anti-human MASP-1 antibodies (SGMI-1)
    - Omeros Corporation Seatle (USA)
- Anti-human MASP-2 antibodies (OMS721 and SGMI-1)
    - Omeros Corporation Seatle (USA)

- FITC-conjugated rabbit anti-human C3c
    - Dako UK Ltd

2.1.3 Kit

- Wizard genomic DNA purification kit
    - Promega

2.1.4 Streptococcus pneumoniae strain

Streptococcus pneumoniae serotype D39, the model strain used in infection experiments, was provided by Professor Peter Andrew, University of Leicester.

2.1.5 3MC Sera

3MC (patient 2) serum was kindly provided by Omeros Corporation Seatle (USA) and 3MC (patient 3) serum was kindly provided by Dr Scott Hackett (Consultant in paediatric Allergy, Immunology and Infectious Diseases. Birmingham Heartlands Hospital.

2.1.6 Mouse sera

Mouse sera used for *in vitro* study were obtained from C57BL/6 mice, wild type and deficient in different complement components types.

2.2 Methods

2.2.1 Haemolytic assays

2.2.1.1 Preparation of the human sera

Human blood was collected by venepuncture from healthy volunteer, 3MC patients (patient 2 and patient 3) and parent of 3MC patient 3 and was placed immediately on ice for 4 hours to allow clotting. Then blood was centrifuged in a cooled centrifuge at 4°C in a speed of 8000 rpm for 5 minutes. Finally 200 µl aliquot of serum was placed in pre-labelled eppendorf tube and stored in -80 °C.

2.2.1.2 Preparation of mouse sera

Mouse blood was collected from wild type and different transgenic mice (C57BL/6 strain) at the age of 8 – 10 weeks. The bleeding was performed via cardiac puncture under general anaesthesia. The collected blood was then placed directly on ice for 6 hours to allow clotting. Then blood was centrifuged in a cooled centrifuge at 4°C in a speed of 5000 rpm for 5 minutes. Finally 200 µl aliquot of serum was placed in pre-labelled eppendorf tube and stored at -80°C.

2.2.1.3 Tube method haemolytic assay using barbital buffered saline

1. Two millilitres of whole rabbit blood (Harlan) were split into two 1.5 ml eppendorf tubes and centrifuged for 3 minutes at 8000 rpm (approximately 5.9 rcf) in the refrigerated centrifuge at 4°C.
2. The RBCs pellet was washed 3 times with ice-cold barbital buffered saline (4 mM barbital, 145 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4).
3. After the third wash, the RBCs pellet was re-suspended in 5 ml of ice cold barbital buffered saline containing 0.1% gelatin and stored at 4°C.
4. 100 µl of suspended RBCs were diluted with 1.4 ml of nano water and spun down at 8000 rpm (approximately 5.9 rcf) for 3 minutes.
5. The OD of the supernatant was adjusted to 0.7 at 541 nm (an OD_{541} of 0.7 at corresponds to approximately 10^9 erythrocytes/ml). After that the re-suspended RBCs were diluted with ice cold barbital buffered saline to a final concentration of 10^8 /ml.
6. Dilutions of the test sera were prepared in ice cold barbital buffered saline and 100 µl of each serum dilution were pipetted into the corresponding well of round-bottomed plate. At the same time, 100 µl of each nano-water (to produce positive control (100% lysis) and barbital buffered saline (as a negative control) were added to corresponding well.
7. 100 µl of appropriately diluted RBCs (10^8/ml) were added to each well.
8. The plate was then incubated for 1 hour at 37°C.
9. Round-bottomed plate was then spun down at 3000 rpm for 5 minutes at room temperature.
10. 100 µl of the supernatant of each well were transferred into the corresponding wells of a flat-bottomed plate and the OD was then read at 415-490 nm.

2.2.1.4 *Tube method haemolytic assay under the alternative pathway specific condition*

All steps of 2.2.1.3 were repeated but alternative pathway specific buffer (4 mM barbital, 145 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, pH 7.4) was used instead of barbital buffered saline.

2.2.1.5 *Haemolytic diffusion plate assay using barbital buffered saline*

1. Erythrocytes are prepared by centrifuging 1ml of blood for 5 minutes at 3000 rpm in eppendorf centrifuge precooled to 4°C. Then, the supernatant was discarded and the pellet was re-suspended with 1 ml of ice-cold barbital buffered saline (4 mM barbital, 145 mM NaCl, 2 mM $CaCl_2$, 1mM $MgCl_2$, pH 7.4) and this wash repeated 2 times (until the supernatant looks clear).
2. After the final wash, the pelleted RBC was diluted in 10 × the volume of pelleted RBCs in the barbital buffered saline.
3. 2% agarose gel (w/v) was prepared by using distilled water and boiled. After that, melted agarose was cooled down in 56°C water bath.
4. Two times barbital buffered saline (8 mM barbital, 300 mM NaCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4) was prepared and 4.5 ml of 2 × barbital buffered saline was put in universal tubes and incubated in a 37°C water bath.
5. Half millilitre of prepared re-suspended RBC solution was added to the universal tube and left the complex temperature to reach 37°C.
6. After that, 5ml of prepared agarose was added to the universal tube and carefully shake to mix with the agarose and poured the Ery-Agarose on the glass plates (5cm × 5cm). The glass plates were left until the agarose gel formed (this step may take approximately 2 minutes). The plates then were incubated inside moisturised humid chamber and placed for about 1 hour in a 4°C fridge.

7. After 1 hour, holes to load the serum sample were punched into the solidified Ery-Agar and 10 µl of prepared serum was added to each hole and returned them back to the fridge for overnight incubation.
8. In the following day, the plates in the humidity chamber were incubated at 37°C and the degree of haemolysis was checked on an hourly basis (for human serum it takes about 1-2 hours for lysis to occur while mouse serum needs more time). Finally, a ring of haemolysis was measured in millimetres and these measurements were used to produce a graph.

2.2.1.6 Haemolytic diffusion plate assay under the alternative pathway specific condition

All steps of 2.2.1.5 were repeated but alternative pathway specific buffer (4 mM barbital, 145 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, pH 7.4) was used instead of barbital buffered saline.

2.2.2 Haemolysis of PNH patient erythrocytes

1. PNH blood was collected from venepuncture and directly put on ice. After that, the blood was centrifuged for 3 minutes at 8000 rpm and washed twice with the alternative pathway buffer (4 mM barbital, 145 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, pH 7.4).
2. 50 µl of pelleted RBCs were added to 1.5ml eppendorf tubes.
3. Ice cold alternative pathway buffer was used to prepare different concentrations of 3MC (patient 3) serum and the diluted serum was then added to pre-labelled eppendorf tubes containing PNH patient RBCs.
4. 50 µl of 0.2 M HCL (for acidified serum) was added to each tube and 25 mM of $MgCl_2$ was added only to the tube containing undiluted serum (100% serum). Heat-inactivated human serum was used as a negative control.
5. Tubes were then incubated at 37°C for at least 1 hour.
6. To a 96 well round bottom plate add 10 µl of FACs flow per well, appropriate to number of tests, followed by the following antibodies: 5 µl CD235a (1/400 Facsflow), 2.5 µl anti-C3d biotin, 5 µl CD71 FITC and 1.5 µl CD59.
7. Add 1.5 µl of blood from each test tube to the appropriate wells and mix.
8. Put in fridge at 4°C for 1 hour, mixing every 15 minutes.
9. Prepare one FACs tube per test with 600 µl of FACs flow.
10. After 1 hour add 150 µl FACs flow to each well and spin plate at 2000 rpm for 30 seconds.
11. Tip off supernatant.
12. Add 20 µl of FACs flow and resuspend by pipetting up and down, add 10 µl streptavidin APC and mix.
13. Put in fridge at 4°C for 30 minutes, mixing every 15 minutes.
14. Add 150 µl FACs flow to each well and spin plate at 2000 rpm for 30 seconds, tip off supernatant, repeat wash step.

15. Add 150 µl of the prepared FACs flow to each well, pipette up and down and transfer to FACs tube.
16. Analyse immediately which was performed by Dr Stephen J Richards at the national PNH centre in Leeds.

2.2.3 C3 deposition assays

2.2.3.1 C3 deposition under alternative pathway specific condition

To measure C3 deposition under alternative pathway specific condition i.e. under conditions that permitted alternative pathway activation but excluded activation of the lectin and classical pathways. Nunc Maxisorb microtiter ELISA plates were coated with 100 µl/well of 10 µg/ml zymosan (Sigma) in coating buffer (15 mM Na2CO3, 35 mM NaHCO3, 0.02% sodium azide, pH 9.6) and plates were incubated overnight at 4°C. The next day, residual protein binding sites were blocked with 250 µl of 1% bovine serum albumin in TBS (10 mM Tris, 140 mM NaCl, pH 7.4). After incubation for 2 hours at room temperature, the wells were washed three times with washing buffer (TBS with 0.05% tween-20). Serum was diluted seven folds in the AP buffer (4 mM barbital, 145 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, pH 7.4) with the initial dilution of 1/5. Diluted serum was added to corresponding wells except the wells from last row of each column, which only received buffer were used as negative control and plates were incubated at 37°C for 60 minutes. After that, plates were washed three times with washing buffer and the bound C3b was detected by adding 100 µl of rabbit anti-C3c (Dako) diluted 1:5000 in wash buffer to each well and incubated for 90 minutes at room temperature. Plates were washed thrice followed by adding of 100 µl of alkaline phosphatase conjugated goat anti-rabbit antibodies (Sigma-Aldrich) diluted 1:5,000 for each well and incubated at room

temperature for 90 min. Plates were washed again, and the extent of C3b deposition was determined by adding 100 µl of colorimetric substrate *p*-nitrophenyle phosphate (*p*NPP) (Sigma-Aldrich). After incubation at room temperature, absorbance was measured at 405 nm using BioRadmicrotitre plate reader.

2.2.3.2 C3 deposition under lectin pathway specific condition

To measure C3 deposition under lectin pathway specific condition, Nunc Maxisorb microtiter ELISA plates were coated with 100 µl/well of 10 µg/ml mannan (Sigma-Aldrich) in coating buffer (15 mM Na2CO3, 35 mM NaHCO3, 0.02% sodium azide, pH 9.6) and incubated overnight at 4°C. The next day, residual protein binding sites were blocked by adding 250 µl of 1% bovine serum albumin in TBS buffer (10 mM Tris, 140 mM NaCl, pH 7.4). After incubation for 2 hours at room temperature, the wells were washed three times by washing buffer (TBS with 0.05% tween-20 and 5mM CaCl2). Serum was diluted seven folds in Barbital buffered saline (4 mM barbital, 145 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) with the initial dilution of 1/40. Diluted serum was added to corresponding wells except the wells from the last row of each column, which just received buffer without serum and were used as negative control and the experiment was carried out as described in 2.2.3.1.

2.2.4 Streptococcus pneumoniae infection study

2.2.4.1 In vitro study

2.2.4.1.1 Preparation of non-passaged bacteria

Bacteria from bead stocks were streaked on blood agar plates and in order to confirm that the bacteria are sensitive to Optochin, Optochin disks were placed in the plates. After overnight incubation of plates at 37°C in a candle jar, in the next afternoon, sweep of colonies from plates was inoculated in 10 ml of brain heart infusion (BHI) broth (Oxoid) and then incubated for 18 hours statically. Next morning, the broth was centrifuged at 3000 rpm, and pellet was re-suspended in 1 ml of BHI serum broth, BHI with 20% (v/v) heat inactivated foetal calf serum (Sigma). 700 µl of this re-suspended pellet in BHI serum broth were added to 10 ml of fresh BHI broth to OD_{500} of 0.6 and incubated at 37°C statically. Frequent OD_{500} checks of bacterial growth were performed and when OD_{500} of bacterial growth reached 1.6, 500 µl of bacterial growth were aliquoted to 1.5ml sterile eppendorf tubes. After at least 24 hours of storage at -80°C, viability of the stocks was determined by Miles and Misra count and checking for Optochin sensitivity was performed (Miles *et al.,* 1938). Briefly the prepared aliquot was thawed and were serially diluted in PBS and plated onto the blood agar and incubated at 37°C anaerobically in a candle jar for overnight. Next day the number of colonies was calculated and viable count were obtained.

2.2.4.1.2 C3 fixation on the surface of S. pneumoniae

An aliquot of non-passaged *Streptococcus pneumoniae* D39 (above suspension) was thawed and centrifuged at 3000 rpm at room temperature for 2 minutes and washed thrice with PBS and re-suspended in 0.5% formalin (Sigma Aldrich) in PBS for 2-3 hours at room temperature. The bacteria were then washed twice with TBS buffer (10 mM Tris, 140 mM NaCl, pH 7.4). After the second wash, the bacteria were re-suspended in the AP buffer to a final concentration of 1×10^6 CFU/ml. 100 µl of suspended bacteria were added to 1.5ml eppendorf tube containing pooled normal human serum at a final concentration of 5% (v/v) with and without addition of recombinant human properdin (5 µg/ml (w/v) final concentration) or pooled wild type sera at a final concentration of 15% (v/v) with and without addition of recombinant murine properdin (10 µg/ml (w/v) final concentration). Tubes were incubated for 1 h at 37°C. After that, the samples were washed twice with TBS. Then FITC-conjugated rabbit anti-human C3c (Dako) diluted 1:5000 in TBS buffer were added to each tube and incubated for 1 hour at room temperature. The opsonised bacteria were then washed thrice and resuspended in TBS and the fluorescence intensity was measured by a FACSCalibur cell analyzer (BD Biosciences).

2.2.4.1.3 Serum bactericidal assay

A modified method of bactericidal assay was used to assess the sensitivities of *S. pneumoniae* to human serum. Stocks of non-passaged *S. pneumoniae* were re-suspended in barbital buffered saline to obtain required bacterial concentration. A known concentration of *S. pneumoniae* prepared in barbital buffered saline was mixed with desired concentration of mouse serum sample in an eppendorf tube. Before incubating the mixture, samples from each tube were taken and

serially diluted in PBS and plated onto blood agar and incubated at 37°C anaerobically for overnight and this shows the count of bacteria that been used. Then the tubes were incubated for 2 hours at 37°C with shaking at 120 rpm. Samples from each tube were taken at different time points, 15, 30, 60, 90 and 120 minutes, which were serially diluted in PBS and plated onto the blood agar and incubated at 37°C anaerobically in a candle jar for overnight. Next day the number of colonies was calculated and viable count were obtained.

2.2.4.2 In vivo study

All the mice used in infection study were 8-10 weeks old C57BL/6 female mice background obtained from Charles River Laboratories, UK. Mice were housed in Division of Biomedical Service (DBS) in the University of Leicester for seven days prior to starting the infectious study to acclimatise them to the new environment. All the procedures used in study were in accordance with guidelines from UK Home Office.

2.2.4.2.1 Animal passage of Streptococcus pneumoniae

An aliquot of non-passaged *Streptococcus pneumoniae* D39 was thawed and streaked onto blood agar plate. After overnight incubation of plates at 37°C in a candle jar, a sweep of colonies from plates was inoculated in 10 ml brain heart infusion (BHI) broth (Oxoid) and then incubated at 37°C statically. Next morning, the broth was centrifuged at 3000 rpm, and pellet was re-suspended in 5 ml of phosphate buffered saline (PBS, pH 7.4). 100 µl of *S. pneumoniae* suspension were injected intraperitoneally (i.p.) in 8-10 week old C57BL/6 mice. After 20-26 hours of introducing bacteria to the mice, symptoms of the illness in

mice reached ++ starry coat. At this stage, they were anaesthetised with 5% v/v isoflurane and 1.6-1.8 L O_2 /min in an anaesthetic chamber and blood was collected by cardiac puncture. 50 µl of this blood was inoculated in 10 ml of BHI and incubated overnight at 37°C statically. In the next morning, the suspension was separated from sediments of erythrocytes and centrifuged at 3000 rpm for 10 minutes. After that, the pellet was re-suspended in 1 ml of BHI serum broth (80% BHI and 20% fetal calf serum). 700 µl of this re-suspended pellet in BHI serum broth were added to 1 ml of BHI broth with final OD_{500} of 0.6 and incubated at 37°C statically. When OD_{500} of bacterial growth reached 1.6 (usually within 5-6 hours), 500 µl of bacterial growth were aliquoted to 1.5ml sterile eppendorf tubes. After at least 24 hours of storage at -80 °C, viability of the stocks were determined by Miles and Misra count and checking for Optochin sensitivity was performed (Miles *et al.,* 1938) as described in 2.2.4.1.1.

2.2.4.2.2 Virulence testing of Streptococcus pneumoniae D39

Prior to infection study, virulence testing of the animal passaged bacteria was performed in 8-10 week old C57BL/6 mice. An aliquot of passaged bacteria was thawed at room temperature and then centrifuged at 13000 rpm at room temperature for 2 minutes. Pellet was then washed by re-suspending in 500 µl sterile PBS and centrifuged again. Then diluted in PBS to achieve the $2.5x10^6$ CFU per 50 µl. Five C57BL/6 mice were anaesthetised with 2.5% v/v isoflurane and 1.6-1.8 L O2/minute and mice were infected with $2.5x10^6$ CFU in 50 µl of PBS. Mice were monitored for signs of illness. When the mice reached +/+ lethargic stage, mice were culled by cervical dislocation. Dose was considered virulence when the time of survival of all mice was within 34-50 hours.

2.2.4.2.3 Determination of blood bacterial burden

After introducing infectious dose of the bacteria to the mice intranasally, the course of infection was monitored by the time-course of bacteraemia. After 6, 12, 24 hours of infection, blood was collected from mice via tail bleed. Mice were placed in an incubator at 37°C for 20 minutes. By this incubation, the veins in the tails of the mice become dilated. Then, 15 µl of blood was collected from the tail of each mouse and 10 µl of this blood was serially diluted in PBS ten folds and plated onto agar supplemented with 5% (v/v) horse blood (oxoid) and incubated overnight at 37°C in CO_2 gas jar. Day after, the numbers of colonies in the plates were counted and through this CFU/ml was obtained.

2.2.4.2.4 Infection of mice

After successful virulence testing, dose was used in infection experiment. 8-10 week old female C57BL/6 mice were used in this study. Before introducing bacteria, the mice were lightly anaesthetised with 2.5% v/v isoflurane and 1.6-1.8 L O2/minute. 50 µl PBS containing 2.5×10^6 CFU of *Streptococcus pneumoniae (*D39) were administered intranasally. 20 µl of introducing inoculum were serially diluted in PBS ten folds and plated onto agar supplemented with 5% (v/v) horse blood (Oxoid) and incubated overnight at 37°C in CO_2 gas jar to confirm the dose of inoculum. After introducing bacteria to the mice, mice were moved to special cages assigned for infected animals and placed on separated infectious racks. These infected mice were closely monitored every three hours after clinical symptoms appeared around 24 hours (hunched appearance, starry coat, lethargy, moribund). Mice showing ++ lethargic were euthanized by cervical dislocation according to Home Office/UK guideline. Survival was recorded for 7 days. Any animal survived after 75 hours of infection survived until the end of experiments.

To investigate the effect of properdin in fighting *S. pneumoniae* infection, half of the mice were injected with intraperitoneal injection of 100 μg of recombinant mouse properdin in 50 μl PBS and control group mice were injected with 50 μl of PBS intraperitoneally. Time points for the injection of recombinant murine properdin were either at the same time of introducing bacteria or 6 hours before infection. 100 μg of recombinant mouse properdin was injected as previous experiment in my supervisor laboratory showed this concentration is effective in fighting *Neisseria meningitidis* infections.

2.2.5 Genotyping of MASP-1/3 deficient mice

2.2.5.1 Isolation of genomic DNA from mouse ear snips

Wizard gDNA Purification Kit (Promega) was used to isolate genomic DNA from mouse ear snips according to manufacturer's instructions.

0.3 -0.5 cm of fresh mice ear snips were incubated overnight at 55°C with gentle shaking in 310 μl of lysis buffer (250 μl of Nuclei Lysis Solution + 60 μl of 0.5 M EDTA solution (pH 8.0) plus 10 μl of 20 mg/ml Proteinase K, Qiagen). On the next morning, 1.5 μl of RNase solution (4 mg/ml) was added to the mixture and mixed by inverting the tube 2-5 times. Then the mixture was incubated for 20-30 minutes at 37°C in a water bath. After that, the samples were left at room temperature for 5 minutes in order to cool to room temperature. 100 μl of protein precipitation solution was added to the room temperature sample and vortex was done at high speed for 20 seconds. Following incubation the tubes on ice for 5 minutes, the samples were centrifuged in a refrigerated centrifuge for 4 minutes at 14,000 rpm. The supernatant, which contains the DNA was removed carefully (avoiding the protein pellet) and transferred to a labelled clean 1.5 ml eppendorf tube. 300 μl of room temperature isopropanol were

added to the tubes and the tubes were inverted several times and then centrifuged for 5 minutes at 13000 rpm. The supernatant was carefully removed and the precipitated DNA was washed with 300 µl of 70% room temperature ethanol by inverting the tubes several times. The samples were centrifuged for 1 minute at 13000 rpm at room temperature. The tubes were inverted on to the clean tissue and the DNA pellet was air dried for 20-30 minutes and 100 µl of DNA re-hydration solution were added and stored at 4°C in a fridge.

Prepared genomic DNA was used to identify MASP-1/3$^{-/-}$, heterozygous (MASP-1/3 $^{+/-}$) and their wild type littermates (MASP-1/3$^{+/+}$) by using polymerase chain reaction (PCR).

2.2.5.2 Polymerase chain reaction

Prepared genomic DNA was used to identify MASP1/3$^{-/-}$, heterozygous (MASP1/3 $^{+/-}$) and their wild type littermates (MASP1/3$^{+/+}$) by using polymerase chain reaction (PCR):

Primer name	**Primer sequence 5′---->3′**
NeoU	CAT CGC CTT CTA TCG CCT TCT TGA
M1U	CTC CCT GCC TCA GAC TGT TTG ATA
Mil	GCT GAT GCT GAT GTT AGG ATG GTA TTC

Reaction mixture of the PCR as following:

Reagents	**Volumes**
Taq DNA polymerase (5U/µl, Thermo)	0.12 µl
Reaction buffer (10x, Thermo)	1.5 µl
dNTP mix (10mM, Promega)	0.3 µl
MgCl2 (25mM, Thermo)	1.5 µl
NeoU	1.5 µl
M1U	1.5 µl
Mil	1.5 µl
Nano pure water	6.08 µl
DNA (200ng/µl)	1 µl

The cycling programme as following:

Step	**Temperature (°C)**	**Duration (second)**	**Step repeat**
Initiation	98	98	
Denaturation	98	30	
Annealing	60	30	
Elongation	72	30	29
Final elongation	72	300	
Hold	4		

Amplification products were finally run on a 1% agarose gel.

2.2.6 Statistical analysis

Statistical significance of differences in the haemolytic assay of difference sera, average illness scores and bacterial loads at different time points were determined by using Student`s unpaired t-test. Survival data was analysed by Mantel–Cox log-rank test. All statistical analyses were done using Graphpad Prism, Version 6.0 (Graphpad Software). Differences were considered significant at P values of <0.05.

Chapter 3: Complement mediated haemolysis

3.1 Results

The complement system can be activated through three different activation pathways, the classical pathway, the lectin pathway and the alternative pathway. Complement activation leads to MAC formation which can lyse non-nucleated erythrocytes as well as bacteria and some nucleated cells. Overshooting complement activation and cell lysis through autologous complement activation is strictly regulated through complement regulators. Deficiencies of any of these regulators may lead to autologous cell damage through uncontrolled complement activation. To identify which complement pathway plays a role in the activation of complement system on erythrocytes, the abilities of different transgenic mice with the deficiency of different complement pathways and human serum with deficiency of MASP-3 and/or MASP-1 that could mediate the lysis of erythrocytes were tested. Erythrocytes from three different species (rabbit, guinea pig and chicken) were used as target cells for the serum lysis by human and mouse sera.

3.1.1 Analysis of mouse sera with targeted complement deficiencies in vitro

3.1.1.1 The classical pathway of complement activation appears not to mediate haemolysis in non-immune mouse serum

Classical pathway is primarily activated after the binding of C1q to immune complexes on the surface of targeted cells. C1q also can activate the classical

pathway even in the absence of immune complex (Navratil *et al.,* 2001). Absence of C1q or C4 abolishes the classical pathway ability to activate the complement system (Botto, 1998; Navratil *et al.,* 2001). To assess the role of the classical pathway in the complement mediated haemolytic activity, different concentrations of mouse sera with deficient C1q and mouse sera with deficient C4, diluted in barbital buffered saline , were used to run haemolytic assay on rabbit erythrocytes (see figure 3.1). In barbital buffered saline the activity of all complement pathways are preserved. In relates to erythrocyte lysis in nano water (100% lysis), the serum concentration of 12.5% of C1q deficient, pooled wild type and C4 deficient show 48%, 46% and 50% lysis respectively. While at serum concentration of 6.25%, they showed 27%, 25% and 29% respectively. At serum concentration of 3.5%, they showed about 13%, 12% and 11% respectively. These differences (at serum concentrations, 12.5%, 6.25% and 3.15%) between C1q deficient, C4 deficient and wild type sera are statistically not significant. These results indicate complement mediated haemolytic activity does not involve the classical pathway in non-immune mouse serum.

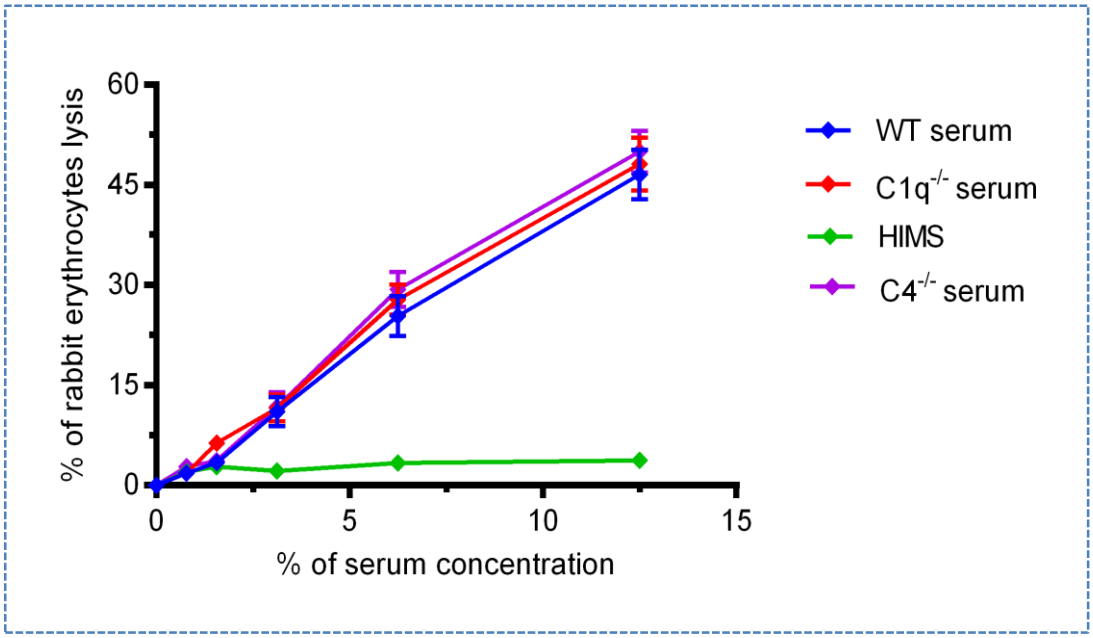

Figure 3.1: Deficiency of the classical pathway does not diminish haemolytic activity of mouse serum on rabbit erythrocytes

Haemolytic assay (tube method) by using C1q deficient, C4 deficient and wild type mouse sera against rabbit erythrocytes. Barbital buffered saline (BBS/Ca^{+2}/Mg^{+2}) was used to run the assay. % of lysis showing in relates to lysis in nano water (100% lysis). An un-paired student t-test was performed: P value at 12.5% of $C1q^{-/-}$ versus pooled wild type is equal 0.789 ($P > 0.05$) and of pooled WT versus $C4^{-/-}$ is equal 0.524 ($P > 0.05$). Heat inactivated mouse serum was used as a negative control. Results are mean (±SEM) of three independent experiments.

3.1.1.2 The lectin pathway effector enzyme MASP-2 does not play a role in the complement mediated haemolytic activity

The lectin pathway initiates the complement cascade upon binding of LP recognition molecules (MBL, CL-11 and ficolins) to the targeted cell surface. Binding of these recognition molecules to the surface of erythrocytes from other species initiates the activation of MASPs which form a complex with the LP recognition molecules. The role of each of these LP recognition molecules in complement mediated lysis was assessed individually (see figures 3.2, 3.3 and 3.4). As can be seen in figure 3.2, using haemolytic diffusion plate assay, there

are no statistically significant differences between the ability of MBL deficient and pooled wild type mouse sera in both 100% and 50% serum concentrations in lysing rabbit erythrocytes. At a mouse serum concentration of 100%, they show a circle of lysis with a diameter of 6.5 and 6 mm, respectively. At a mouse serum concentration of 50%, both show a circle of lysis about 4 mm. In figure 3.3, using tube method haemolytic assay, triple knockout (MBL null/Ficolin A -/-) and wild type mouse sera used against rabbit erythrocytes. As can be seen, at serum concentration of 15%, wild type and triple knock-out sera achieve 56% and 62% of lysis (in nano water lysis is set as 100%), a difference that is statistically not significant. Similarly, at serum concentration of 7.5%, no difference between their abilities in lysis, both show about 28% lysis. Similarly, running haemolytic diffusion plate assay between CL-11 deficient and wild type mouse sera shows no statistical difference in their abilities to lyse chicken erythrocytes (see figure 3.4). Thus it can be seen clearly that the LP recognition molecules have no role in complement mediated haemolysis in non-immune mouse sera.

MASP-2 is a key component of the LP and a deficiency of MASP-2 leads to the complete loss of the lectin pathway C3 convertase complex C4bC2a (Schwaeble *et al.,* 2011). To assess the role of MASP-2 in complement mediated haemolysis, MASP-2 deficient mouse serum was used to lyse rabbit erythrocytes and compared that of pooled wild type serum (see figure 3.5). Running haemolytic assay, tube method, in barbital buffered saline shows that no statistically significant difference can be achieved between the ability of MASP-2 deficient mouse serum and pooled wild type serum in lysing rabbit erythrocytes at serum concentrations of 12.5% and 6.25%.

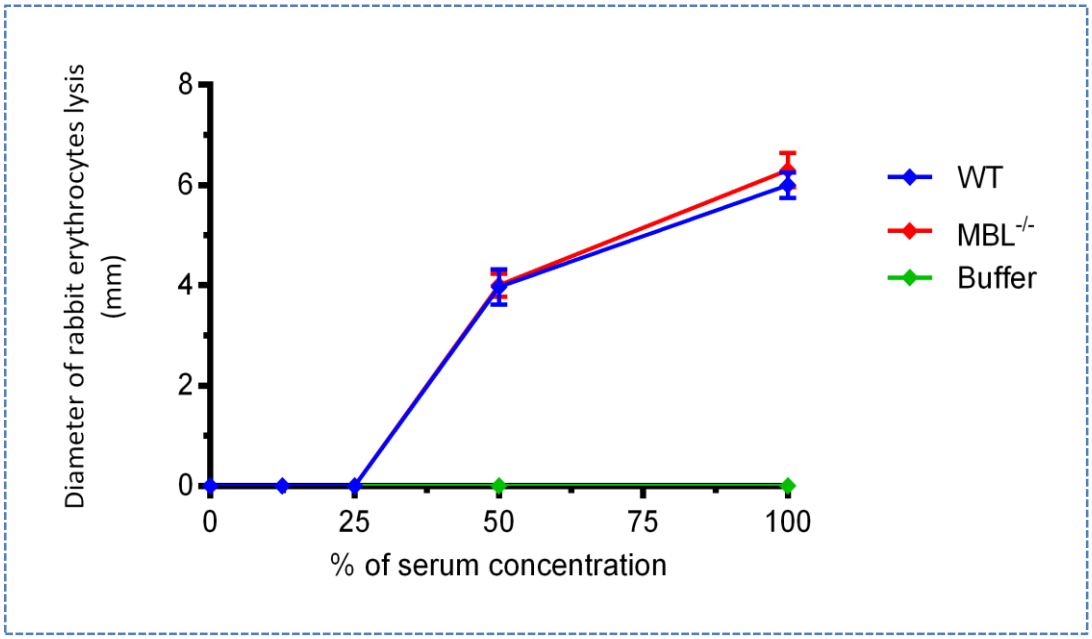

Figure 3.2: MBL deficiency does not affect the haemolytic activity of moue serum on rabbit erythrocytes

Haemolytic diffusion plate assay using MBL deficient and pooled wild type mouse sera against rabbit erythrocytes. Barbital buffered saline (BBS/Ca^{+2}/Mg^{+2}) was used to run the assay. An un-paired student t-test was performed. P values are > 0.05 at both serum concentrations, 100% and 50%, between MBL-/- and WT type mouse sera. Heat inactivated mouse serum was used as a negative control. Results are mean (±SEM) of three independent experiments.

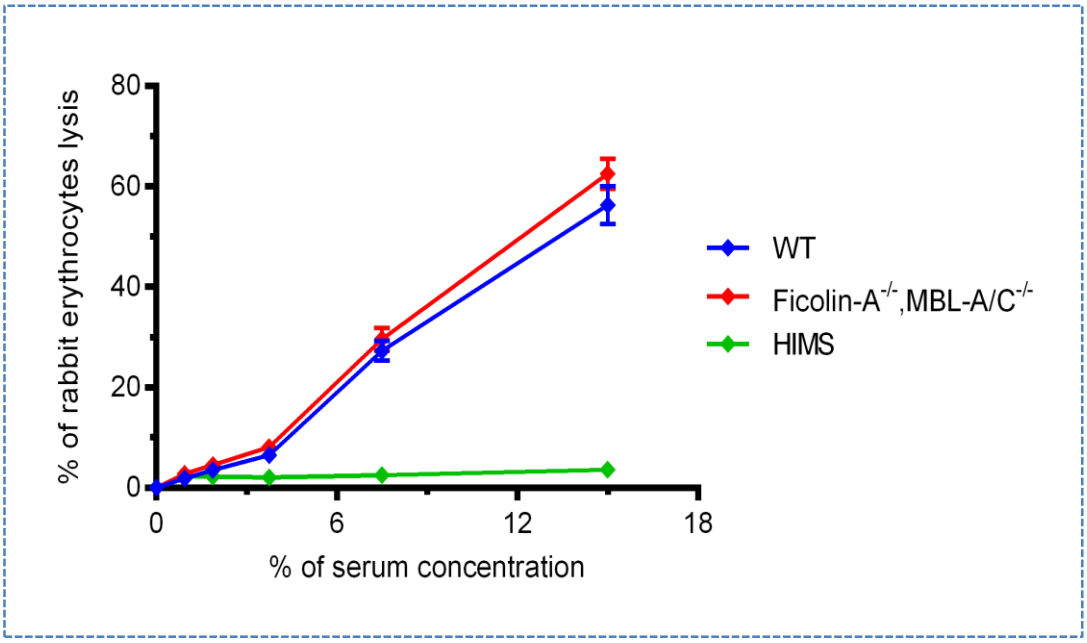

Figure 3.3: Deficiency of Ficolin A and, MBL A and C does not affect the haemolytic activity of mouse serum on rabbit erythrocytes

Haemolytic assay, tube method, using Ficolin A, MBL A and C deficient and pooled wild type mouse sera against rabbit erythrocytes. Barbital buffered saline (BBS/Ca^{+2}/Mg^{+2}) was used to run the assay. % of lysis showing in relates to lysis in nano water (100% lysis). An un-paired student t-test was performed: P value at serum concentrations of 12.5% and 6.25% of ficolin-$A^{-/-}$, MBL-$A/C^{-/-}$ and pooled WT equal 0.266 ($P>0.05$) and 0.478 ($P>0.05$) respectively. Heat inactivated mouse serum was used as a negative control. Results are mean (±SEM) of three independent experiments.

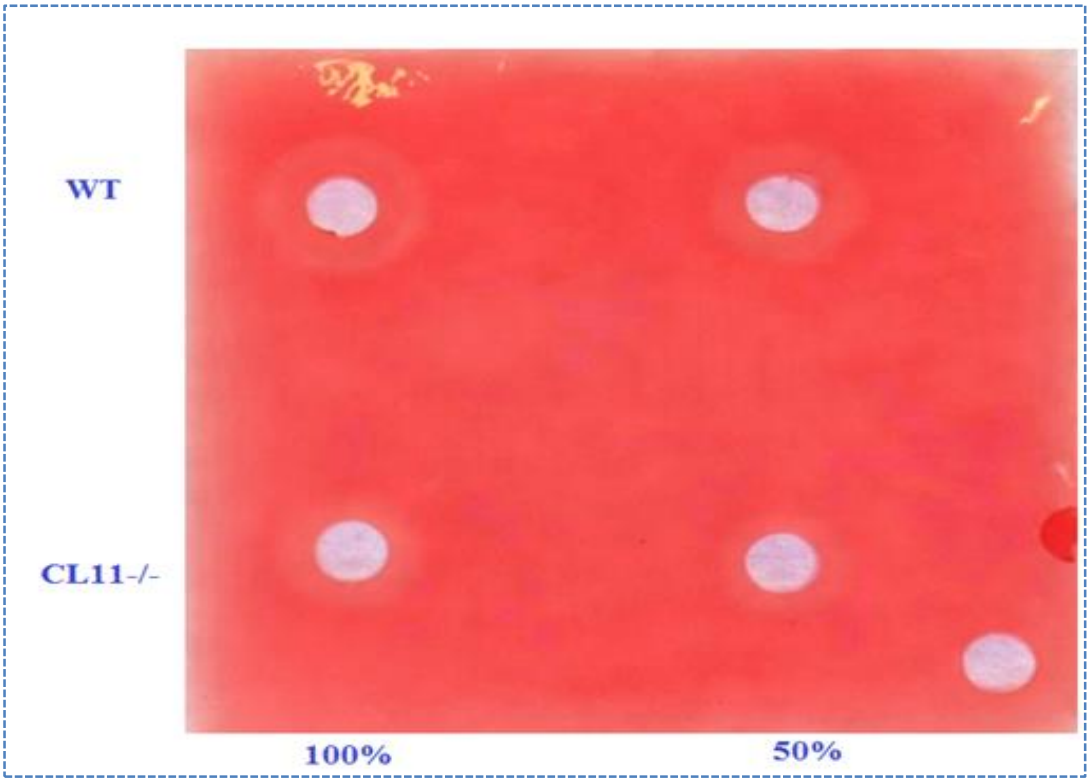

Figure 3.4: Deficiency of CL11 does not affect the haemolytic activity of mouse serum on chicken erythrocytes

Haemolytic diffusion plate assay using pooled wild type and CL-11 deficient sera on chicken erythrocytes. Barbital buffered saline (BBS/Ca^{+2}/Mg^{+2}) was used to run the assay. Heat inactivated mouse serum was used as a negative control. An un-paired student t-test was performed. P values are > 0.05 at both serum concentrations, 100% and 50%, between $CL11^{-/-}$ and WT type mouse sera. The shown result is representative of three independent experiments with the same or very similar experimental outcome.

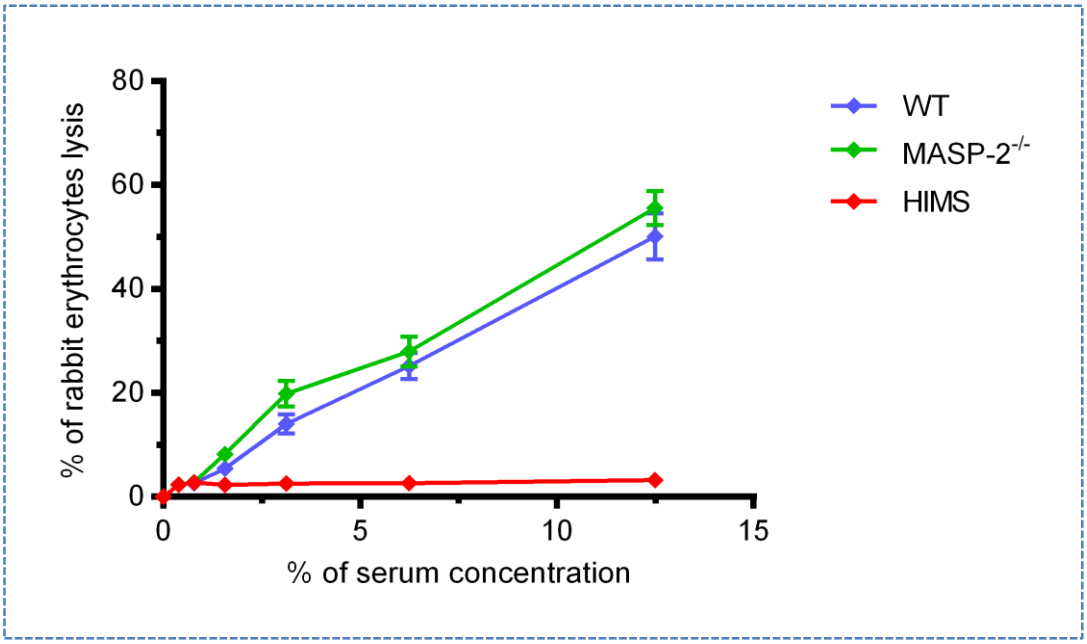

Figure 3.5: Deficiency of MASP-2 does not affect haemolytic activity of mouse serum on rabbit erythrocytes
Haemolytic assay (tube method) comparing the haemolytic activity of MASP-2 deficient and pooled wild type mice sera against rabbit erythrocytes. Barbital buffered saline (BBS/Ca^{+2}/Mg^{+2}) was used to run the assay. % of lysis showing in relates to lysis in nano water (100% lysis). An un-paired student t-test was performed: P values at 12.5% and 6.25% of MASP-$2^{-/-}$ versus WT are statistically not significant (P= 0.383 and 0.502 respectively). Heat inactivated mouse serum was used as a negative control. Results are mean (±SEM) of three independent experiments.

3.1.1.3 The alternative pathway in mouse plays a crucial role in complement mediated haemolytic activity

The main components of the alternative pathway of complement are C3, Factor B, properdin and factor D. The absence of any of these components leads to the complete loss of alternative pathway functional activity. Similarly, recent data indicated that a deficiency of the lectin pathway serine proteases MASP-1 and MASP-3 renders the AP deficient (Takahashi *et al.*, 2010). I therefore tested and compared the ability of Factor D deficient mouse serum, Factor B deficient mouse serum and MASP-1/3 deficient mouse serum to lyse rabbit erythrocytes

and compared to wild type serum (see figures 3.6 and 3.7) and (tables 3.1 and 3.2.). As can be seen in figure 3.6, running tube method haemolytic assay in barbital buffered saline, at serum concentrations of 12.5%, in relates to lysis in nano water (100% lysis), pooled wild type serum produces 40% of lysis while $fB^{-/-}$, $fD^{-/-}$ sera produce only 13% and 16% lysis respectively. These differences between pooled wild type and $fD^{-/-}$ sera and pooled wild type and $fB^{-/-}$ sera are statistically significant (see table 3.1). Similar results were obtained when using serum concentrations of 6.25% (see table 3.2).

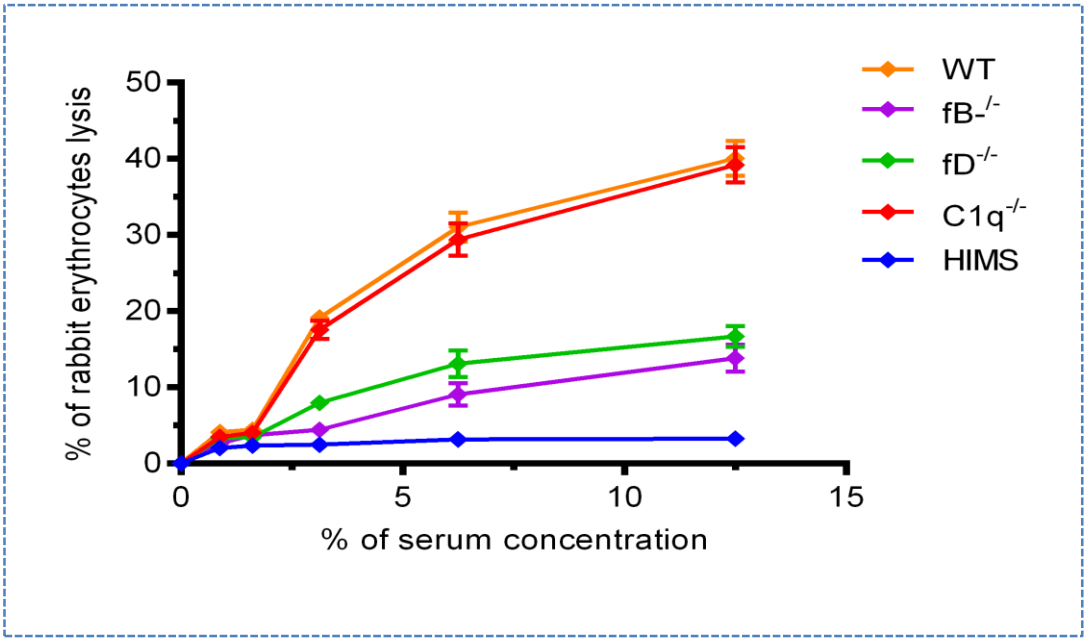

Figure 3.6: Deficiency of the alternative pathway abolishes haemolytic activity of mouse serum toward rabbit erythrocytes

Haemolytic assay, tube method, by using factor D deficient, factor B deficient and pooled wild type mouse sera against rabbit erythrocytes. Barbital buffered saline (BBS/Ca^{+2}/Mg^{+2}) was used to run the assay. % of lysis showing in relates to lysis in nano water (100% lysis). Heat inactivated mouse serum was used as a negative control. Results are mean (±SEM) of three independent experiments.

Table 3.1: Statistical significant assessed between the haemolytic activity of different mouse sera against rabbit erythrocytes using Student`s t-test

Students t test (At 12.5% serum concentration)		
	Statistically significant	P value summary
WT vs $fB^{-/-}$	Yes	*** (0.0008)
WT vs $fD^{-/-}$	Yes	*** (0.001)
WT vs $C1q^{-/-}$	No	(0.801)

Table 3.2: Statistical significant assessed between the haemolytic activity of different mouse sera against rabbit erythrocytes using Student`s t-test

Student`s t test (At 6.25% serum concentration)		
	Statistically significant	P value summary
WT vs $fB^{-/-}$	Yes	*** (0.0008)
WT vs $fD^{-/-}$	Yes	** (0.0023)
WT vs $C1q^{-/-}$	No	(0.604)

This indicates an important role of the alternative pathway in complement mediated haemolytic activity. Similarly, as can be seen in figure 3.7, the ability of MASP-1/3 deficient serum to lyse rabbit erythrocytes is significantly impaired in comparison to wild type serum control. Running tube method haemolytic assay in barbital buffered saline, at serum concentration of 10%, pooled wild type, MASP-2 deficient and MASP1/3 deficient sera show 45%, 50% and 12% lysis respectively and at serum concentration of 5%, wild type, MASP-2 deficient and MASP1/3 deficient show 26%, 30% and 10% respectively. These differences between MASP1/3 deficient and wild type sera are statistically significant (see tables 3.3 and 3.4).

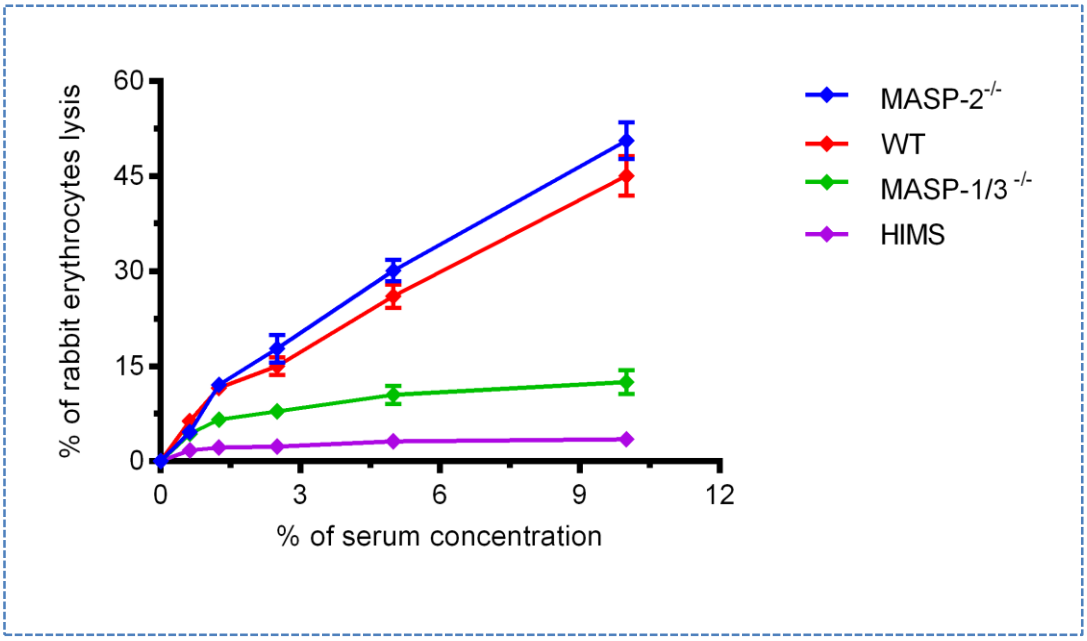

Figure 3.7: Deficiency of MASP-1/3 abolishes haemolytic activity of mouse serum towards rabbit erythrocytes

Haemolytic assay, tube method, by using pooled wild type, MASP-2$^{-/-}$, and MASP1/3$^{-/-}$ mouse sera against rabbit erythrocytes. Barbital buffered saline (BBS/Ca^{+2}/Mg^{+2}) was used to run the assay. % of lysis showing in relates to lysis in nano water (100%). Heat inactivated mouse serum was used as a negative control. Results are mean (±SEM) of three independent experiments.

Table 3.3: Statistical significant assessed between the haemolytic activity of different mouse sera against rabbit erythrocytes using Student`s t-test

Students t test (At 10% serum concentration)		
	Statistically significant	P value summary
WT vs MASP-2$^{-/-}$	No	(0.261)
WT vs MASP-1/3$^{-/-}$	Yes	*** (0.0009)

Table 3.4: Statistical significant assessed between the haemolytic activity of different mouse sera against rabbit erythrocytes using Student`s t-test

Students t test (At 5% serum concentration)		
	Statistically significant	P value summary
WT vs MASP-2$^{-/-}$	No	(0.190)
WT vs MASP-1/3$^{-/-}$	Yes	** (0.0026)

3.1.1.4 MASP-3 plays an important role in complement mediated haemolysis in mouse

The previous result showed that MASP-1/3 deficient serum lacks the ability to lyse rabbit erythrocytes. In order to clarify whether MASP-3 or MASP-1 or both are required for complement mediated lysis, recombinant full length human MASP-3 (FL MASP-3) was preincubated with MASP-1/3 deficient serum for 30 minutes on ice and then used to run the haemolytic assay. This reconstitution restored haemolytic activity of MASP-1/3$^{-/-}$ serum to a level that was even higher than that seen in pooled wild type serum (see figure 3.8). However, this difference is not statistically significant. Running tube method haemolytic assay in barbital buffered shows MASP-1/3$^{-/-}$ serum at a serum concentration of 10% produces about 10% lysis. Following reconstitution of MASP-1/3 deficient serum with recombinant FL MASP-3, the serum haemolytic activity increased in a concentration dependent manner. When adding recombinant FL MASP-3 at a final concentration of 3.12 μg/ml (w/v), haemolysis increased to 30% lysis, same amount as see with pooled wild type serum. Adding recombinant FL MASP-3 at a final concentration of 6.25 μg/ml achieved 38% lysis. No more lysis was achieved by adding higher concentrations of recombinant protein. Similar result achieved by running haemolytic diffusion plate assay on chicken

erythrocytes (see figure 3.9). At a serum concentration of 100% and 50%, MASP1/3$^{-/-}$ serum does not show any lysis and addition of recombinant FL MASP-3 to MASP1/3$^{-/-}$ serum restores haemolytic activity of this serum. This restoration of haemolytic activity of MASP-1/3$^{-/-}$ serum with recombinant FL MASP3 indicates MASP-3 plays important role in complement mediate haemolytic activity and MASP-1 dose not has role (when using activated MASP-3) as restoration of haemolytic activity is complete. Furthermore, chicken serum which has MASP-3 and not MASP-1 does not compromise in the ability to lyse rabbit and guinea pig erythrocytes (see figures 3.10 and 3.11). Another interesting point is that the ability of chicken serum to lyse both rabbit and guinea pig erythrocytes dose not affected by the absence of calcium i.e. running assay under alternative pathway specific condition by using AP buffer, which inhibits both the classical and lectin pathways` functions and allows the AP full functioning.

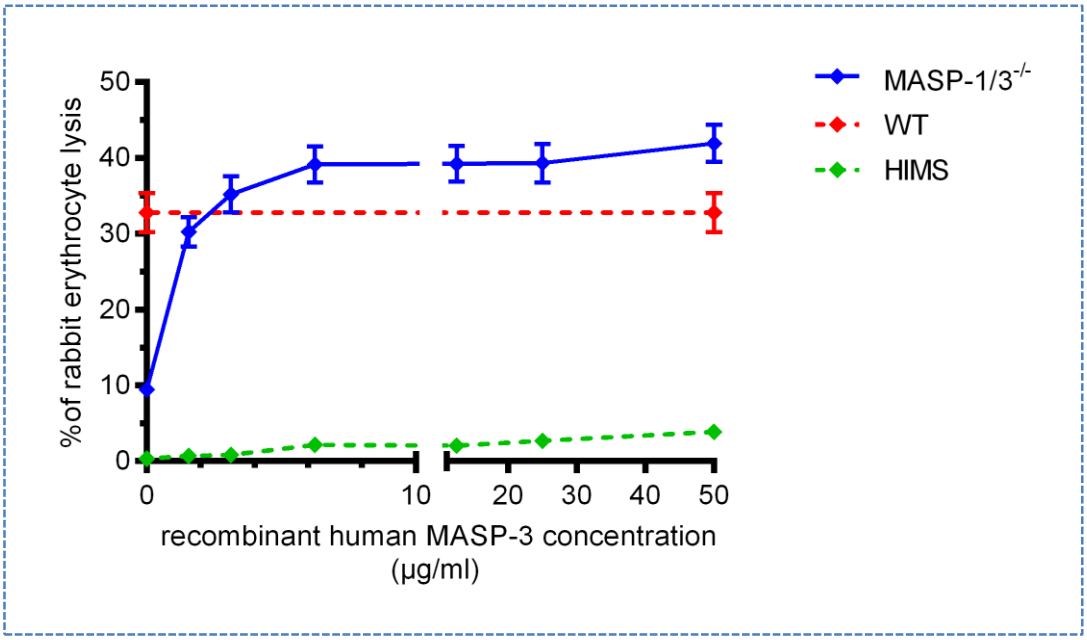

Figure 3.8 : Human full length MASP-3 restores haemolytic activity of MASP-1/3 deficient mouse serum towards rabbit erythrocytes

Haemolytic assay, tube method, using 10% MASP-1/3$^{-/-}$ mouse serum against rabbit erythrocytes. The assay was run in barbital buffered saline (BBS/Ca^{+2}/Mg^{+2}). Prior to the addition of erythrocytes, the serum was reconstituted with increasing concentrations of recombinant human full length MASP-3 (w/v). % of lysis showing in relates to nano water lysis (100%). Heat inactivated mouse serum was used as a negative control. Results are mean (±SEM) of three independent experiments.

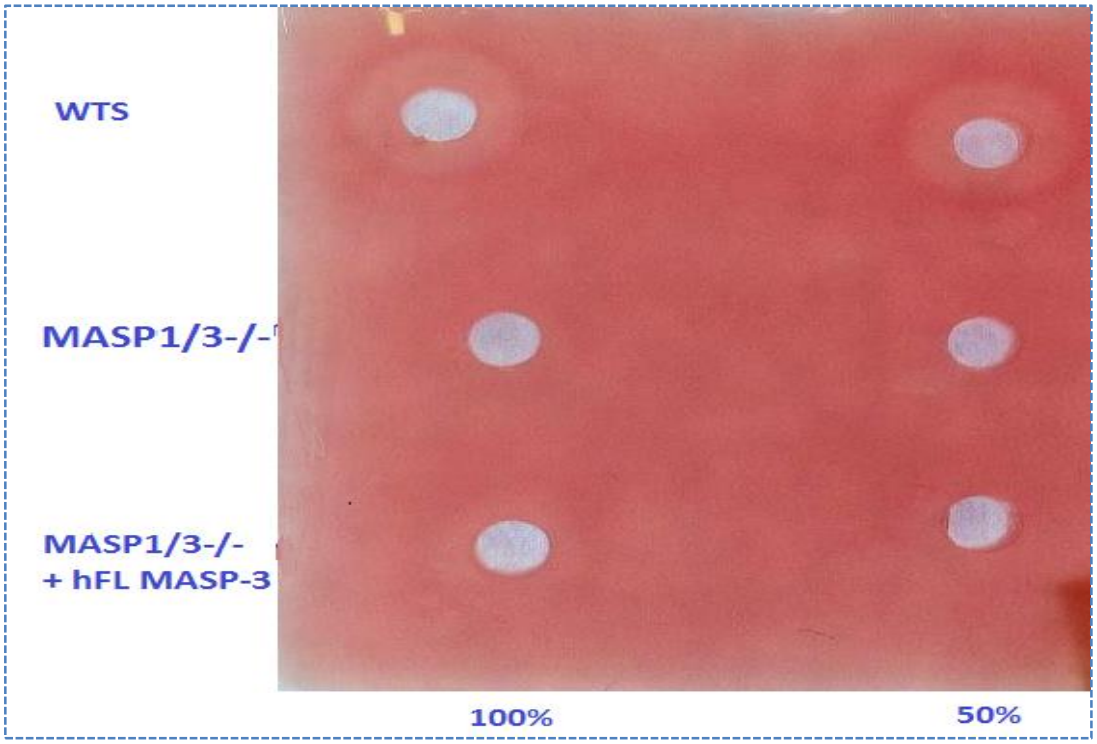

Figure 3.9: Human full length MASP-3 restores haemolytic activity of MASP-1/3 deficient mouse serum towards chicken erythrocytes

Haemolytic diffusion plate assay using wild type, MASP1/3 deficient and reconstituted MASP1/3 deficient serum with recombinant human full-length MASP-3 in a final concentration of 20 µg/ml (w/v) on chicken erythrocytes. Barbital buffered saline (BBS/Ca^{+2}/Mg^{+2}) was used to run the assay. MASP1/3 deficiency heat inactivated mouse serum was used as a negative control. Result represents three independent experiments with the same or very similar experimental outcome.

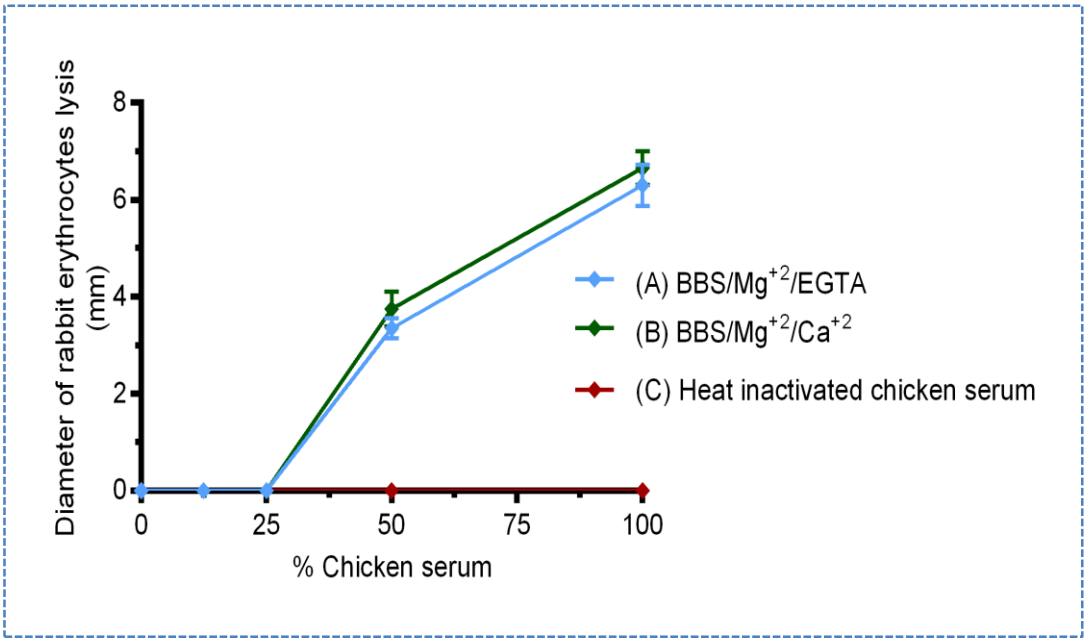

Figure 3.10: Absence of calcium does not diminish haemolytic activity of chicken serum towards rabbit erythrocytes

Haemolytic diffusion plate assay using chicken serum on rabbit erythrocytes. (A) The assay was run under alternative pathway-specific condition (BBS/Mg^{+2}/EGTA). (B) The assay was run in the barbital buffered saline (BBS/Ca^{+2}/Mg^{+2}). P value between A and B at different chicken serum concentrations, 100% and 50%, are > 0.05. Heat inactivated chicken serum was used as a negative control. Results are mean (±SEM) of three independent experiments.

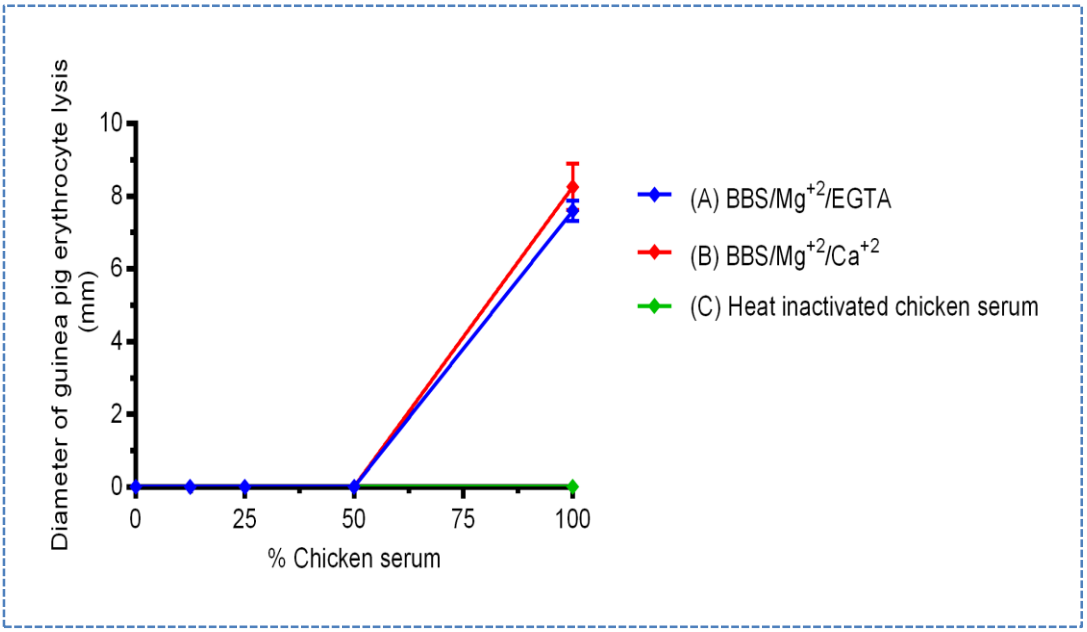

Figure 3.11: Absence of calcium does not diminish haemolytic activity of chicken serum towards guinea pig erythrocytes

Haemolytic diffusion plate assay using chicken serum on guinea pig erythrocytes. (A) The assay was run under alternative pathway specific condition by using AP buffer (BBS/Mg^{+2}/EGTA). (B) The assay was run in the barbital buffered saline (BBS/Ca^{+2}/Mg^{+2}). P value between A and B at different chicken serum concentrations, 100% and 50%, are > 0.05. Heat inactivated chicken serum was used as a negative control. Results are mean (±SEM) of three independent experiments.

3.1.1.5 Addition of external recombinant murine properdin increases haemolytic activity of wild type mouse serum

Previous results show that the AP plays an important role in the complement mediated haemolytic activity in unimmunised mouse and chicken sera. To assess whether addition of external recombinant properdin affects haemolytic activity in mouse sera, 20 µg/ml of recombinant murine properdin was added to wild type mouse serum. This concentration of recombinant murine properdin was chosen after serious of experiments to determine the concentration that has the highest effect on pooled wild type serum (data not shown). This addition enhances haemolytic activity of wild type mouse serum

dramatically (see figure 3.12). In relates to lysis in nano water (set to 100% lysis) at a serum concentration of 7.5%, wild type shows 16% lysis and serum ability increases to about 30% lysis by addition of recombinant murine properdin. This difference is statistically significant. Similarly, statistically significant enhancement of WT serum haemolytic activity was achieved by this addition at the serum concentration of 3.75% and 1.85%.

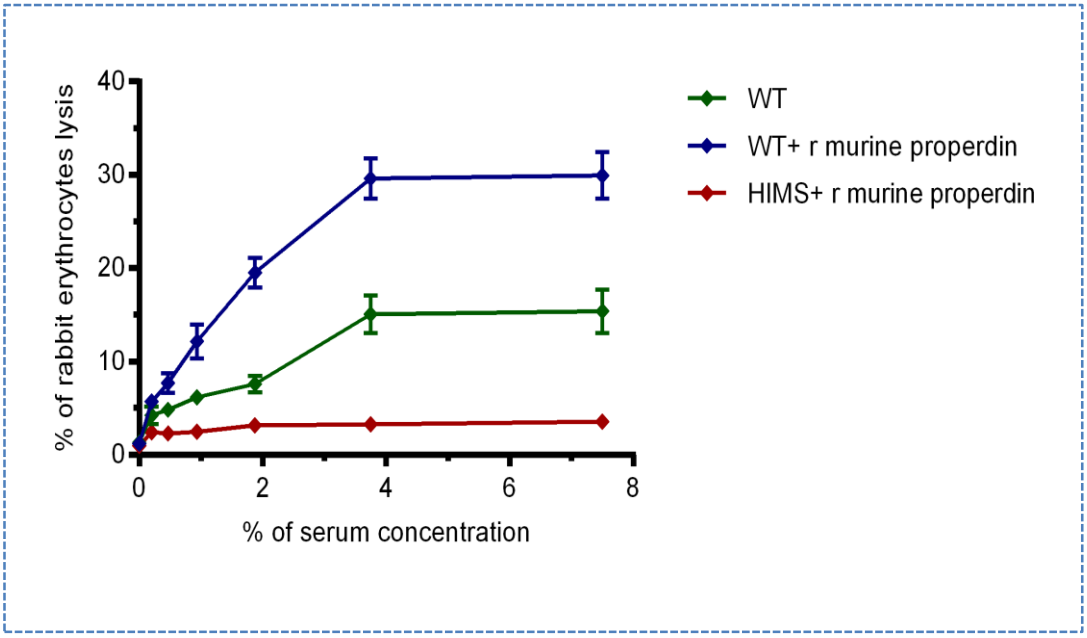

Figure 3.12: Extrinsic recombinant murine properdin enhances haemolytic activity of mouse serum towards rabbit erythrocytes

Haemolytic assay, tube method, against rabbit erythrocytes comparing the haemolytic activity of pooled wild type mouse serum before and after addition of 20 µg/ml (w/v) of recombinant murine properdin. Barbital buffered saline (BBS/Ca^{+2}/Mg^{+2}) was used to run the assay. % of lysis showing in relates to lysis in nano water (100% lysis). An un-paired student t-test was performed: P value at 7.5% of mouse serum versus mouse serum with recombinant properdin is equal 0.0084 ($P<0.05$). At 3.75% and 1.85% serum concentration is equal 0.0074 and 0.0029 respectively. Heat inactivated mouse serum with 20 µg/ml of recombinant murine properdin was used as a negative control. Results are mean (±SEM) of three independent experiments.

3.1.2 Analysis of human sera with either MASP-3 or combined MASP-3 and MASP-1 deficiency, i.e. sera of 3MC patients

The previous result by using mouse serum clearly showed that MASP-3 plays important role in the complement mediated haemolysis. To assess the role of MASP-3 in human complement mediated haemolysis, 3MC sera from two patients were used to lyse erythrocytes from three different species which are rabbit, guinea pig and chicken erythrocytes. The sera from two 3MC patients have been used in in this thesis. Patient 2 has a mutation in exon 12 of the *MASP1* gene. Because this exon encodes only the light chain of MASP-3 (serine protease domain of MASP-3), the patient still has MASP-1 (Rooryk *et al.*, 2011). While, there is mutation in codon 4 in patient 3. This mutation causes insertion of a stop codon within the leader peptide sequence, leading to premature termination of protein biosynthesis. As a result of this, patient has deficiency in both serine proteases of MASP-1 and MASP-3 and truncated third gene product, MAp44 (mutation detected by sequencing; West Midlands Genetics Service). Patient 3 is also MBL deficient (Professor Wilhelm Schwaeble laboratory work).

3.1.2.1 3MC sera significantly impaired complement mediated haemolytic activity through alternative pathway

The complement mediated haemolytic activity is significantly impaired in 3MC sera while running assay under the alternative pathway specific condition i.e. using AP buffer (BBS/Mg^{+2}/EGTA) (see figure 3.13). s can be seen in figure 3.13, in relates to lysis in nano water (100%), pooled normal human serum at the concentration of 12.5% showed lysis of 75% of rabbit erythrocytes while 3MC sera, patient 2 and patient 3, at the same concentration showed lysis of 27% and 32% of rabbit erythrocytes, respectively. These differences between NHS and

3MC sera are statistically significant (see table 3.5). Similarly, there are statistically significant differences in lysis at the serum concentration of 6.25% between the NHS and 3MC sera (see table 3.6). As pooled normal human serum at the concentration of 6.25% showed lysis of 37% of rabbit erythrocytes while 3MC sera at this concentration, patient 2 and patient 3, showed lysis of 12% and 16% respectively. Statistically significant differences also achieved at the serum concentration of 3.12% (see table 3.7). Similar result obtained by using guinea pig erythrocytes (see figure 3.14). MBL deficient human plasma was used because 3MC (patient 3) is deficient also in MBL and result showed MBL deficiency does not affect the serum ability in lysing guinea pig erythrocytes. Both father and mother of patient 3 have heterogeneous mutation in the *MASP1* gene but full functioning MASP-1 and MASP-3 (mutation was detected by sequencing; West Midlands Genetics Service). Furthermore, both of them are also deficient in MBL (Professor Wilhelm Schwaeble laboratory work). As can be seen in figure 3.14, at the plasma concentration of 100%, pooled normal, father and mother of patient 3 and MBL deficient plasma show about 9.5 mm lysis while patient 3 plasma just shows 2.5 mm of lysis. This difference is statistically significant. At the plasma concentration of 50%, patient 2 plasma does not show any lysis while others show 8.8 mm of lysis. Patient 2 is deficient in MASP-3 and has MASP-1 while patient 3 is deficient in both MASP-3 and MASP-1. The patient 2 has MASP-1 and lacks the ability to lyse rabbit erythrocytes in the AP assay. This indicates that MASP-3 plays a pivotal role in the alternative pathway mediated haemolytic activity in human.

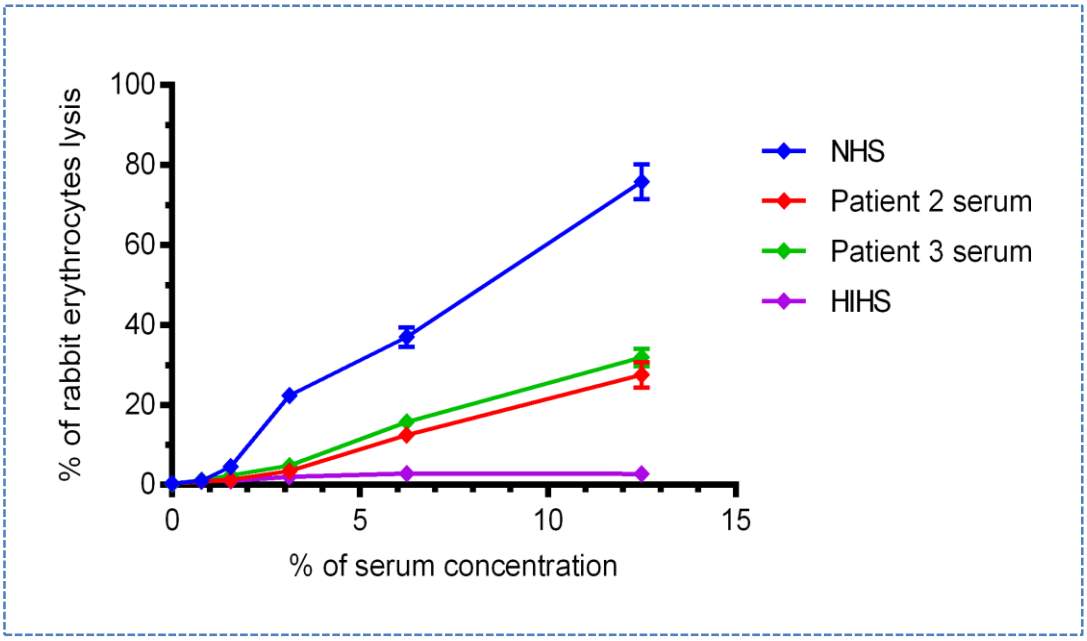

Figure 3.13: 3MC sera are significantly compromised in their haemolytic activities toward rabbit erythrocytes

Haemolytic assay (tube method) by using pooled normal human serum (NHS) and 3MC sera (patient 2 and patient 3) against rabbit erythrocytes. The assay was run under alternative pathway specific condition i.e. using AP buffer (BBS/Mg^{+2}/EGTA). % of lysis showing in relates to lysis in nano water (100%). Heat inactivated human serum was used as a negative control. Results are mean (±SEM) of three independent experiments.

Table 3.5: Statistical significant assessed between the haemolytic activity of pooled human and 3MC sera against rabbit erythrocytes using Student`s t-test

Student`s t test (At 12.5% serum concentration)		
	Statistically significant	P value summary
NHS vs patient 2 serum	Yes	*** (0.0009)
NHS vs patient 3 serum	Yes	*** (0.0009)

Table 3.6: Statistical significant assessed between the haemolytic activity of pooled human and 3MC sera against rabbit erythrocytes using Student`s t-test

Student`s t test (At 6.25% serum concentration)		
	Statistically significant	P value summary
NHS vs patient 2 serum	Yes	** (0.0014)
NHS vs patient 3 serum	Yes	** (0.0015)

Table 3.7: Statistical significant assessed between the haemolytic activity of pooled human and 3MC sera against rabbit erythrocytes using Student`s t-test

Student`s t test (At 3.12% serum concentration)		
	Statistically significant	P value summary
NHS vs patient 2 serum	Yes	*** (0.0002)
NHS vs patient 3 serum	Yes	*** (0.0005)

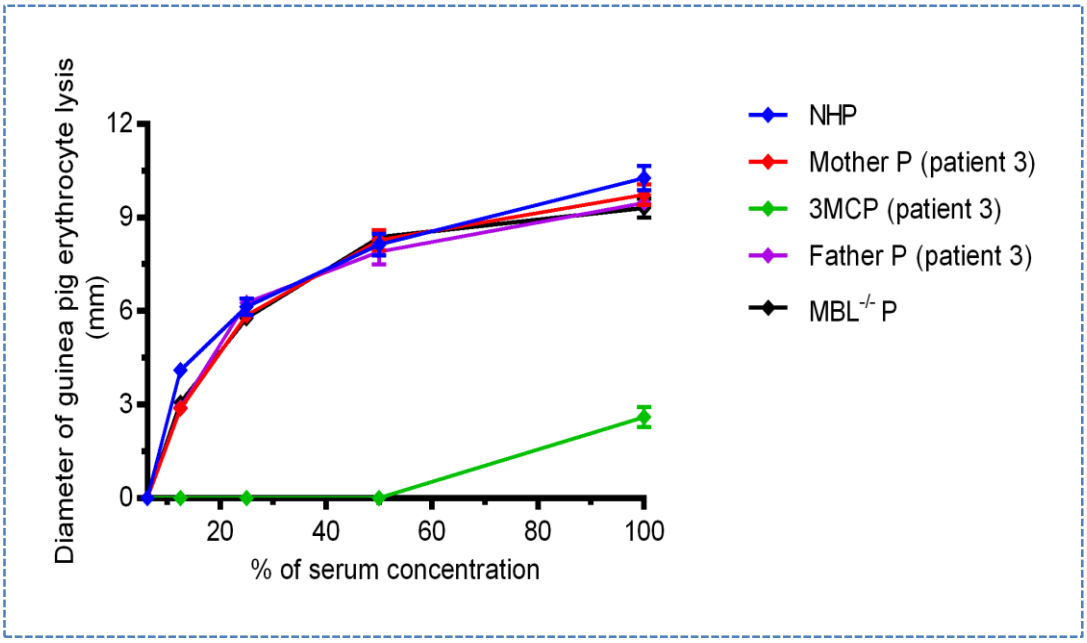

Figure 3.14: 3MC serum is significantly compromised in their haemolytic activity toward guinea pig erythrocytes

Haemolytic diffusion plate assay using normal human plasma (NHP), $MBL^{-/-}$ plasma, 3MC plasma (patient 3), and father and mother of patient 3 plasma against guinea pig erythrocytes. The assay was run under alternative pathway specific condition i.e. using AP buffer (BBS/Mg^{+2}/EGTA). An un-paired student t-test was performed: P value at a 100% serum concentration of NHS with Patient 3 is equal 0.0002. Heat inactivated human serum was used as a negative control. Results are mean (±SEM) of three independent experiments.

3.1.2.2 Supplementation of 3MC sera with recombinant MASP-3 restores haemolytic activity

MASP-3 deficient sera are impaired complement mediated lysis under the alternative pathway specific assay condition. To identify whether this impairment is due to the deficiency of MASP-3 or some other condition within the 3MC sera, recombinant human MASP-3 was pre-incubated with 3MC sera (patient 2 and patient 3) for 30 minutes on ice before running the assay. This restoration reconstitutes the haemolytic activity of 3MC sera. The assay was run

under alternative pathway specific condition. By reconstituting 3MC (patient 2 and patient 3) sera or plasma with recombinant human MASP-3 the haemolytic activities of 3MC sera toward rabbit (see figures 3.15, 3.16 and 3.17), chicken (see figure 3.18) and guinea pig (see figures 3.19 and 3.20) erythrocytes were restored in a concentration dependent manner. As seen in figure 3.15, the haemolytic activity of 7% plasma of 3MC patient 2 and patient 3 are about 15% and 13% respectively compared to the lysis in nano water which is set as 100%. Addition of recombinant full length human MASP-3 (FL MASP-3) was restored haemolytic activity in a concentration dependent manner. At a final concentration of 25 μg/ml (w/v) of extrinsically added FL MASP-3, the haemolytic activity of patient 2 reaches about 68% and in 3MC patient 3 about 58%. At a final concentration of 100 μg/ml (w/v) of the recombinant FL MASP-3, the plasma of patient 2 shows higher haemolytic activity (101%) than that seen in 3MC plasma of patient 3 which reaches about 84% of lysis. These differences in haemolytic activity between the two MASP-3 reconstituted 3MC plasma samples are statistically not significant. Haemolytic activity of 3MC sera was also restored when using enzymatically active truncated form of recombinant human MASP-3 (composed of the two C-terminal domains CCP1/ CCP2 of the heavy chain and the MASP-3 specific serine protease domain). 3MC sera were preincubated with increasing concentrations of recombinant either full-length or truncated human MASP-3 (tr MASP-3) for 30 minutes on ice before adding erythrocytes. Running haemolytic assay (tube method) under alternative pathway specific condition, both truncated and FL MASP-3 enhanced the lytic activity of 3MC sera in a dose dependent manner (see figure 3.16). In direct comparison full-length MASP-3 (expressed in a eukaryotic expression system) was more effective in restoring serum haemolytic activity than the truncated MASP-3 expressed in a bacterial expression system, purified from bacterial inclusion bodies and refolded. As shown in figure 3.16, adding FL MASP-3 to 7% 3MC serum at a final concentration of 6.25 μg/ml achieves 53% of the lysis

seen in nano water (100% lysis) while adding the same quantity of tr MASP-3 increased lysis to only 25%. This difference is statistically significant (see table 3.8). Similarly, statistically significant differences can be achieved by adding these recombinant protein preparations at a final concentration of 12.5, 25 and 50 μg/ml (w/v). At the recombinant protein concentration of 12.5 μg/ml, FL MASP-3 increased lysis up to 62%, while addition of the same quantity of tr MASP-3 increased lysis to 30%. At the final concentrations of 25 μg/ml, they showed lysis of 70% and 45% respectively. While the lysis stays steady after addition of 50 μg/ml of FL MASP-3 (70%) but by adding this concentration of tr MASP-3, the lysis increased to 58%.

Using haemolytic diffusion plate assay, it can be seen that the serum of 3MC patient 3 does not lyse any rabbit erythrocytes at all, even at 100% serum concentration when running assay under alternative pathway specific condition .However, after pre-incubating 3MC serum with a final concentration of 40 μg/ml (w/v) of recombinant truncated human MASP-3, the haemolytic activity of patient 3 serum restored to approximately the same level of NHS as both show the circle of haemolysis with the diameter of about 8.5 mm in a serum concentration of 100% and 6.5 mm in a serum concentration of 50%. Similar result is obtained by using both chicken (see figure 3.18) and guinea pig erythrocytes (see figures 3.19 and 3.20).

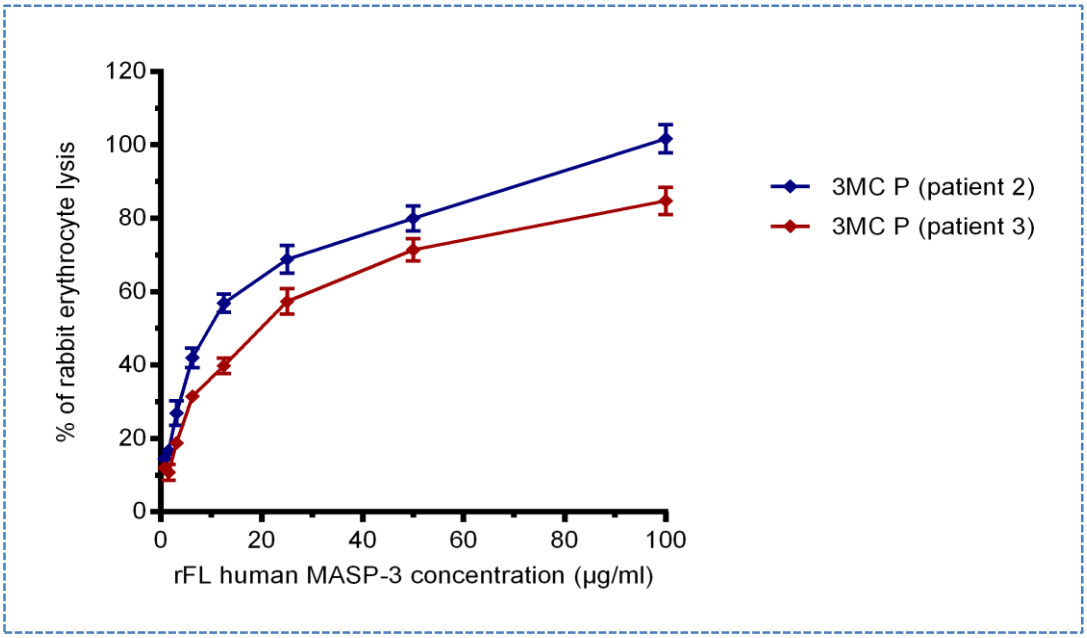

Figure 3.15: Full length human MASP-3 restores haemolytic activity of 3MC sera toward rabbit erythrocytes

Haemolytic assay (tube method) is performed by using 7% 3MC (patient 2 and patient 3) sera against rabbit erythrocytes. The assay was run under alternative pathway specific condition by using AP buffer. Prior to the addition of RBC, the sera were reconstituted with increasing concentrations of recombinant human full length MASP-3. % of lysis showed in relates to lysis in nano water (100% lysis). Results are mean (±SEM) of three independent experiments.

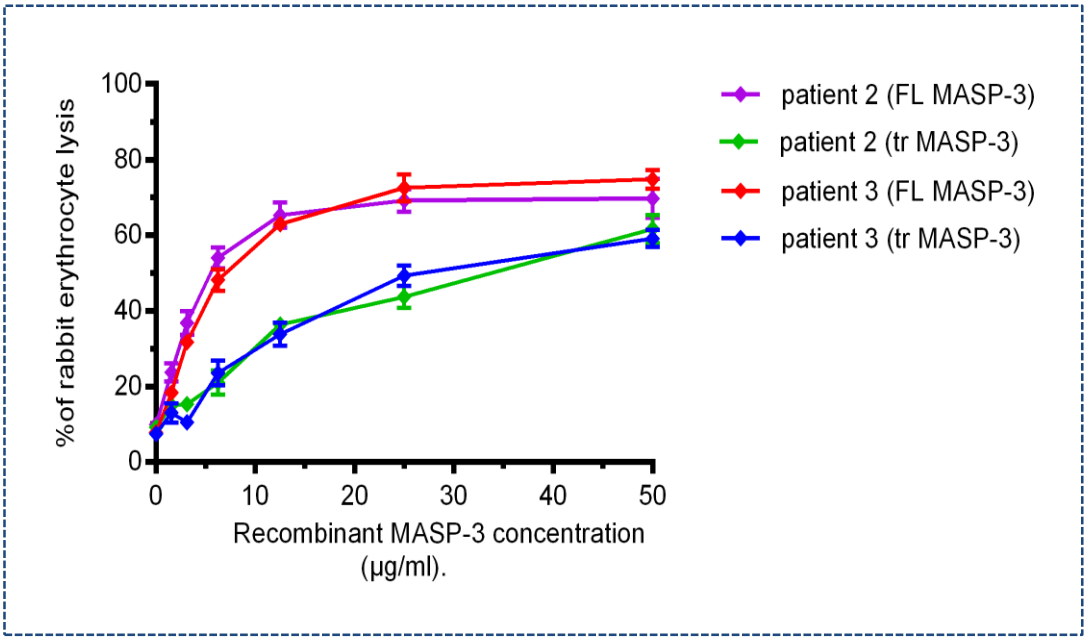

Figure 3.16: Both truncated and full length human MASP-3 restore haemolytic activity of 3MC sera towards rabbit erythrocytes

Haemolysis of rabbit erythrocytes in 7% 3MC sera (tube method). The assay was run under alternative pathway specific condition by using AP buffer (BBS/Mg^{+2}/EGTA). Prior to the addition of erythrocytes, the sera were reconstituted with increasing concentrations of recombinant full length human MASP-3 (FL MASP-3) and recombinant truncated human MASP-3 (tr MASP-3). % of lysis showed in relates to lysis in nano water (100% lysis). Results are mean (±SEM) of three independent experiments.

Table 3.8: Statistical significant assessed between the haemolytic activity of reconstituted 3MC sera (patient2 and patient 3) with FL MASP-3 and tr MASP-3 against rabbit erythrocytes using Student`s t-test

Recombinant concentration (µg/ml)	Statistically significant	P value summary
6.25	Yes	*** (0.0001)
12.5	Yes	*** (0.0001)
25	Yes	*** (0.0001)
50	Yes	** (0.0065)

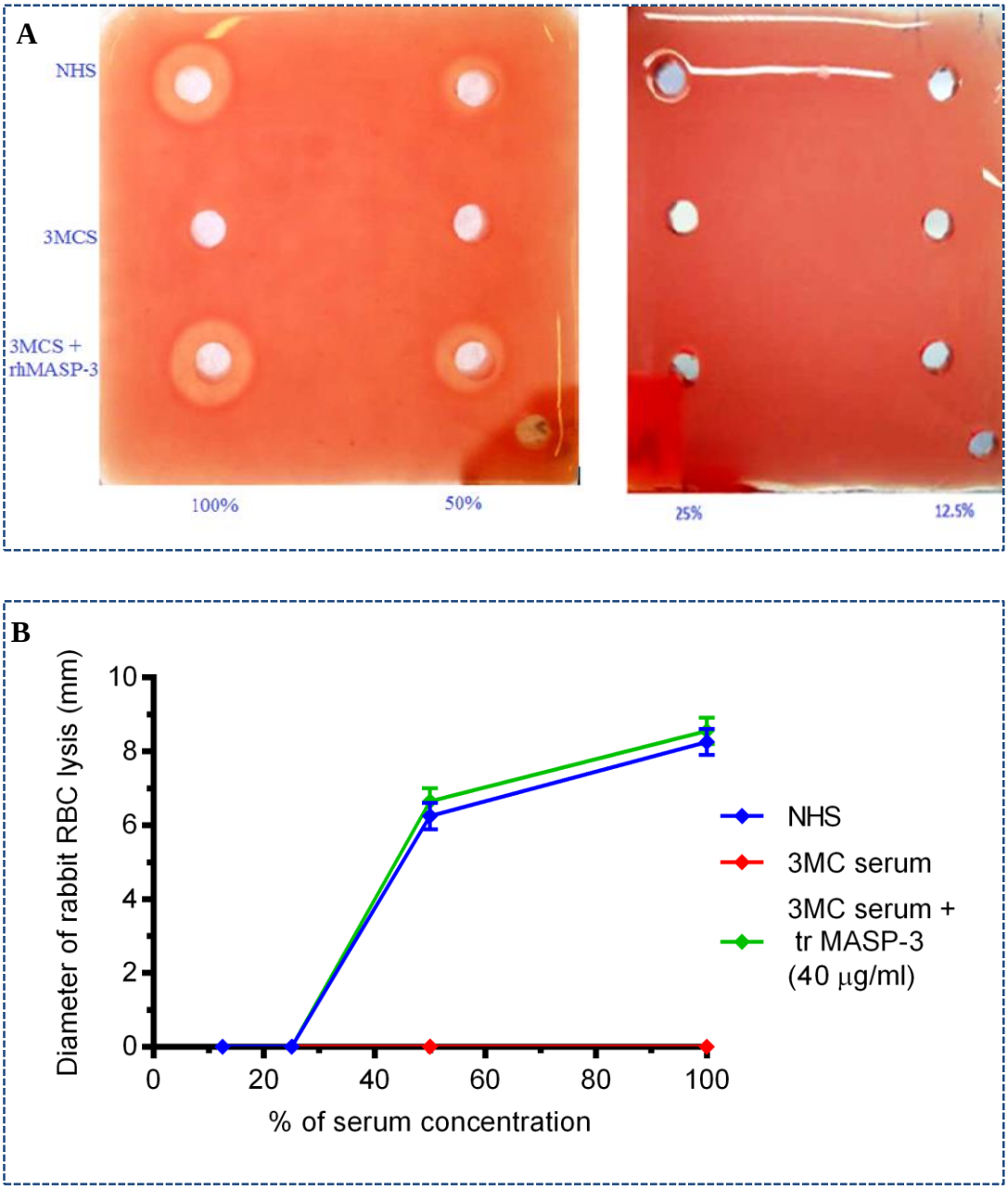

Figure 3.17: Truncated human MASP-3 restores haemolytic activity of 3MC serum toward rabbit erythrocytes

Haemolytic diffusion plate assay by using 3MC (patient 3) serum against rabbit erythrocytes. The assay was run under alternative pathway specific condition in the AP buffer (BBS/Mg^{+2}/EGTA). Prior to the addition of the serum, the sera were reconstituted with 40 μg/ml (w/v) of recombinant truncated human MASP-3 (tr MASP-3) for 30 minutes on ice. Buffer without serum was used as a negative control. **A**: is the image of the plate. B: is the diagram of the diameter of the lysis. Results are mean (±SEM) of three independent experiments.

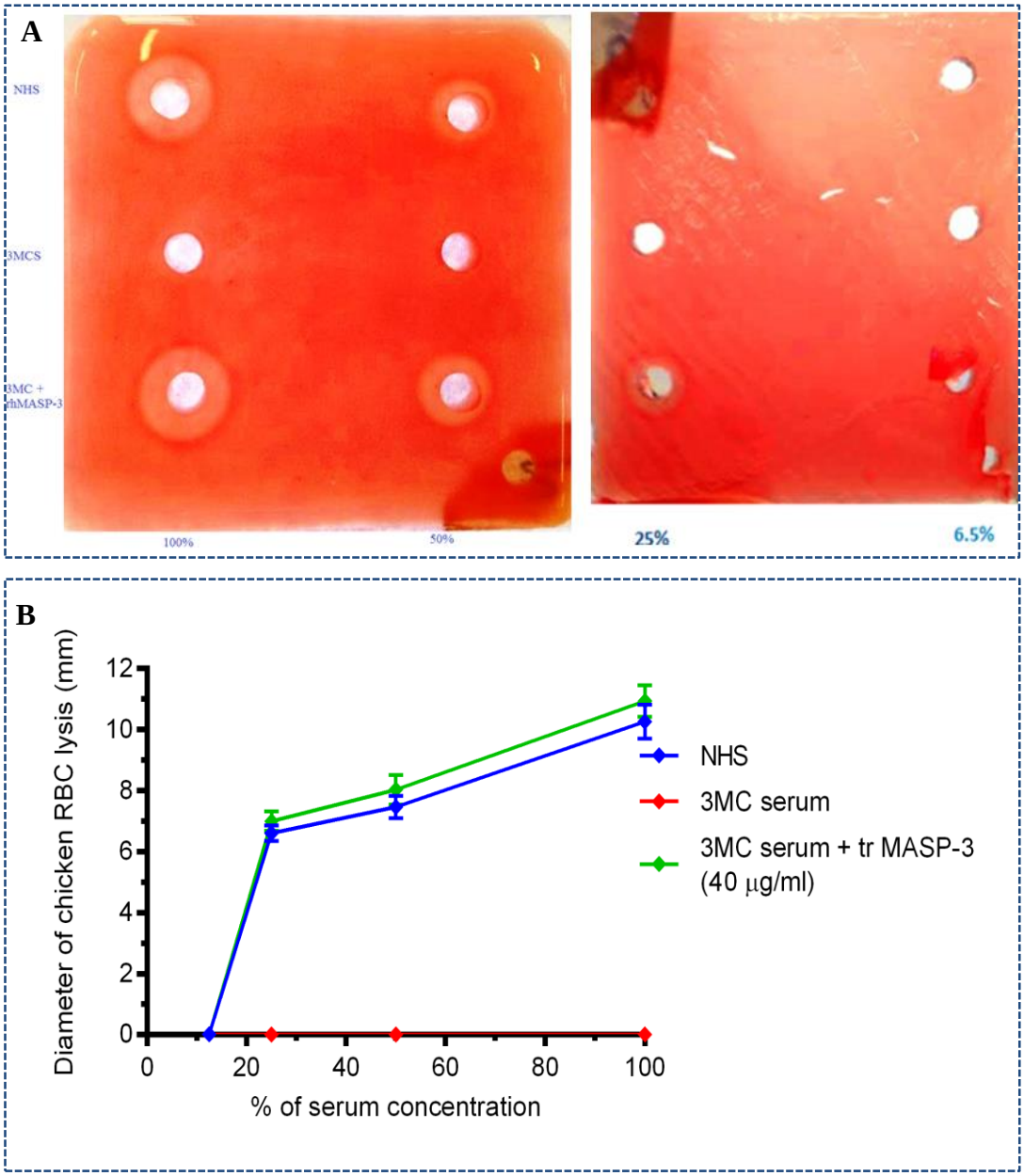

Figure 3.18: Truncated human MASP-3 restores haemolytic activity of 3MC sera toward chicken erythrocytes

Haemolytic diffusion plate assay using 3MC (patient 3) serum on chicken erythrocytes. The assay was run under alternative pathway-specific condition in the AP buffer (BBS/Mg^{+2}/EGTA). Prior to the addition of the serum, the sera were reconstituted with 40 μg/ml (w/v) of recombinant truncated human MASP-3 (tr MASP-3) for 30 minutes on ice. Buffer without serum was used as a negative control. **A**: is the image of the plate. B: is the diagram of the diameter of the lysis. Results are mean (±SEM) of three independent experiments.

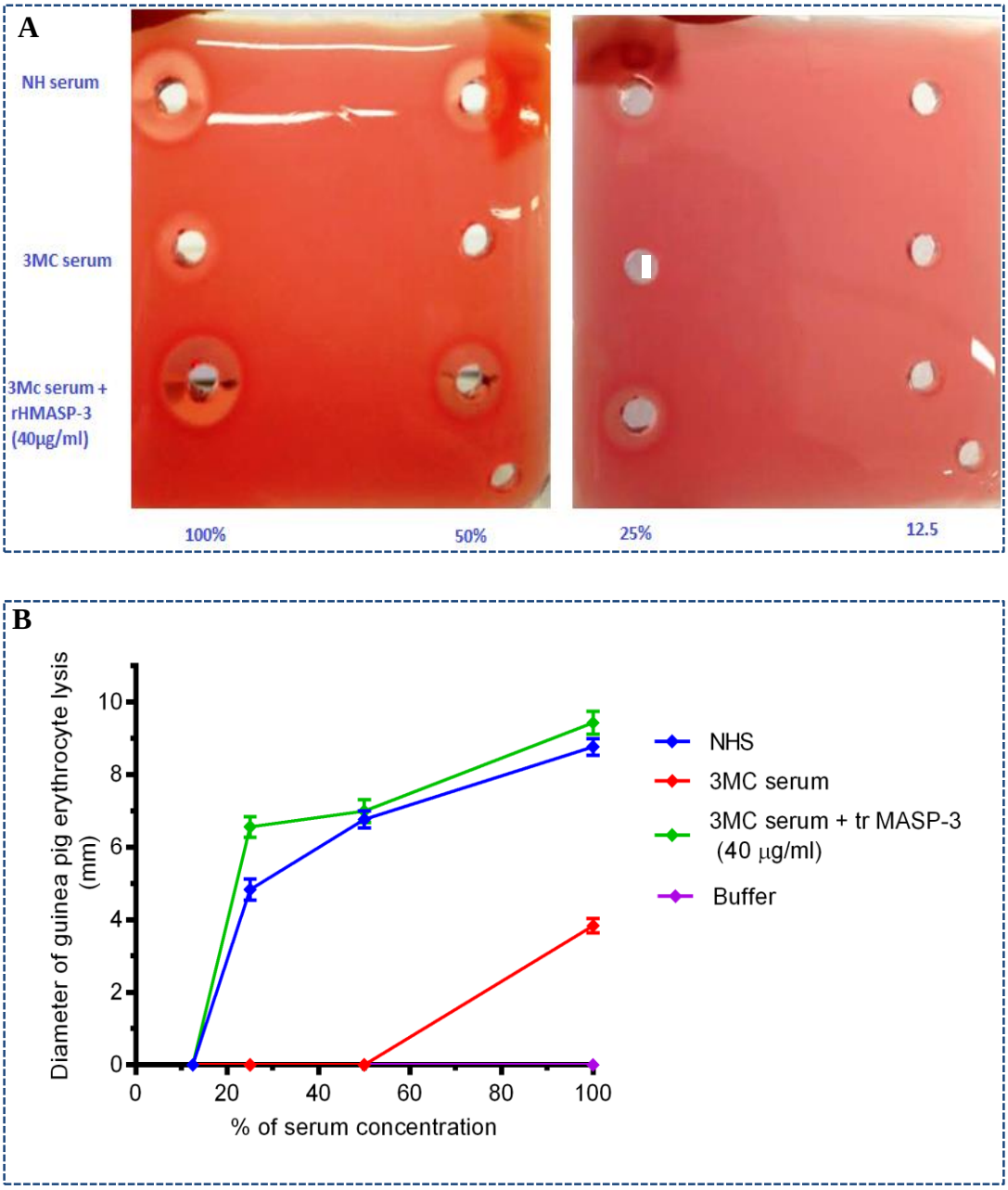

Figure 3.19: Truncated human MASP-3 restores haemolytic activity of 3MC serum towards guinea pig erythrocytes

Haemolytic diffusion plate assay using 3MC (patient 3) serum against guinea pig erythrocytes. The assay was run under alternative pathway specific condition in the AP buffer (BBS/Mg^{+2}/EGTA). Prior to the addition of the serum, the sera were reconstituted with 40 µg/ml (w/v) of recombinant truncated human MASP-3 (tr MASP-3) for 30 minutes on ice. Buffer without serum was used as a negative control. **A**: is the image of the plate. **B**: is the diagram of the diameter of the lysis. Results are mean (±SEM) of three independent experiments.

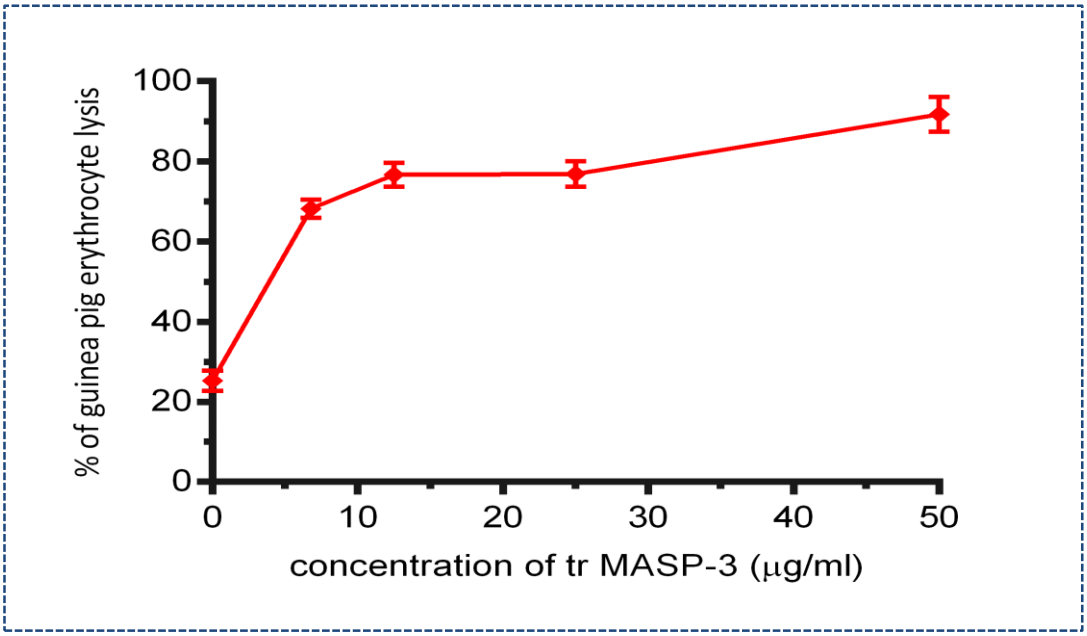

Figure 3.20: Truncated human MASP-3 restores haemolytic activity of 3MC serum toward guinea pig erythrocytes

Haemolytic assay (tube method) on guinea pig erythrocytes using 10% 3MC (patient 2) serum. The assay was run under alternative pathway specific condition in the AP buffer (BBS/Mg^{+2}/EGTA). Prior to the addition of RBC, the sera were reconstituted with increasing concentrations of recombinant truncated human MASP-3. % of lysis showing in relates to in nano water lysis (100% lysis). Results are mean (±SEM) of three independent experiments.

3.1.2.3 3MC haemolytic functional activity through both the classical and the lectin pathway

The classical pathway is primarily activated through binding of C1q to the immune complex on the targeted cell. In the presence of calcium in a buffer that used to run the assay, all pathways of the complement system are functioning. The previous results show that 3MC sera lack ability to lyse erythrocytes in the AP assay. To identify whether there is any difference between 3MC and normal human sera haemolytic activity when all complement pathways are functioning, haemolytic assays were run in the buffer that support all three complement pathways i.e. barbital buffered saline (see figure 3.21). As seen in

the figure 3.21, in relates to nano water lysis (100% lysis), at serum concentration of 12.5%, 3MC sera (patient 2 and patient 3) show about 73% lysis while pooled normal human serum shows 81% lysis and this difference is not significant statistically. At 6.25% serum concentration, 3MC sera show about 54% lysis while NHS shows 60% lysis and this difference also is not statistically significant. Similarly, as can be seen in (see figure 3.22) there is no difference between 3MC and normal human sera haemolytic activity when using buffer that supports all complement pathways while there is statistically significant differences between 3MC and normal human sera in the assay that run under alternative pathway specific condition. Also, there are statistically significant differences between human serum haemolytic activity in the condition that preserve only AP in comparison to the condition that support all complement pathways (see tables 3.9 and 3.10). Similarly, there is statistically significant difference in the haemolytic activity of 3MC serum between conditions mentioned above. These results show that in the condition that support all complement pathways, the classical pathway is initiated through the binding of acquired antibodies in the human serum to antigens on the rabbit erythrocytes surface. Contrary to using human serum, using both unimmunised mouse and unimmunised chicken serum to lyse rabbit erythrocytes do not show any differences between the condition that preserves only AP in comparison to the condition that support all complement pathways (see figures 3.10 and 3.11). This shows that haemolytic activity of 3MC sera against rabbit erythrocytes in the presence of calcium is likely acquired antibody mediated lysis. To assess whether blocking the lectin pathway decrease haemolytic activity of human serum, different types of anti-human MASP-2 antibodies were used. These antibodies were preincubated with pooled normal human serum for 15 minutes at 37°C before adding erythrocytes (see figure 3.23). Running haemolytic assay in barbital buffered saline even in a concentration of 0.012 mg/ml of these antibodies, the ability of normal human serum to lyse erythrocytes does not

diminished. This indicates that MASP-2 dependent lectin pathway activity does not play a significant role in the complement mediated haemolytic activity and similar result with mouse serum obtained. To assess the role of the classical pathway in this activity high salt buffer should be used to dissociate the C1q complex but this assay is not suitable method for haemolytic assay because high salt buffer cause osmotic disequilibrium and induce erythrocyte lysis. Furthermore, I used anti-human C1sa antibody to block the classical pathway and result show it does not affect the haemolytic activity of normal human and 3MC sera (results are not shown). We did further evaluation of the efficiency of this antibody in blocking the classical pathway and results show that this antibodies lacks ability to block the classical pathway.

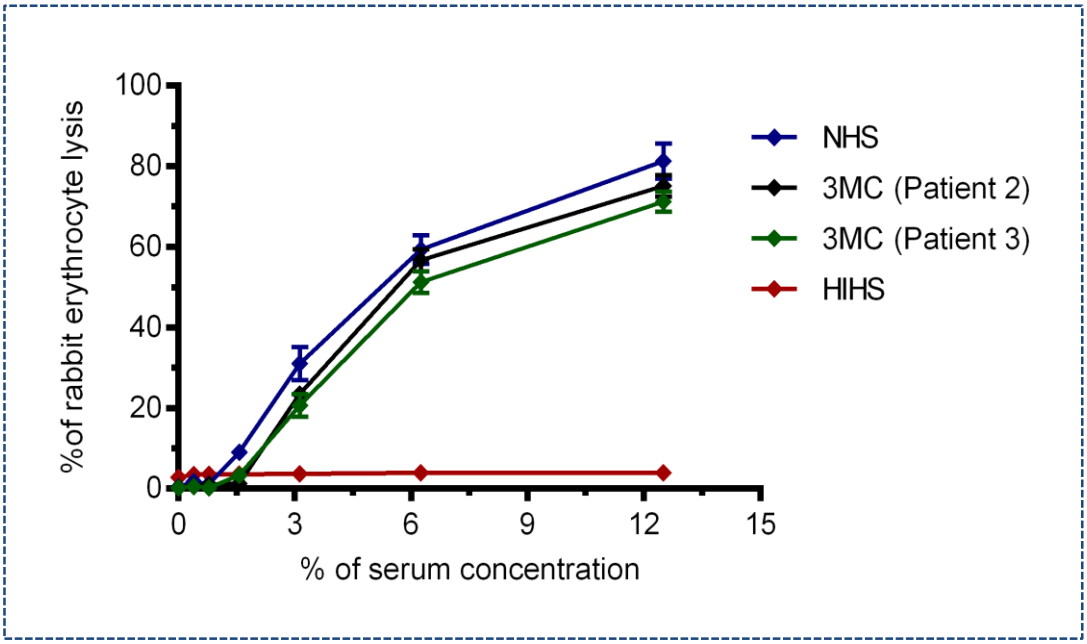

Figure 3.21: Haemolytic activity of the 3MC sera towards rabbit erythrocytes in the presence of calcium

Haemolytic assay (tube method) by using normal human serum (NHS) and 3MC sera (patient 2 and patient 3) against rabbit erythrocytes. Barbital buffered saline (BBS/Ca^{+2}/Mg^{+2}) was used to run the assay. An un-paired student t-test was performed: P value at a 12.5% and 6.25% serum concentration of NHS with Patient 2 is > 0.05. Similarly, P value of NHS with patient 3 in mentioned serum concentration is > 0.05. Heat inactivated human serum was used as a negative control. Results are mean (±SEM) of three independent experiments.

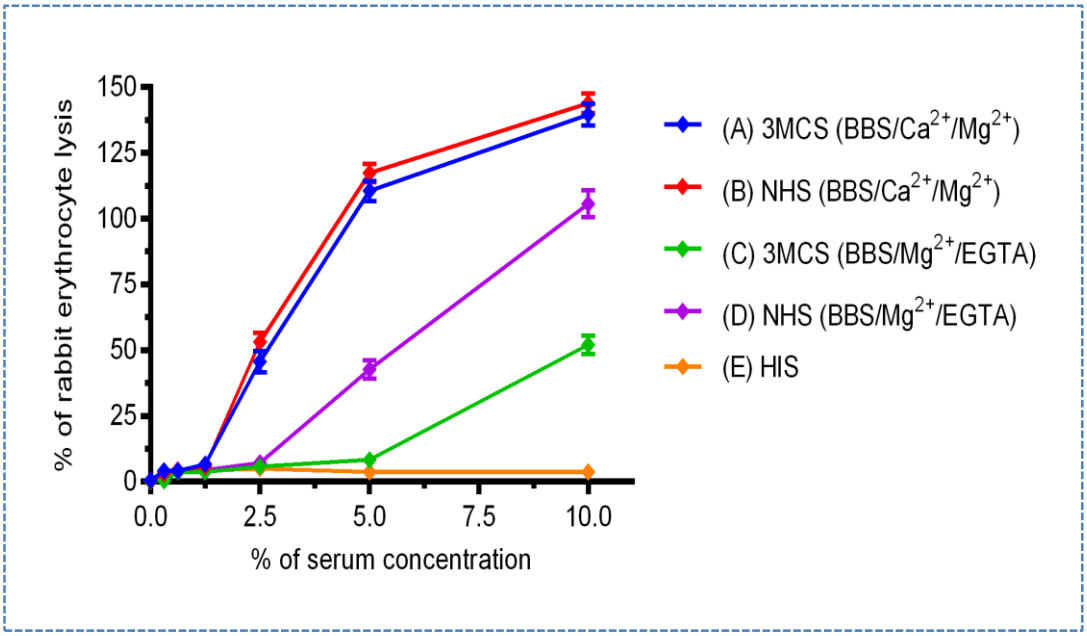

Figure 3.22: Haemolytic activity of 3MC (patient 3) serum in the presence and absence of calcium

Haemolytic assay (tube method) using pooled normal human and 3MC (patient 3) sera on rabbit erythrocytes. A &C run under alternative pathway specific condition using (BBS/Mg^{+2}/EGTA). B & D run in barbital buffered saline (BBS/Ca^{+2}/Mg^{+2}). % of lysis showing in relates to lysis in nano water (100% lysis). Heat inactivated human serum was used a negative control. Results are mean (±SEM) of three independent experiments.

Table 3.9: Statistical significant assessed between the haemolytic activity of NHS and patient 3 against rabbit erythrocytes using Student`s t-test

Students t test (At 10% serum concentration)		
	Statistically significant	P value summary
A vs C	Yes	*** (0.0002)
B vs D	Yes	** (0.039)
D vs C	Yes	***(0.001)

Table 3.10: Statistical significant assessed between the haemolytic activity of NHS and patient 3 sera against rabbit erythrocytes using Student`s t-test

Students t test (At 5% serum concentration)		
	Statistically significant	P value summary
A vs C	Yes	*** (0.0001)
B vs D	Yes	*** (0.0001)
D vs C	Yes	***(0.0006)

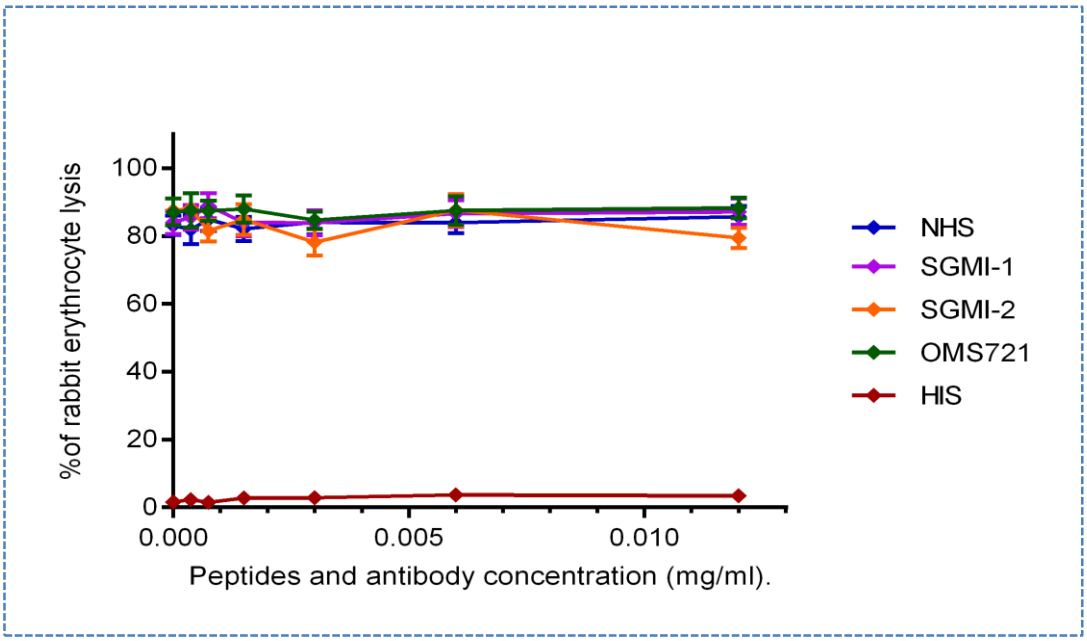

Figure 3.23: Anti human MASP-2 and MASP-1 antibodies do not diminish haemolytic activities of human serum towards rabbit erythrocytes

Haemolytic assay (tube method) by using 6.25% concentration of pooled normal human serum (NHS). Barbital buffered saline (BBS/Ca^{+2}/Mg^{+2}) was used to run the assay. Different antibodies were preincubated in different concentrations with 6.25% NHS and incubated for 15 minutes at 37°C before adding erythrocytes. % of lysis showing in relates to lysis in nano water (100% lysis). Heat inactivated human serum was used as a negative control. Results are mean (±SEM) of three independent experiments.

3.1.3 Role of MAp44 in complement mediated haemolytic activity

MAp44 is an alternative splicing product of *MASP-1* gene. It is composed of N-terminal CUB1 domain, epidermal growth factor-like domain (EGF), a second CUB domain (CUB2), complement control protein 1 (Deng *et al*., 2009). It competes with both MASP-2 and MASP-3 in binding to mannan and thus down regulates the complement activation (the mean of its serum concentration is 1.5 μg/ml) (Deng *et al*., 2009). To assess whether external MAp44 affect the complement mediated haemolytic assay, different concentrations of recombinant MAp44 were added to 9% human serum and the assay was run in barbital buffered saline (see figure 3.24). In the same time different concentration of recombinant MAp44 was also added to 12% normal human serum and the assay was run under alternative pathway specific condition. By using barbital buffered saline, 9% pooled normal human serum show 100% lysis and this level of lysis stay steady even in the addition of 50 μg/ml (w/v) of recombinant MAp44/MAP-1 concentration. Similarly, the ability of normal human serum to lyse rabbit erythrocytes stays unchanged under alternative pathway specific condition even after adding 50 μg/ml (w/v) of recombinant MAp44, showing 83% lysis.

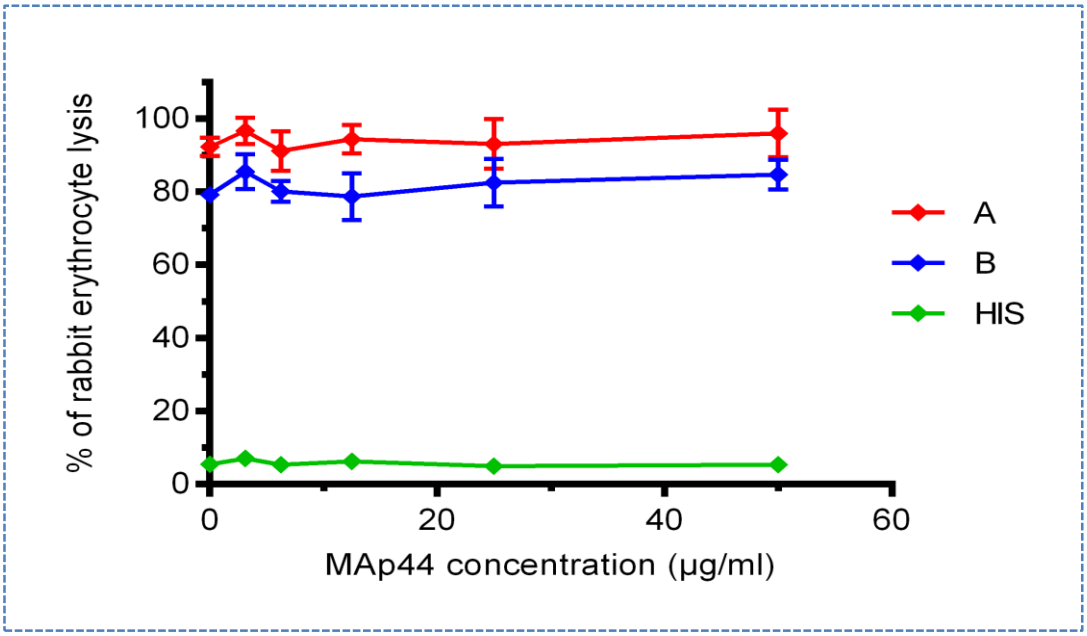

Figure 3.24: Addition of recombinant MAp44 does not inhibit haemolysis of rabbit erythrocytes in human serum

Haemolytic assay (tube method) on rabbit erythrocytes by using normal human serum. A: 9% human serum used and the assay run in barbital buffered saline (BBS/Ca^{+2}/Mg^{+2}). B: 12% human serum used and the assay run under alternative pathway-specific condition by using (BBS/Mg^{+2}/EGTA). Prior to the addition of erythrocytes, the sera were reconstituted with increasing concentrations of recombinant human MAp44. Heat inactivated human serum was used as a negative control. Results are mean (±SEM) of three independent experiments.

3.1.4 Addition of external recombinant MASP-3 does not increase the haemolytic activity of normal human serum

My data clearly show that MASP-3 plays pivotal role in the complement-mediated haemolysis but it was not clear whether addition of external MASP-3 increases haemolytic activity of NHS. Different concentration of recombinant full length human MASP-3 or MASP-3A (which consists of only the heavy chain of MASP-3 and has no light chain) was added to 5% pooled normal human serum concentration and the assay was run in the barbital buffered saline and

at the same time different concentration of recombinant full length human MASP-3 or MASP-3A was added to 7% pooled normal human serum concentration and the assay was run under alternative pathway specific condition (see figure 3.25). Result shows the haemolytic activity of NHS in both types of buffer does not change statistically even in the presence of 100 µg/ml (w/v) of recombinant proteins.

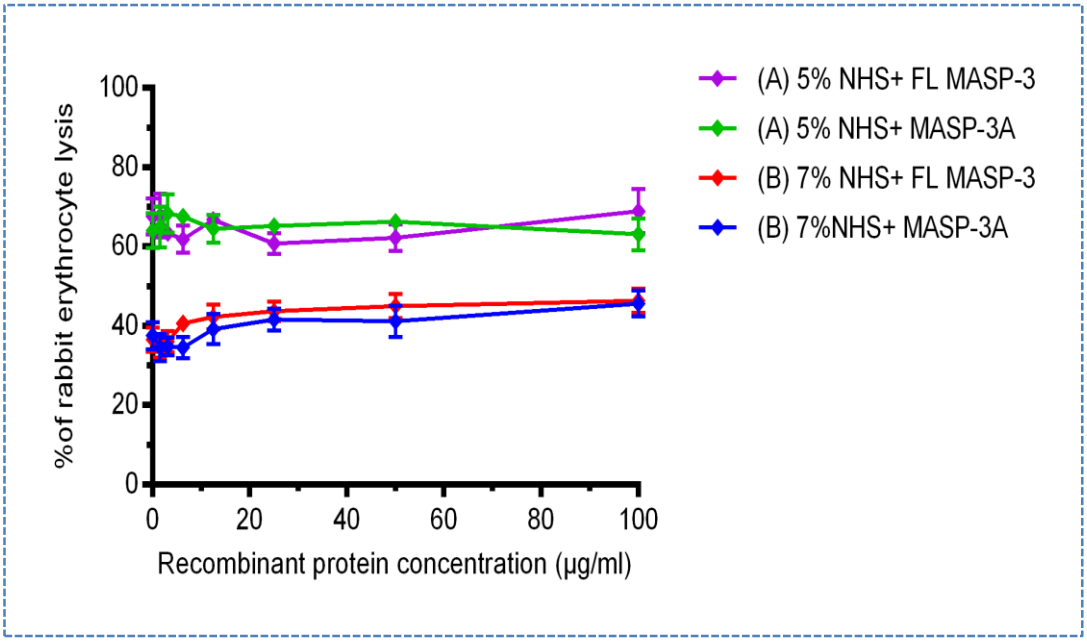

Figure 3.25: Extrinsic recombinant MASP-3 does not increase haemolytic activity of human serum towards rabbit erythrocytes

Haemolytic assay (tube method) on rabbit erythrocytes using normal human serum. A: 5% pooled NHS used and the assay run in the barbital buffered saline (BBS/Ca^{+2}/Mg^{+2}). B: 7% NHS used and the assay run under alternative pathway specific condition (BBS/Mg^{+2}/EGTA). Prior to the addition of erythrocytes, different concentration of recombinant proteins (either FL MASP-3 or MASP-3A) was added to the serum. Results are mean (±SEM) of three independent experiments.

3.1.5 MASP-3 is essential to convert pro factor D to factor D in human serum

Recent data showed that the physiological activator of factor D is MASP-3 as it directly activates factor D *in vitro* study (Iwaki *et al.*, 2011). To assess the role of MASP-3 in activating pro factor D in human, factor D depleted human serum was reconstituted with recombinant truncated human MASP-3 (see figure 3.26). 10% of fD depleted human serum was preincubated with constant recombinant human pro factor D (rPro-fD) (4 μg/ml, w/v) and increasing concentrations of recombinant truncated human MASP-3 at 37°C for 20 minutes before running the assay. At the same time, same serum concentration of fD depleted serum was preincubated with 15 μg/ml (w/v) of recombinant tr MASP-3 without recombinant pro fD at 37°C for 20 minutes and the same concentration of fD depleted human serum was also pre incubated with recombinant human pro factor D (2 μg/ml, w/v) and recombinant truncated human MASP-3 (15 μg/ml, w/v) at 37°C for 20 minutes. The assay was run under alternative pathway specific condition. As can be seen in figure 3.26, fD depleted serum at the concentration of 10% shows 20% of lysis and addition of tr MASP-3 (15 μg/ml, w/v) or rPro-fD (4 μg/ml, w/v) to the fD depleted serum does not increase the haemolytic activity of the serum. While addition of rPro-fD (4 μg/ml, w/v) together with different concentration of tr MASP-3 increases the haemolytic activity of fD depleted serum in a concentration manner. At the MASP-3 concentration of 2.5, 5 and 15 μg/ml, the serum shows 95%, 100% and 110% of lysis respectively. Addition of 15 μg/ml (w/v) of FL-MASP-3 together with 2 μg/ml (w/v) of rPro-fD to the fD depleted serum increased the lysis to 60%.

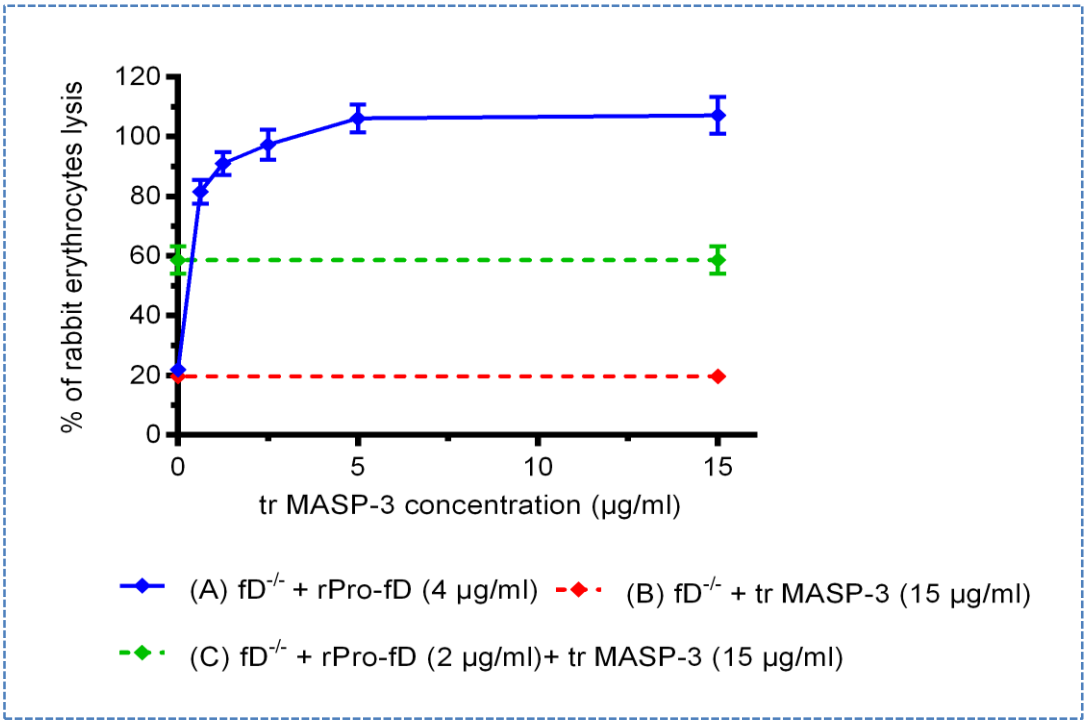

Figure 3.26: MASP-3 converts pro factor D to active factor D

Haemolytic assay (tube method) using factor D depleted human serum against rabbit erythrocytes**.** A: 10% of fD depleted serum ($fD^{-/-}$) was preincubated with constant recombinant human pro factor D (rPro-fD) (4 µg/ml) and different concentrations of truncated human MASP-3 (tr MASP-3) at 37°C for 20 minutes before adding erythrocytes. B: 10% of $fD^{-/-}$ serum was pre-incubated only with truncated human MASP-3 (15 µg/ml, w/v) at 37°C for 20 minutes before adding erythrocytes. C**:** 10% of $fD^{-/-}$ serum was preincubated with rPro-fD (2 µg/ml, w/v) and tr MASP-3 (15 µg/ml, w/v) at 37°C for 20 minutes before adding erythrocytes. The assay was run under alternative pathway specific condition (BBS/Mg^{+2}/EGTA). Results are mean (±SEM) of three independent experiments.

3.1.6 C3 deposition assay

Some pathology mediated by the AP is through C3 deposition on the targeted cell. To identify the ability of 3MC sera to deposit C3 on the targeted cells, 96 microtitre wells was coated with zymosan and different concentration of 3MC (patient 2 and patient 3) and normal human sera were added to the corresponding wells. The assay was run under alternative pathway specific condition (see figure 3.27). As can be seen in the figure 3.27, the 3MC sera abilities to deposit C3 are diminished significantly in a 20%, 10% and 5% serum concentration in comparison to pooled normal human serum. $MBL^{-/-}$ serum also was used to exclude any effect of MBL as the 3 MC (patient 3) is also deficient in MBL. AP_{50} achieved in NHS, $MBL^{-/-}$ and $C4^{-/-}$ sera at serum concentration of 4.5%, 5.7% and 5.1% respectively. While in patient 2 and patient 3 sera, it was achieved at a serum concentration of 12.3% and 8.2% respectively (table 3.11). This differences are statistically significant (table 3.12). This result indicates 3MC ability to cleave C3 through the AP is impaired significantly. To identify whether this diminishing is due to MASP-3 or something else in the 3MC sera, 3MC (patient 3) serum was reconstituted with different concentration of recombinant FL MASP-3 (figure 3.28). Prior to run the assay, 10% 3MC (patient 3) serum was reconstituted with different concentration of recombinant human FL MASP-3 for 30 minutes on ice. Similarly, 5% of the 3MC serum was reconstituted with different concentration of recombinant human FL MASP-3 and at the same time 5% also reconstituted with the different concentration of MASP-3A for 30 minutes on ice prior to run the assay. Result clearly shows that different 3MC sera concentrations in the presence of recombinant FL MASP-3 restored their ability to cleave C3 in to C3b while the 3MC serum reconstituted with recombinant human MASP-3A still lacks ability to cleave C3 to C3b.

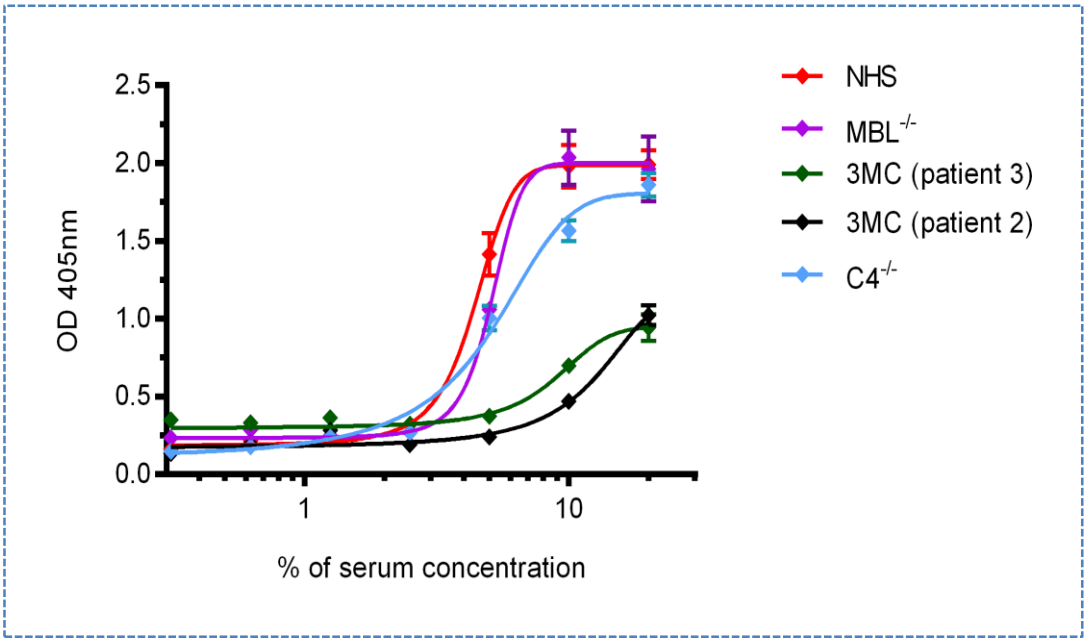

Figure 3.27: 3MC sera are significantly compromised in C3 deposition under alternative pathway specific condition

AP-driven C3b deposition on zymosan-coated microtitre plates as a function of serum concentration. The assay was run under alternative pathway specific condition (BBS/Mg^{+2}/EGTA). Results are mean (±SEM) of three independent experiments.

Table 3.11: showing acquired serum concentration for different types of human sera to achieve AP_{50}

Serum	AP_{50}
NHS	4.5%
$MBL^{-/-}$	5.7%
$C4^{-/-}$	5.1%
3MC (patient 2)	12.3%
3MC (patient 3)	8.2%

Table 3.12: Statistical significant assessed between the C3 deposition abilities of different human sera using Student`s t test

	5% of serum concentration	10% of serum concentration	20% of serum concentration
NHS vs patient 2	0.0011(***)	0.0004 (***)	0.0009 (***)
NHS vs patient 3	0.0018 (**)	0.0008 (***)	0.0011 (***)
NHS vs $C4^{-/-}$	0.0591	0.0619	0.9070
NHS vs $MBL^{-/-}$	0.0602	0.08227	0.9074

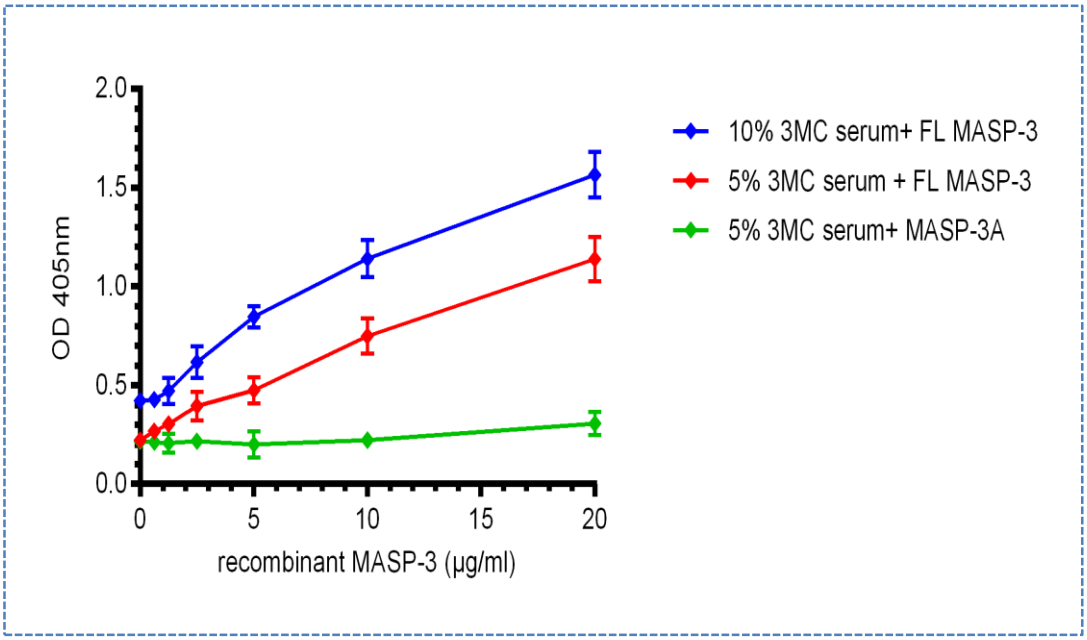

Figure 3.28: Full length human MASP-3 restores 3MC sera ability to cleave C3 under alternative pathway specific condition

AP-driven C3b deposition on zymosan-coated microtitre plates using 5% and 10% 3MC serum. The assay was run under alternative pathway specific condition (BBS/Mg^{+2}/EGTA). Prior to run the assay, increasing concentrations of recombinant full length human MASP-3 were added to 10% and 5% 3MC serum and different concentration of MASP-3A added to 5% 3MC serum. Student`s t test was performed. P value between $MSP1/3^{-/-}$ reconstituted with FL MASP-3 and 3MC (patient 3) serum reconstituted with MASP-3A at a concentration of 20 µg/ml, 10 µg/ml and 5 µg/ml equal 0.0029, 0.0055 and 0.0425 respectively (P value of < 0.05 is significant). Results are mean (±SEM) of three independent experiments.

Similar result archived by using MASP-1/3 deficient mouse serum (see figure 3.29). Running C3 deposition assay under alternative pathway specific condition clearly show that MASP-1/3 deficient mouse serum lacks ability to cleave C3 even in a serum concentration of 20% and pre-incubation of MASP-1/3 deficient mouse serum with recombinant full length human or mouse MASP-3 (5 µg/ml, w/v) restored MASP-1/3 deficient mouse serum ability to cleave C3 to the same level of pooled wild type serum control.

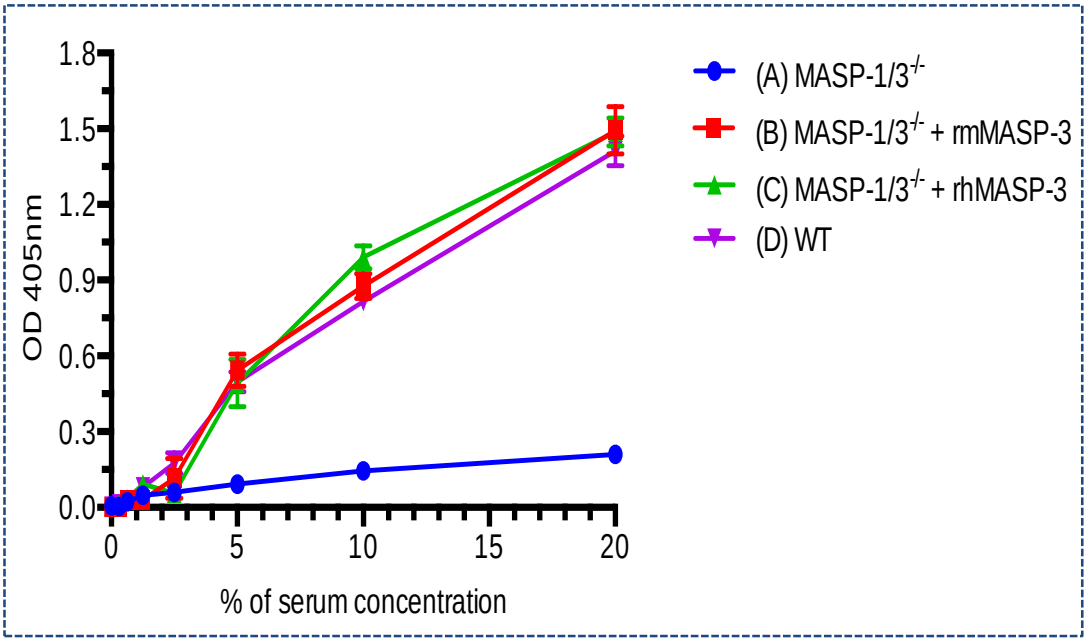

Figure 3.29: MASP-1/3 deficient mouse serum lacks ability in C3 deposition under alternative pathway specific condition and recombinant human and murine MASP-3 restores its ability to cleave C3

AP-driven C3b deposition on zymosan-coated microtitre plates using MASP-1/3$^{-/-}$ serum. The assay was run under alternative pathway specific condition (BBS/Mg^{+2}/EGTA). Prior to run the assay, 5 µg/ml (w/v) of recombinant full length human MASP-3 (C) or recombinant full length murine MASP-3 (B) were added to MASP-1/3$^{-/-}$ serum and incubated for 30 minutes on ice. Student`s t test was performed. P value between MSP1/3$^{-/-}$ reconstituted with rmMASP-3 and MSP1/3$^{-/-}$ at a serum concentration of 20%, 10% and 5% equal 0.0027, 0.0022 and 0.0098 respectively (P value of < 0.05 is significant). Results are mean (±SEM) of three independent experiments.

To identify the role of MASP-3 in the lectin pathway mediated C3 cleavage, C3 cleavage assay was run under lectin pathway specific conditions (see figure 3.30). 96 microtitre wells was coated with mannan and different serum concentration of wild type, MASP-2 deficient, MASP-1/3 deficient and reconstituted MASP-1/3 deficient mouse serum with recombinant mouse MASP-3 were added to corresponding wells. The assay was run in barbital buffered saline (see figure 3.30). Starting serum concentration was 2.5% to stop alternative pathway function as properdin concentration at this concentration is not enough to maintain alternative pathway activity. Result show MASP-1/3 deficient serum ability to cleave C3 is about half ability of wild type serum control. Furthermore, restoration of MASP-1/3 deficient serum with recombinant full length murine MASP-3 does not increase the ability of this serum to cleave C3.

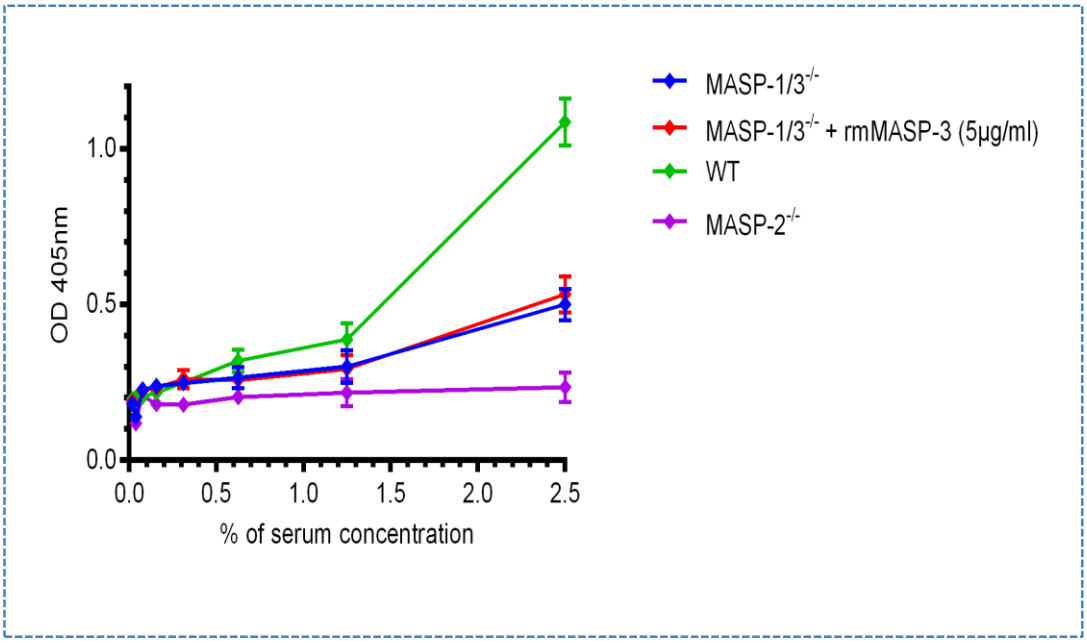

Figure 3.30: Recombinant full length murine MASP-3 does not increase MASP-1/3$^{-/-}$ ability in C3 deposition under lectin pathway specific condition

Lectin pathway driven C3b deposition on mannan-coated microtitre plates using different mouse sera. The assay was run under lectin pathway-specific condition (BBS/Ca^{+2}/Mg^{+2}). MASP-1/3$^{-/-}$ serum was used alone and with preincubation with 5 µg/ml (w/v) of recombinant full length murine MASP-3 for 30 minutes on ice. MASP-2$^{-/-}$ serum used as a negative control. Student`s t test was performed. P value between MSP1/3$^{-/-}$ serum with and without addition of rmMASP-3 is equal 0.722 and between MASP1/3$^{-/-}$ and WT is equal 0.002 (P value of < 0.05 is significant). Results are mean (±SEM) of three independent experiments.

3.2 Discussion

The Complement system is a central component of the humoral and the cellular immune system and represents a key effector mechanism of innate immunity (Reid, 1983). Complement system mediated the coating of the surface of pathogens (i.e. opsonisation) with different complement activation products (e.g. C3b), and the lysis of some pathogens and targeted cells through forming membrane attack complexes (Walport, 2001; Dunkelberger and Song, 2010).

Activation of the classical pathway is mainly antibody driven while initiation of lectin pathway activation depends on carbohydrate recognition processes. In contrast the alternative pathway is initiated through spontaneous hydrolysis of C3. Its activation is held in check by an array of regulatory fluid phase components, which controls a continuous balance between spontaneous low grade activation and negative regulation through potent down-regulatory components. In addition to fluid phase resident complement control proteins in blood plasma and other body fluids, all host cell surfaces are equipped with surface bound negative regulators and complement receptors to avoid cytotoxicity through autologous complement attack.

Deficiencies of complement regulatory components on the surface of host cells can render these cells susceptible to autologous complement attach and lysis leading to serious pathologies like paroxysmal nocturnal haemoglobinuria. Since erythrocytes are non-nucleated cells which have – compared to nucleated cells – a very low degree of membrane turn over (they have no endoplasmatic reticulum and only few vacuoles), these cells are particularly sensitive to MAC mediated lysis and a single MAC can be sufficient to induce lysis (Platts-Mills and Istlizaka, 1974). As mentioned above, initiation of the different activation pathways of complement system is mediated by different mechanisms and specific pathway may play a role in the pathogenesis of specific disease. It is at

the end the structure of the cell surface that determines which of the different pathways of complement activation drives this process.

To assess the relative contribution of each of the three activation pathways on complement mediated lysis of erythrocytes, I tested human sera and sera of different transgenic mice with targeted complement deficiencies for specific complement activation pathways for their ability to lyse rabbit, chicken and guinea pig erythrocytes. My results were obtained using these different target erythrocytes in human and mouse sera with and without defined complement deficiencies under the specific experimental conditions described. Erythrocytes of the different species (i.e. rabbit and chicken erythrocytes for mouse serum and rabbit, chicken and guinea pig erythrocytes for lysis by human serum) are sensitive to lysis by mouse and/or human serum because either i) their species specific membrane associated complement regulators do not efficiently down regulate activation of the host complement system or ii) their cell surface structures fail to effectively interact with the fluid phase regulators of the host complement system and thereby fail to prevent activation of the host complement system on the surface of the target erythrocytes leading to opsonisation of the target erythrocytes and MAC dependent haemolysis. In case ii) the glycocalyx structures of the xenogenic target erythrocytes fail to interact with either human or mouse factor H in the host serum resulting in low inhibitory activity of host factor H to bind to host C3b and decay the alternative pathway C3 convertase and the C5 convertases (Schmidt *et al.,* 2011).

3.2.1 Complement mediated haemolysis in mouse

3.2.1.1 The classical pathway of complement activation appears not to mediate haemolysis in non-immune mouse serum

Classical pathway is primarily activated after the binding of C1q to immune complexes on the surface of targeted cells. C1q has also been reported to activate the classical pathway through binding directly to bacterial lipopolysaccharides, apoptotic cells, C-reactive protein, viral protein and poly anionic compounds (Navratil *et al.,* 2001). Absence of C1q abolishes all classical pathway functional activity. Similarly, in absence of complement C4 classical pathway functional activity is rendered dysfunctional. My results clearly show that the absence of C1q or C4 do not decrease the ability of mouse serum to lyse rabbit erythrocytes (see figure 3.1). These results indicate that the classical pathway pays a redundant role in the mediation of haemolysis in naïve mouse serum under physiological conditions. However, if erythrocytes were sensitised with antibodies, the classical pathway was shown to be quite effective in mediating haemolysis (Platts-Mills and Istlizaka, 1974). For the majority of my experiments I have chosen to not sensitise erythrocytes with antibodies since sensitisation may override the usual pathophysiological processes leading to haemolytic disease.

3.2.1.2 *The lectin pathway effector enzyme MASP-2 does not play an obvious role in complement mediated haemolytic activity*

Lectin pathway is initiated by a multimolecular lectin pathway activation initiation complex composed of a multimeric pattern recognition subcomponent and usually homodimers of either of the three different lectin pathway specific MASPs. The recognition molecules of the LP are Mannan-binding lectin (MBL), ficolins and CL-11 (Hansen *et al.,* 2010; Schwaeble *et al.,* 2002). Interaction of these recognition molecules with the target cell surfaces initiates the activation of this pathway through the MBL-associated serine proteases (MASP-1, MASP-2 and MASP-3) which bind as dimers to specific MASPs binding sites of the recognition subunits (Schwaeble *et al.,* 2002). Different structures on different cell surfaces vary in their ability to bind to different LP recognition molecules to trigger LP activation. Deficiency of any of this recognition molecule may lead to a partial deficiency of the LP. My results demonstrate that haemolytic activities of different mouse sera with targeted deficiencies of either MBL-A and MBL-C (MBL null double deficient mice), and/or ficolin-A, or CL11 are very similar to those seen wild type mouse control sera (see figures 3.2, 3.3 and 3.4). This indicates that the LP recognition molecules may not have a role in complement mediated haemolytic activity, perhaps because they do not avidly bind to the xenogenic target erythrocytes. MASP-2 is the key enzyme of the lectin pathway and since it is the only LP specific serine protease that can effectively cleave both C4 and C2. Therefore, in absence of MASP-2, the LP C3 convertase complex C4bC2a cannot form (Schwaeble *et al.,* 2011) and MASP-2 deficient mice are deficient of the MASP-2 dependent lectin pathway effector arm 2, LEA-2 (see figure 3.31). Similar to the observation made when testing mouse blood deficient of LP recognition molecules, the deficiency of MASP-2 does not decrease the ability of the mouse serum to lyse erythrocytes (see figure 3.5). These results are in agreement with a previous, but far more restricted

observation reported by Suankratay *et al.* 1998 claiming that the initiation of the lectin pathway on the erythrocytes requires the erythrocytes to be coated with mannan since uncoated RBC does were not lysed. Likewise, this lysis of mannan-coated erythrocytes was absent in MBL deficient serum (Suankratay *et al.,* 1998). In addition, this haemolysis was shown to be alternative pathway dependent since no haemolysis of mannan-coated erythrocytes was seen in factor D deficient serum (Suankratay *et al*., 1998).

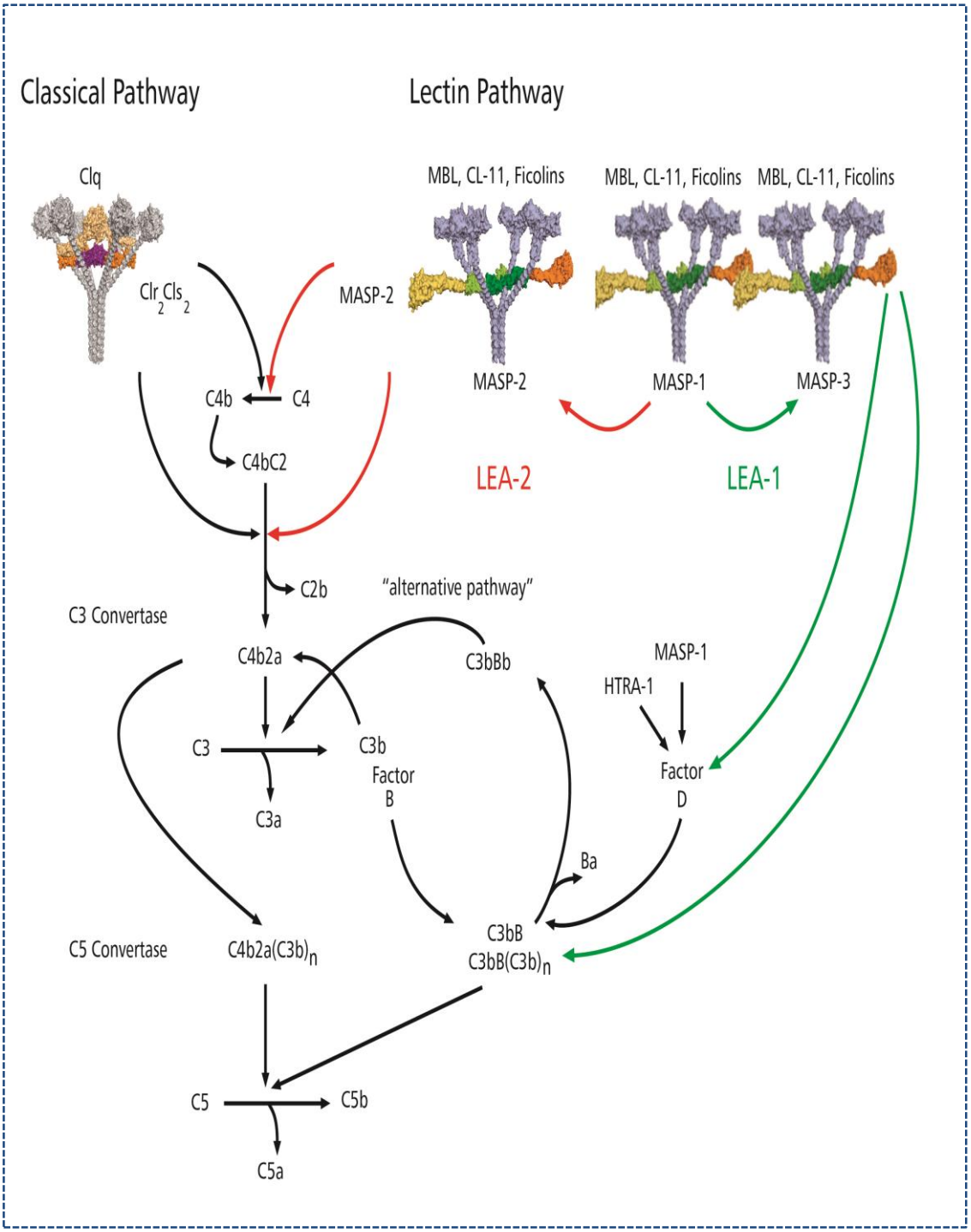

Figure 3.31: New insight of the complement system activation

Complement system activation by the classical, the alternative and the lectin (Lectin effector arm 2, LEA-2, and lectin effector arm 1, LEA1) and the alternative pathway. (Figure courtesy of Professor W. Schwaeble, University of Leicester UK).

3.2.1.3 *The alternative activation pathway plays a key role in complement mediated haemolysis in mouse*

The alternative pathway of complement activation provides a critical first line of defence against many organisms. This pathway has no recognition molecule and sustains a constant low state of activation and the discrimination between self and non self cells is mediated by complement regulators on self-structures and surfaces. The components of the alternative pathway include C3b, factor B, factor D, properdin, (Thurman and Holers, 2006) and recent work implicated an involvement of MASP-1 and MASP-3 (Takahashi *et al.,* 2010). Deficiency of factor B or factor D abolishes or dramatically reduces AP functional activity (Pryzdial and Isenman 1987; Schwaeble and Reid 1999; Volanakis and Narayana, 1996). It is well known and widely accepted that susceptibility of RBCs from different species to serum lysis by complement does not essentially required coating or antibody sensitisation of the RBCs (Lange and Magnadottir, 2003). Under physiological conditions, the haemolytic activity of factor B deficient and factor D deficient mouse sera is nearly absent (see figure 3.6). This highlights the important role of the AP in complement mediated haemolysis in mouse serum under physiological conditions. Most recent work in Prof. Schwaeble's laboratory has showed that addition of recombinant human properdin significantly increases bactericidal activity of human serum against *Neisseria meningitides* (Ali *et al.*, 2014). Similarly, my result showed adding recombinant murine properdin to mouse serum increases haemolytic activity of the mouse serum (see figure 3.12).

3.2.1.4 *Role of MASP-3 in complement mediated haemolysis in mouse*

MASP-3 is a member of the serine protease family (Schwaeble *et al.,* 2002). MASP-3 is composed of an N-terminal CUB1 domain, followed by an epidermal growth factor-like domain (EGF), a second CUB domain (CUB2), followed by two complement control protein domains (CCP1 and CCP2) (synonymously also called short consensus domain of complement regulators, SCRs) and the C-terminal serine protease domains. In recent years, an important role of either MASP-1 or MASP-3 in driving the alternative pathway has been postulated as recent data showed that MASP-1/3 deficient mouse serum lacks AP functional activity (Takahashi *et al.,* 2010). Furthermore, these mice were shown to be deficient in active factor D with profactor D (factor D in its zymogen state) being predominantly found in MASP-1/3 deficient mouse sera. It was shown that the addition of active factor D to MASP-1/3 deficient mouse serum can restore the AP functional activity (Takahashi *et al.,* 2010). Testing MASP-1, MASP-2 and MASP-3 triple knockout mouse serum shows that these mice have significantly lower complement functional activity than wild type mouse serum (Iwaki *et al.,* 2011).

My results indicate that MASP-3 also acts as a critical positive regulator of alternative pathway functional activity and that MASP-3 cleavage and activation is a critical step in the mediation of complement dependent haemolytic activity. MASP-1/3 deficient mouse serum is significantly impaired in its ability to lyse erythrocytes and neither classical pathway nor lectin pathway can maintain complement mediated haemolytic activity in the absence of the MASP-1 and MASP-3 (see figure 3.7). This indicates either MASP-1 or MASP-3 or both are critically involved in the complement mediated haemolytic activity. To clarify this, I reconstituted MASP-1/3 deficient mouse serum with recombinantly expressed human MASP-3 and enzymatically active fragments thereof. I succeeded in demonstrating that reconstitution of MASP-1/3 deficient

mouse serum with recombinant MASP-3 restores haemolytic activity of MASP-1/3 deficient serum to the levels even marginally higher than seen in MASP-3 sufficient wild type serum (see figures 3.8 and 3.9) and this is most probably because of the presence of more profactor D in MASP1/3$^{-/-}$ serum. My results clearly highlight that MASP-3 plays an important role in the complement-mediated haemolysis in mouse.

3.2.2 Complement mediated haemolytic activity in chicken serum is dependent on the alternative pathway

My results show under physiological conditions, complement-mediated haemolytic activity of chicken serum is mediated by the alternative pathway and neither the classical nor the LP appear to play role. Using magnesium containing EGTA buffer, which inhibits both the calcium-dependent classical and lectin activation pathways, does not decrease the haemolytic ability of the chicken serum against both guinea pig and rabbit erythrocytes (see figures 3.10 and 3.11). Chicken serum contains MASP-3, but lacks MASP-1 (Lynch *et al.*, 2005) but the absence of MASP-3 does not appear to affect the haemolytic activity of the chicken serum. So this and previous results showing that restoring haemolytic activity of MASP-1/3 deficient serum with recombinant MASP-3 indicate that MASP-3 plays a critical role in complement-mediated haemolytic activity. These results underline that MASP-3 not MASP-1 is the critical enzyme that is required to maintain the AP functional activity (this work and see also Iwaki *et al.*, 2011).

3.2.3 Complement mediated haemolysis in human serum

Activation of the complement system leads up to the terminal activation cascade resulting in the formation of MAC complexes that form pores through the cell surface membrane of targeted cells which finally may cause cell lysis. It is well established that human serum can lyse erythrocytes of different species (Lange and Magnadottir, 2003; Martin *et al.*, 1976; Platts-Mills and Istlizaka, 1974). Each different pathway of the complement activation has been assessed individually. Erythrocytes from three different species (rabbit, guinea pig and chicken) were used as target cells for lysis by human serum.

3.2.3.1 Role of the classical pathway in complement mediated haemolysis in human serum

Assessing the role of the classical pathway in mediating haemolytic activity in human serum is often difficult because human sera from different individuals have acquired antibodies which can modify the haemolytic towards erythrocytes from different species in human serum. These acquired antibodies in immune sera can form immune complexes on target erythrocytes and initiate classical pathway activation. Therefore, these haemolytic assays lysing erythrocytes from different species do not exactly mimic the physiological processes leading to haemolytic disease under *in vivo* conditions. For example, PNH patients usually do not have autoantibodies against their own erythrocytes unless autoantibody mediated diseases coexist with PNH. Sheep erythrocytes, which are usually not lysed by human serum, can be effectively lysed after sensitising sheep erythrocytes with antibodies indicating that in this case the critical initiation of complement activation is initiated via the classical pathway (Platts-Mills and Istlizaka, 1974). An experimental condition that selectively disables classical pathway functional activity is the use of high salt

buffers which disintegrates the C1 complex while LP complexes stay intact and the AP remains fully functional. This experimental condition, however, cannot be applied for haemolytic assays as it causes erythrocyte lysis.

3.2.3.2 Role of the lectin pathway in complement mediated haemolysis in human

The lectin pathway is initiated by a lectin pathway recognition subcomponent and three different lectin pathway specific enzymes. The recognition molecules of the LP are Mannan-binding lectin (MBL), ficolins and CL-11 (Hansen *et al.*, 2010; Schwaeble *et al.*, 2002). Interaction of these recognition molecules with the target cell surfaces initiates the activation of this pathway through MBL-associated serine proteases (MASP-1, MASP-2 and MASP-3), which bind as dimers to specific MASPs binding sites of the recognition subunits (Schwaeble *et al.*, 2002). MASP-2 is a key enzyme of the lectin pathway and deficiency of this enzyme causes complete deficiency of the lectin pathway C3 convertase C4bC2a (Schwaeble *et al.*, 2011). Under physiological conditions, the anti-human MASP-2 antibody OMS721, which effectively inhibits MASP-2 functional activity, does not decrease the ability of human serum to lyse erythrocytes (see figure 3.23). Similarly, a deficiency of the lectin pathway recognition molecule MBL does not affect the haemolytic activity of human serum (see figure 3.14). This indicates that the MBL may not play a critical role in complement mediated lysis of erythrocytes in human serum as MBL lacks ability to bind to PNH erythrocytes and erythrocytes of different species. This result is in agreement with the results published by Suankratay *et al.* that they show that in order to initiate lectin pathway activation on erythrocytes via MBL, erythrocytes have to be sensitised with mannan (Suankratay *et al.*, 1998).

3.2.3.3 *The alternative activation pathway plays a key role in complement mediated haemolysis in human*

Alternative pathway activation of complement is considered not to be initiated by specific pattern recognition molecules or by immune complexes. Initiation of the alternative pathway is mediated by a so called "tickover" event. According to this hypothesis, the alternative pathway maintains a continuous low state of activation, which can quickly swing from a predominant state of inhibition to a predominant state of activation (where the negative regulators show weaker activity that the positive regulators). The role of the alternative pathway dependence of haemolytic activity in human has been discussed. Factor B and factor D depleted human serum show severely impaired haemolytic activity because their alternative activation pathway is not working. To test the ability of the AP in mediating haemolytic activity the assay was run under alternative pathway specific condition that allows AP functional activity while blocking both the LP and CP by using EGTA, Mg^{+2}. The EGTA buffer's affinity to bind calcium is much higher than its affinity to chelate Mg^{+2} at pH 7.4 (Brayan and David, 1968). This feature of the EGTA buffer results in the Ca^{+2} dependent classical and lectin pathways being inhibited, while the Ca^{+2} independent but Mg^{+2} dependent alternative pathway remains fully functional. My results demonstrate that in human serum the AP is critical in mediating haemolytic activity (see figure 3.26) confirming the previous published results that showed the importance of the AP in lysing rabbit erythrocytes in Mg^{+2} dependent manner in human serum (Martin *et al.,* 1976; Platts-Mills and Istlizaka, 1974). However, I observed significant differences when running haemolytic assays under conditions selecting for each of the three complement activation pathways (see figure 3.22). The differences observed can easily be explained by the presence or absence of specific acquired antibody populations that are present in human sera. Dependent on the presence or absence of

immunoglobulins that can bind antigenic structures on target erythrocytes from different species, individual antibody profiles can initiate classical pathway activation on target erythrocytes. In contrast, when using non-immunised mouse and chicken sera, such individual differences can be excluded, because of the maintenance of these animal in pathogen free environments limits the presence of acquired antibodies in non-immune sera.

My result on mouse serum show addition of recombinant murine properdin increases haemolytic activity of the wild type mouse serum. In contrary to this, addition of recombinant human MASP-3 to normal human serum does not increase haemolytic activity in human serum (see figure 3.25) and this indicates most likely that the concentration of MASP-3 in the serum in full saturation.

3.2.3.4 3MC sera are compromised in the complement mediated haemolytic activity toward rabbit, guinea pig and chicken erythrocytes

My results by using mouse serum show MASP-3 plays important role in complement mediated haemolysis in mouse (see figures 3.7, 3.8 and 3.9). In line with that, I tested the haemolytic activity of MASP-3 deficient sera of patients presenting with an inherited developmental defect termed 3MC syndrome. Sera from two different 3MC patients (patient 2 and patient 3) have been used in my assays. Patient 2 has three SNPs within the exon 12 of the *MASP1* gene that encodes the serine protease of MASP-3 leading to the expression of functionally inactive MASP-3 while the MASP-1 expression is unaffected. Patient 2 therefore has functionally active MASP-1 and MAp44 in his plasma. Patient 3, has SNP inserting a stop codon into the coding sequence for the signal peptide shared by MASP-1, MASP-3 and MAp44 and is therefore deficient of MASP-1, MASP-3 and MAp44 (Professor Wilhelm Schwaeble personal communication). In

addition to this, patient 3 is deficient of MBL. The 3MC sera ability to lyse rabbit erythrocytes (see figure 3.13), guinea pig erythrocytes (see figure 3.14) and chicken erythrocytes (see figure 3.18) are compromised significantly under alternative pathway specific condition. Furthermore, addition of recombinant human MASP-3 restores their functional haemolytic activities in a concentration dependent manner even to a level that marginally higher than normal human serum (see figures 3.15, 3.16, 3.17, 3.18, 3.19 and 3.20). This is most probably because of the presence of pro factor D in 3MC sera in a high concentration. Furthermore, addition of recombinant human MASP-3 with profactor D to the factor D depleted human serum restores the serum ability to mediate haemolytic activity while adding pro factor D alone without recombinant MASP-3 just restores half of the haemolytic activity of the serum (see figure 3.26). This is because of the presence of some activated factor D in the recombinant pro factor D protein. On the other hand, addition of recombinant human MASP-3 to the factor D depleted human serum alone does not show any restoration of the haemolytic activity of the serum (see figure 3.26). These results concur data that show MASP-3 is a physiological activator of factor D (Iwaki *et al.,* 2011).

These results by using MASP1/3 deficient mouse serum and 3MC patient sera show that MASP-3 plays a pivotal role in the complement mediated haemolysis and this may be a beneficial target in the therapy of the disease that caused by complement mediated haemolysis like PNH.

3.2.3.5 Role of MAp44 in complement mediated haemolytic activity in human

MAp44 is an alternative splicing product of *MASP1* gene. It shared the 5 N-terminal domains forming the heavy chain of MASP-1 and MASP-3 with these

serine proteases (i.e. CUB1/EGF/CUB2/CCP1/CCP2 domains and then continues with an enzymatically inert C-terminal sequence with no homology to the C-terminal serine proteases of MASP-1 and MASP-3. Since the heavy chain of MASP-1, MASP-2, MASP-3 and MAp44 bind to the serine protease binding site located within the collagenous region of all 5 different human and mouse lectin pathway recognition molecules, MAp44 is considered to compete with MASP-1, MASP-2 and MASP-3 for the binding to their respective recognition subcomponents and thus is considered to down-regulate lectin-pathway mediated complement activation. My own results (see figure 3.24) however indicates that MAp44 exhibits no inhibitory activity in complement-mediated haemolysis, since addition of a molar excess of recombinant human MAp44 does not in any way affect the ability of human serum to lyse erythrocytes.

3.2.4 MASP-3 deficiency decreases C3 deposition activity of both human and mouse sera

Upon activation of the complement system on targeted cells different anaphylatoxins are released and the targeted cells are coated with different complement components (like C3b) which act as opsonin in different stages of the complement cascade. Even blocking the final pathway of complement system to prevent cell mediated lysis leads to continuous accumulation of these opsonin on the target cell surfaces. For example, despite using eculizumab, most of the PNH patients remain anaemic and have evidence of extravascular haemolysis like reticulocytosis, increased bilirubin level and decreased haptoglobin level (Roth *et al.,* 2011; Risitano *et al.,* 2009). This is due to entrapment of the erythrocyte coated C3b in the hepatosplenic macrophages (Hill *et al.,* 2010; Risitano *et al.,* 2009). In contrast to this, my results show in the absence of MASP-3, C3 deposition decreased dramatically in both human and

mouse serum scenarios and restoration of these sera with recombinant human and murine MASP-3 restores their ability to deposit C3b in a concentration dependent manner (see figures 3.27, 3.28 and 3.29). Because the MASP-3 function is to activate pro factor D to factor D, so the absence of MASP-3 causes blockage of the AP in the level before C3b is formed and by this also C3b deposition on the complement targeted cell decrease dramatically.

Chapter 4: Haemolytic assay using 3MC serum against PNH clone erythrocytes

4.1 Results

4.1.1 3MC serum lacks ability to lyse PNH clone erythrocytes

My results clearly show that MASP-3 plays an important role in the complement mediated haemolysis in both human and mouse sera. PNH clone erythrocytes are prone to be lysed by the complement system because their surfaces are deficient in the cell bound complement regulators, CD55 and CD59. These deficiencies seen in some of the erythrocytes from the PNH patients' subject is thought to be the defect that renders that subpopulation of cells hyper-susceptible to complement mediated haemolysis. The final stage of the complement activation, MAC, results in complete loss of cells. To assess the role of MASP-3 in complement mediated haemolysis on PNH clone erythrocytes (CD59 negative), 3MC serum (patient 3) was used against PNH patient erythrocytes. All conditions, except for two control conditions, were run under alternative pathway specific condition. Thus, the observed complement activity is assumed to be lectin independent and reflect the AP. Human serum was used as a positive control and heat inactivated human serum used as a negative control (see figure 4.1). The PNH patient blood group is (A+ve) while 3MC serum (patient 3) is (O+ve), this means 3MC blood group is incompatible with the PNH blood group. As high serum concentrations were used (25% and 50%), it is unclear whether the EGTA concentration used is enough to chelate all calcium and inhibit the classical and lectin pathway. RBCs from a subject with PNH were exposed to various levels (25 to 100%) of either acidified normal human serum (NHS) or acidified serum from 3MC patient 3. Flow cytometry was used to measure surface expression of CD59 and cell surface deposition of C3d. The staining of surface CD59 reveals CD59-positive and CD59-negative

subpopulations of patient RBCs. Analysis of how the two subpopulations differ in the extent of C3d surface deposition was used to measure their relative sensitivities to the complement activation. To assess the role of MASP-3 in complement mediated lysis in PNH patients, 3MC serum (patient 2) was used. Recombinant truncated human MASP-3 was added to the 3MC serum to create a "normal" control sample with an intact AP. Two concentrations of serum, 25% and 50%, were separately tested. The two different serum levels allowed the experimenters to observe different stages of the progression from complement deposition to cell loss (haemolysis). Analysis of the relative levels of C3d staining on CD59-positive and CD59-negative RBCs is a measure of the relative level of complement activation on the subpopulations. The greater the level of C3d deposition (more events in Q2 in dot-plot), indicates the greater the activity of the complement pathway. A significant decrease in the number of events in a particular gate relative to a control sample is interpreted as haemolysis. In other words, cells "disappear" from the gate because they have undergone cell death and no longer contribute to the event count for the entire population.

In figure 4.1, 50 μl of washed PNH patient RBCs were added to heat inactivated serum together with 25 mM $MgCl_2$ (for optimal complement lysis) and 50 μl of 0.2 M HCL (for acidified serum tests) (see figure 4.1).

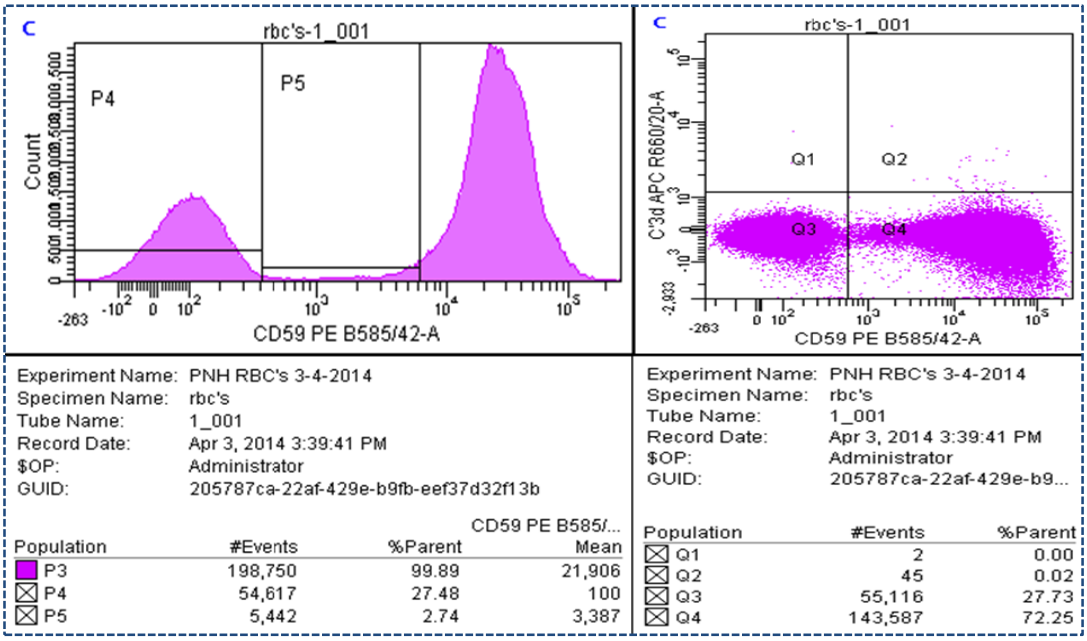

Figure 4.1: Heat inactivated serum does not lyse PNH clone erythrocytes
50 µl of washed PNH patient RBCs incubated in 400 µl of heat inactivated normal human serum. 25 mM $MgCl_2$ was added and 50 µl of 0.2 M HCL (for acidified serum).

As can be seen in figure (4.1), the total PNH clone RBC size is 27.48% in P4 in histogram and Q3 in dot-plot. This is total number of CD59 negative RBCs as the same amount of patient RBCs were used in other tubes (50 µl). For assessing haemolysis this number is used as a total number of PNH RBC clone, i.e. 100% amount of CD59 negative RBCs. There is no C3 deposition on CD59 negative and positive RBCs, Q1 and Q2 respectively and this indicates there is no complement activation in the heat inactivated human serum.

In figure 4.2, PNH patient RBCs incubated in 100% NHS.

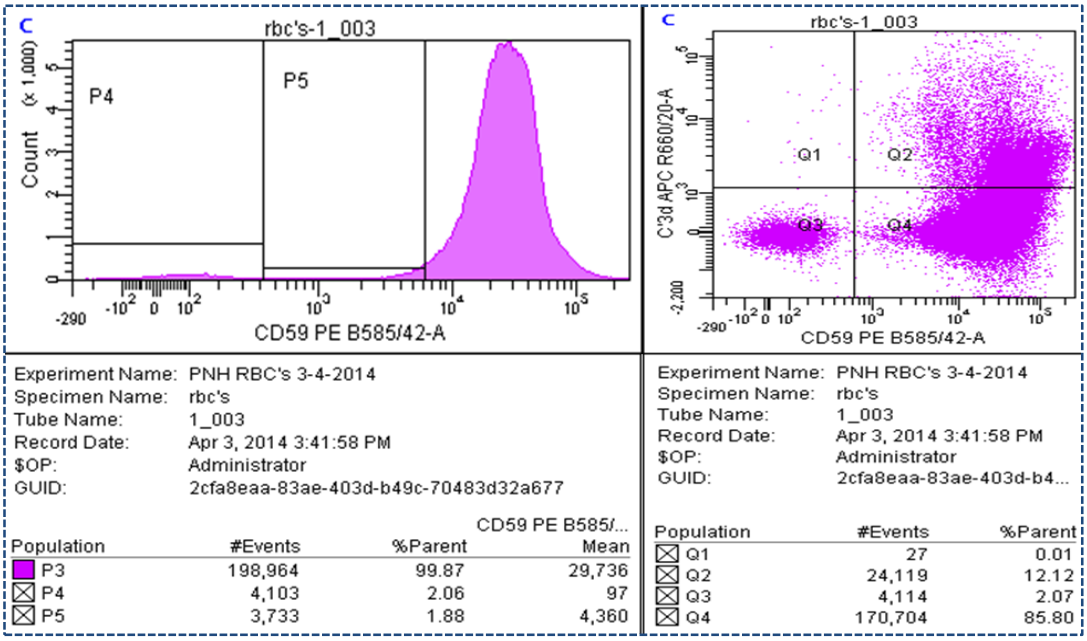

Figure 4.2: 100% human serum lyses PNH clone erythrocytes completely
50 µl of washed PNH patient RBCs incubated in 400 µl of normal human serum. 25 mM $MgCl_2$ was added and 50 µl of 0.2 M HCL (for acidified serum).

As seen in figure (4.2) by using 100% NHS, more than 93% of PNH clone RBCs are lysed (just 2.06% remain form CD59-negative RBCs, P4 in histogram and Q3 in dot spot) and there is significant C3d deposition on CD59-positive RBCs (Q2 in dot spot). These indicate complement system activation on the both groups of RBCs and this activation cause cell lysis in CD59-negative cells and C3b deposition on CD59-positive cells. In this tube EGTA was not used, so presumably complement activation comes from all three pathways. No C3b deposition can be seen on CD59-negative RBCs (Q1 in dot spot) as the activation of complement system on this group of RBCs continue and they have undergone cell death and no longer contribute to the event count for the entire population.

In figure 4.3, 50% NHS was used and the assay run under alternative pathway specific condition to inhibit both the classical and lectin pathways and let the AP full functioning.

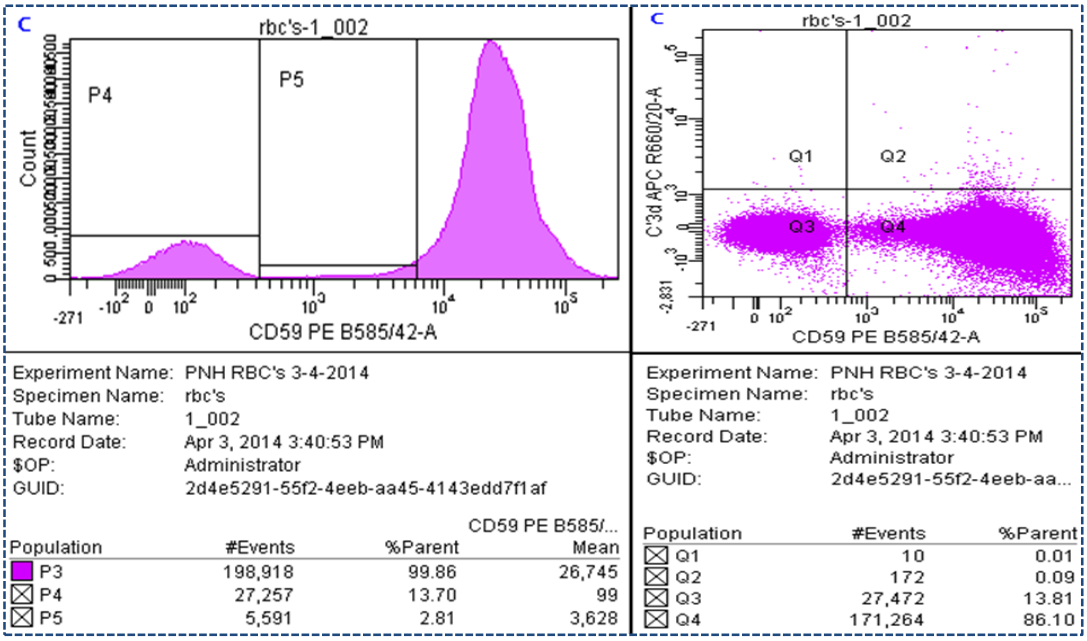

Figure 4.3: 50% human serum lyses about 50% of PNH clone erythrocytes under AP specific condition

50 µl of washed PNH patient RBCs incubated in 200 µl of normal human serum (50% serum). 50 µl of 0.2 M HCL was added (for acidified serum). The assay was run under alternative pathway specific condition (BBS/Mg^{+2}/EGTA).

As can be seen in figure 4.3, addition of EGTA to PNH RBCs incubated in (50% NHS) shows diminishing complement activity. There is significant inhibition of the haemolysis seen in the CD59-negative subpopulation by the addition of EGTA in comparison to 100% serum (49% lysis versus 98% lysis, respectively) and only a trace amount of C3d deposition in CD59-pos subpopulation (0.1% in Q2). This diminishing in complement activity is because the amount of serum is half that used in the previous two conditions and the EGTA added here is capable of significantly reducing the level of complement activity and cell lysis caused by ABO incompatibility. Interestingly, in this particular case, the EGTA was not as successful at preventing haemolysis of CD59-negative cells as heat-inactivation with twice the concentration of serum in figure 4.1 (49% lysis versus 2% lysis respectively). So this indicates the AP functional activity in this concentration.

In (see figure 4.4), 25% NHS is used and the assay run under alternative pathway specific condition.

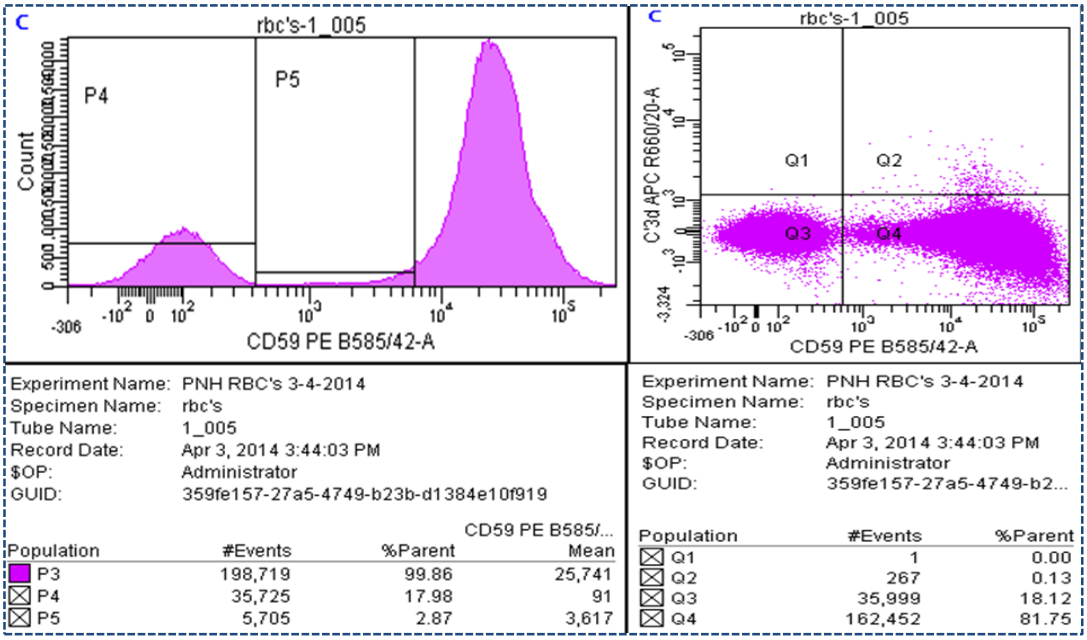

Figure 4.4: 25% human serum lyses 35% of PNH clone erythrocytes under AP specific condition

50 µl of washed PNH patient RBCs incubated in 100 µl of normal human serum (25% serum). 50 µl of 0.2M HCL was added (for acidified serum). The assay was run under alternative pathway-specific condition (BBS/Mg^{+2}/EGTA).

As can be seen in figure 4.4, incubating PNH patient RBCs in 25% concentration of NHS reduces complement activation on CD59-negative cells in comparison to 50% and 100% NHS. It shows 35% lysis of the total CD59-negative subpopulation (17.98% total population, P4 or Q3 + Q1). There is no C3d deposition in CD59-negative subpopulations (0%, Q1). Despite the presence of EGTA, some C3d deposition on CD59-positive cells can be seen (0.13%, Q2). There may be residual complement activity is due to ABO incompatibility even in the presence of EGTA.

In (see figure 4.5), 25% 3MC (patient 3) serum is used and assay run under alternative pathway-specific condition.

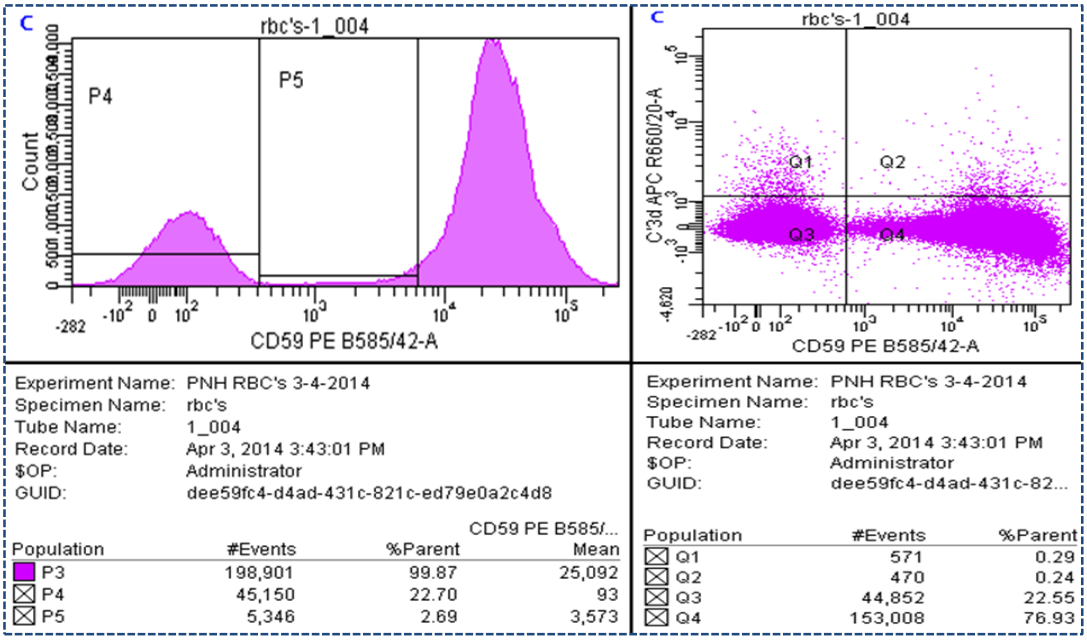

Figure 4.5: 25% 3MC serum is compromised dramatically in the haemolytic activity toward PNH clone erythrocytes

50 µl of washed PNH patient RBCs incubated in 100 µl of 3MC (patient 3) serum (25% serum). 50 µl of 0.2 M HCL was added (for acidified serum). The assay was run under alternative pathway specific condition (BBS/Mg^{+2}/EGTA).

As can be seen in figure 4.5, by incubating PNH patient RBCs in 25% concentration of 3 MC (patient 3) serum causes decrease in the haemolysis of CD59-negative cells (23% total population, P4 or Q3 + Q1) comparable to what was observed in the same concentration of NHS (17% lysis versus 35% lysis, respectively). Some C3d deposition in CD59-neg subpopulation (0.3%, Q1) and on CD59-positive cells (0.2%, Q2), suggesting that despite the presence of EGTA, there is some residual complement activity is due to ABO incompatibility. In order to clarify this decrease in haemolytic activity of 3MC serum is due to MASP-3 deficiency or something else in the 3MC serum, 40

µg/ml (w/v) of recombinant truncated human MASP-3 was added to the serum prior to addition of RBCs (see figure 4.6).

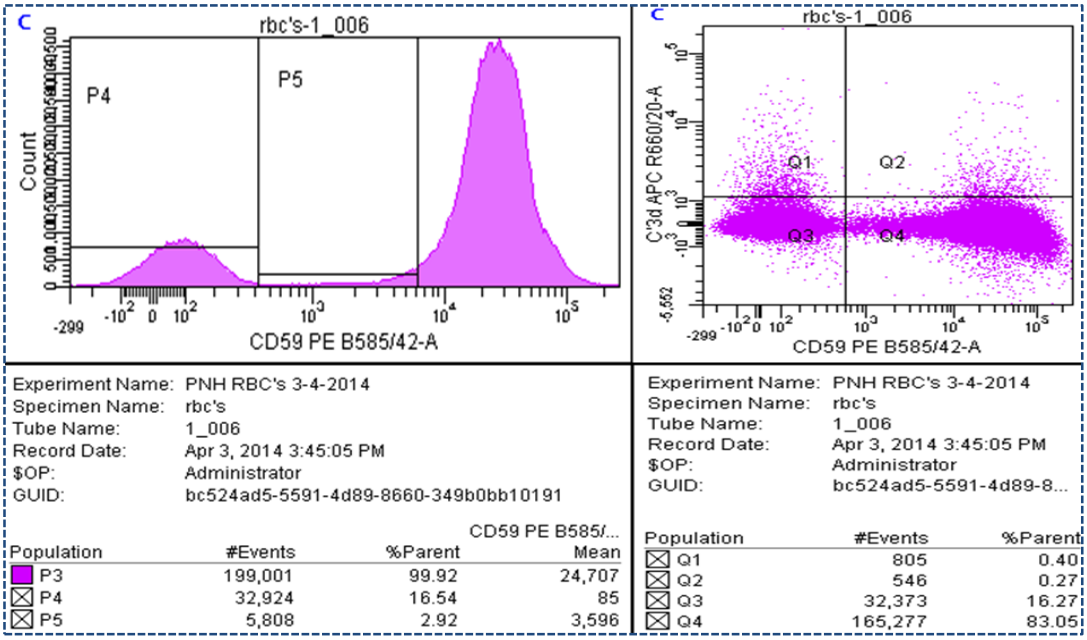

Figure 4.6: Truncated MASP-3 restores haemolytic activity of 3MC serum towards PNH clone erythrocytes

25% 3MC serum was preincubated with 40 µg/ml (w/v) of recombinant truncated human MASP-3 and incubated for 30 minutes on ice prior to addition of PNH patient RBCs. 50 µl of 0.2 M HCL was added (for acidified serum). The assay was run under alternative pathway specific condition (BBS/Mg^{+2}/EGTA).

As can be seen in figure 4.6, 25% 3MC serum supplemented with recombinant tr MASP-3 restores complement activation on CD59-negative cells. The CD59-negative subpopulation lysis in 3MC serum reconstituted with tr MASP-3 is increased dramatically comparable to what was observed in the same concentration of 3MC serum lacking recombinant protein (38% lysis versus 17% lysis, respectively) and this haemolytic activity is comparable with the NHS at the same concentration (38% versus 35%). Thus, the presence of recombinant MASP-3 has dramatic effect on haemolysis at the concentration of 25% serum. While C3d deposition cannot be seen on the CD59-negative subpopulation of

figure 4.5, the addition of recombinant MASP-3 does not increase C3d deposition on CD59-neg cells (0.29 and 0.40 respectively) as the complement activation on CD59-negative RBCs end up with cell lysis. Despite the presence of EGTA, see some C3d deposition on CD59-pos cells (0.3%, Q2 in dot spot).

In (figure 4.7), 50% 3MC (patient 3) serum is used and assay was run under alternative pathway specific condition.

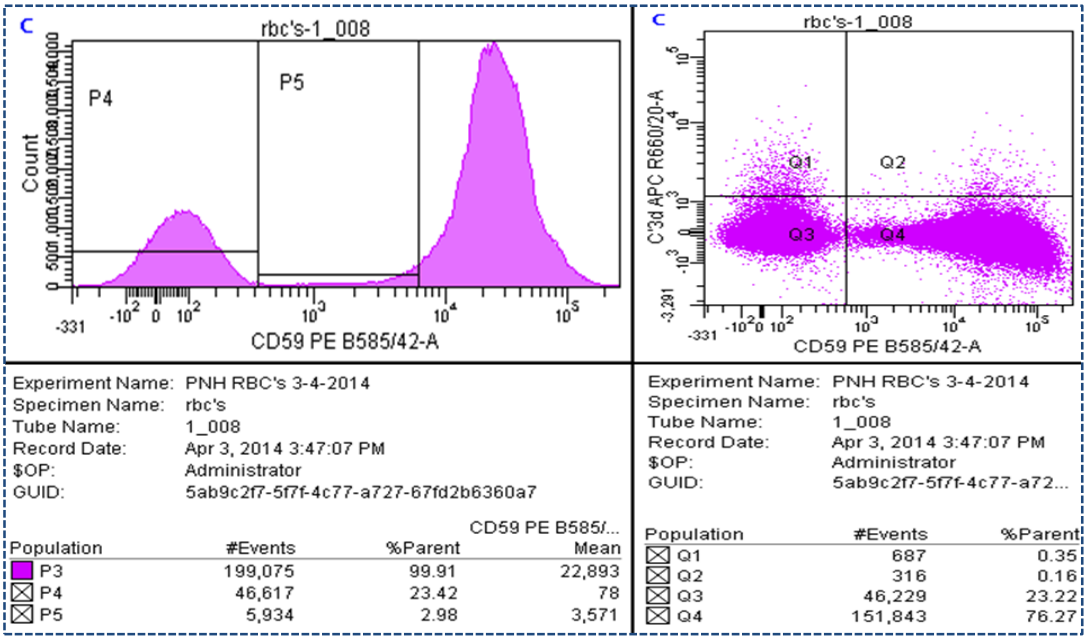

Figure 4.7: 50% 3MC serum is compromised dramatically in the haemolytic activity toward PNH clone erythrocytes

50 µl of washed PNH patient RBCs incubated in 200 µl of 3MC (patient 3) serum (50% serum). 50 µl of 0.2 M HCL was added (for acidified serum). The assay was run under alternative pathway specific condition (BBS/Mg^{+2}/EGTA).

As can be seen from figure 4.7, incubating PNH patient RBCs in 50% 3MC serum shows reduced haemolysis on CD59-negative population dramatically in comparison to NHS at the same concentration (16% lysis versus 51% lysis, respectively). Thus, it is clear that the MASP-3 deficiency of the 3MC serum causes a reduction in haemolysis. Interestingly, despite the absence of MASP-3

in the 3MC serum and the presence of EGTA, there is still clear C3d deposition within CD59-negative subpopulation (0.35% of total events). This deposition was less observed in the lower 3MC serum level (25%) of (figure 4.5) (0.29% C3d-pos events). So, this may be most probably due to ABO incompatibility. In order to clarify whether this decrease in haemolytic activity of 3MC serum is due to MASP-3 deficiency or something else in the 3MC serum, 40 µg/ml (w/v) of recombinant truncated human MASP-3 was added to the serum prior to addition of RBCs (see figure 4.8**).**

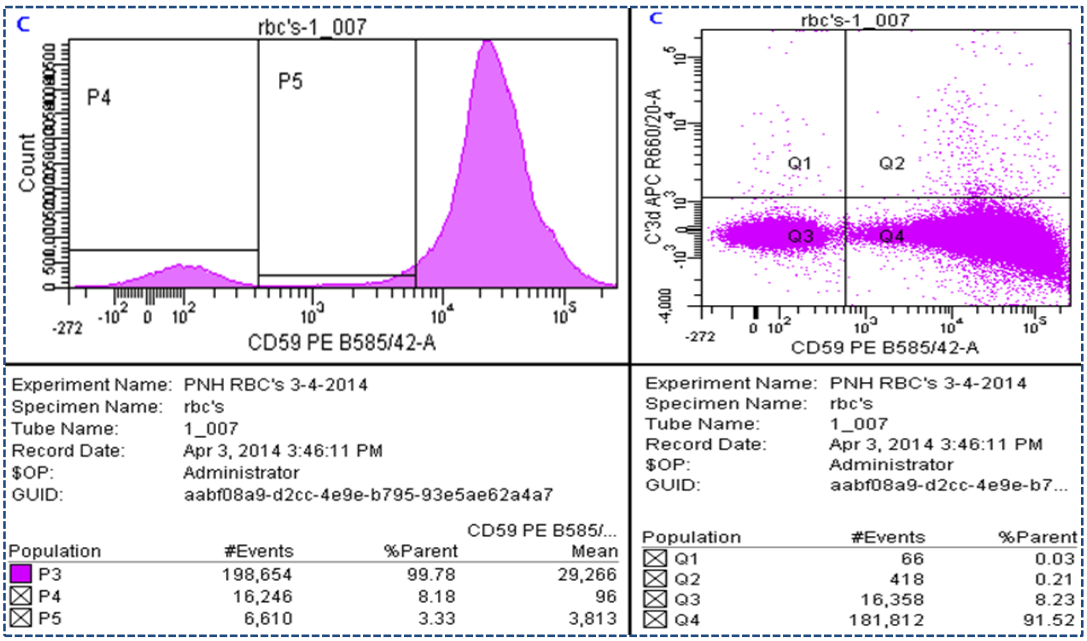

Figure 4.8: Truncated MASP-3 restores haemolytic activity of 3MC serum towards PNH clone erythrocytes

50% 3MC serum was preincubated with 40 µg/ml of recombinant truncated human MASP-3 and incubated for 30 minutes on ice prior to addition of PNH patient RBCs. 50 µl of 0.2 M HCL was added (for acidified serum). The assay was run under alternative pathway specific condition (BBS/Mg^{+2}/EGTA).

It can be seen clearly 50% 3MC serum that has been supplemented with recombinant MASP-3 restores the haemolysis of CD59-negative cells. See clear decrease in the CD59-negative sub-population, haemolysis, in 3MC serum that

reconstituted with trMASP-3 in comparison to 3MC sera with the same concentration (71% lysis versus 16% lysis, respectively). Interestingly, this level of lysis is more than even normal human serum in same concentration (71% versus 51% respectively) and this is may be either due to different degree of ABO incompatibility between sera or presence of more profactor D in 3MC serum. C3d deposition within CD59-negative subpopulation is decreased dramatically and this is because the CD59-negative subpopulation is quickly lysed. These results clearly clarify the pivotal role of MASP-3 in lysis of PNH clone RBCs in human.

4.2 Discussion

PNH is an acquired haematological disorder which emerges resulting from somatic mutation in the X-linked *phosphatidylinositol glycan class (A)* gene. This causes absence of CD55 and CD59 on the hamatopoeitc stem cells and their progenies (Holguin *et al*., 1989; Holguin *et al*., 1990, Nicholson-Weller *et al*., 1982; Parker, 2007; Parker *et al*., 2005). This makes them vulnerable to lysis by an uncontrolled complement activation.

Eculizumab, which is anti C5 monoclonal antibody, is the only complement system inhibitor drug and becomes a standard treatment for PNH patients with symptoms (Roth *et al*., 2011). Although using eculizumab reduces the risk of thrombus recurrence, the risk of thrombosis still remains high in these patients in comparison to normal population. Despite using eculizumab, the majority of the patients remain anaemic and have evidence of extravascular haemolysis like reticulocytosis, increased bilirubin level and decreased haptoglobin and just half of the patients remains transfusion independent (Roth *et al*., 2011; Risitano *et al*., 2009; Lidorfer *et al*., 2010). This is due to entrapment of the erythrocyte coated C3b in the hepatosplenic macrophages (Hill *et al*., 2010; Risitano *et al*., 2009: Hill *et al*., 2010). This is because of the fact that eculizumab is an anti C5 monoclonal antibody and does not have a role in the deposition of C3b on the surface of PNH erythrocytes. Using FACS analysis on PNH clone erythrocytes, there are no sign of C3 bound to PNH clone erythrocytes. In contrary to this, all patients that receive eculizumab have evidence of C3 bounding to their PNH clone erythrocytes surfaces (Risitano *et al*., 2009). This indicates that in patients that have not received eculizumab PNH clone erythrocytes are lysed through MAC formation on the erythrocyte surface (Lindorfer *et al*., 2010). It is generally accepted that continuous alternative pathway activation is an essential component of the pathophysiology of PNH (Lindorfer *et al*., 2010). Since extravascular haemolysis is not taken care of in this very expensive treatment

with eculizumab (approximate costs in excess of 300,000 GBP per patient per year) blocking the AP at an earlier stage of activation, i.e. before C3 activation is likely to provide a far superior therapeutic approach since this would prevent both intravascular and extra vascular haemolysis (Lindorfer *et al.*, 2010). PNH clone erythrocytes are more prone to lysis in the acidified serum, because this acidification enhances the binding of factor B to the C3b that has already bound to the erythrocyte surface, enhances generation of both AP C3 and C5 convertases and decrease the regulatory functions of both CR1 and factor I on C3b bounding erythrocytes (Fishelson *et al.*, 1987).

My results clearly demonstrate that MASP-3 plays an important role in driving the AP in both human and mouse sera. My work has shown this in both haemolytic assays and C3 deposition assays. In order to establish the proof-of-principal showing that a deficiency of MASP-3 affects the haemolytic activity of human serum, I have used and assessed the lytic activity and the opsonising activity of 3MC serum against the PNH clone erythrocytes. When running my assay under alternative pathway specific conditions, 3MC serum (patient 3), showed markedly impaired in lysing of PNH clone erythrocytes (see figures 4.5 and 4.7). That this impairment is due to the absence of MASP-3 in the 3MC serum used was proven by restoring the defective haemolytic activity and the lack of C3 opsonisation of PNH erythrocytes by recombinant human truncated MASP-3 to the 3MC serum tested (see figures 4.6 and 4.8). The restoration of the ability of 3MC serum to lyse PNH clone erythrocytes has unequivocally shown that the defective serum lytic activity of 3MC serum is caused by the absence of functionally active MASP-3.

A Western blot analysis of immunoprecipitated and deglycosylated factor D preparations (carried out by our collaborator Dr. Minoru Takahashi, Fukushima Medical University, Japan) of the two 3MC patient sera that I have analysed in my study revealed that the most prevalent form of factor D contained in these

sera was pro-factor D (data not shown). These data strongly underline my conclusion that the defective AP function in the 3MC sera analysed in my thesis is caused by the lack of profactor D conversion to active factor D via MASP-3.

Some haemolysis observed in the 3MC serum on PNH clone erythrocytes and this lysis most probably driven by ABO incompatibility as the 3MC patient and PNH patient are ABO incompatible and the EGTA that were used is unable efficiently to chelate calcium in the low PH buffer that were used to run the assay (Bryant and David, 1968). This shows targeting MASP-3 therapeutically may be an effective measure in treating the diseases that are AP mediated. Furthermore, By targeting AP alone and allow the other two complement pathways functioning normally the risk of infections is decreased especially by pathogens that are liable to lysis by complement system like *Neisseria meningitidis.*

Chapter five : *Role of properdin in fighting Streptococcus pneumoniae infection in mice*

5.1 Results

5.1.1 Results of in vitro study

5.1.1.1 Recombinant properdin enhances C3b deposition on the surface of S. pneumoniae

Activation of complement cascades are initiated by recognition of pathogen associated surface structures through specific recognition subcomponents. Complement activation leads to the release of potent anaphylatoxins, the coating of the surface of pathogens (i.e. opsonisation) with different complement activation products (e.g. C3b) and finally to the lysis of some pathogens through membrane attack complexes (Walport, 2001; Dunkelberger and Song, 2010A). Recent work in our laboratory showed recombinant properdin enhances C3b deposition on *Neisseria meninigititis*. In line of that, I tested the ability of recombinant properdin (P_n) to enhance C3b deposition on *S. pneumoniae* by using both human and mouse serum (see figures 5.1 and 5.2**).** The assay was run under conditions that permitted alternative pathway activation but excluded activation of the lectin and classical pathways i.e. using AP buffer (BBS/Mg^{+2}/EGTA). As can be seen in the figure 5.1, using 5% human serum, little C3b deposition can be seen on the bacteria. Pre-incubating human serum with recombinant human P_n at a concentration of (5 μg/ml) for 30 min on ice leads to substantial deposition of C3b or iC3b on the bacteria. Similarly, by using 15% mouse serum no C3b deposition can be seen while pre-incubating mouse serum with recombinant murine P_n at a concentration of (10 μg/ml) for

30 min on ice leads to major C3b and iC3b deposition on the bacteria. This indicates that addition of recombinant P_n leads to increased C3b deposition on the surface of bacteria, thus potentially enhancing phagocytosis.

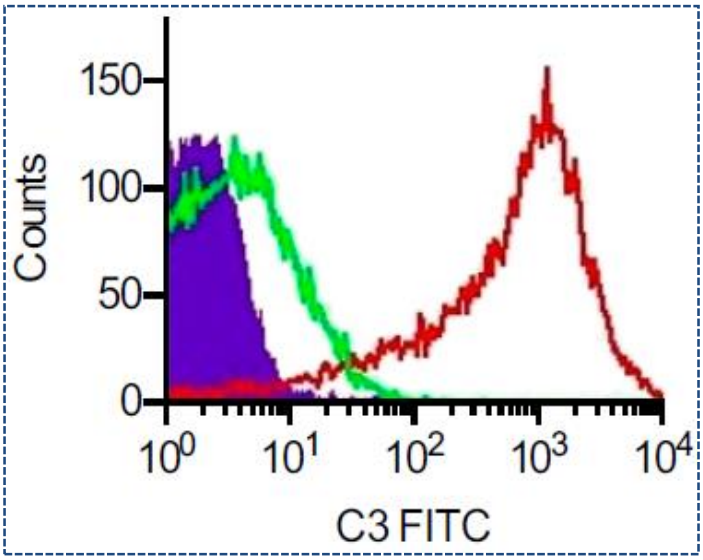

Figure 5.1: Recombinant human P_n enhances *S. pneumoniae* opsonisation with C3b
1×10^5 *S. pneumoniae* D39 cells were opsonised with 5% NHS (v/v), with and without the addition of (5 μg/ml) of recombinant human P_n. The assay was run under alternative pathway specific condition by using AP buffer (BBS/Mg^{+2}/EGTA). FACS analysis was used to assess C3b deposition. Green shows bacteria opsonised with 5% human serum alone; red, bacteria opsonised with 5% human serum plus recombinant human P_n (5 μg/ml); blue, represents unopsonised bacteria. This result represents three independent experiments.

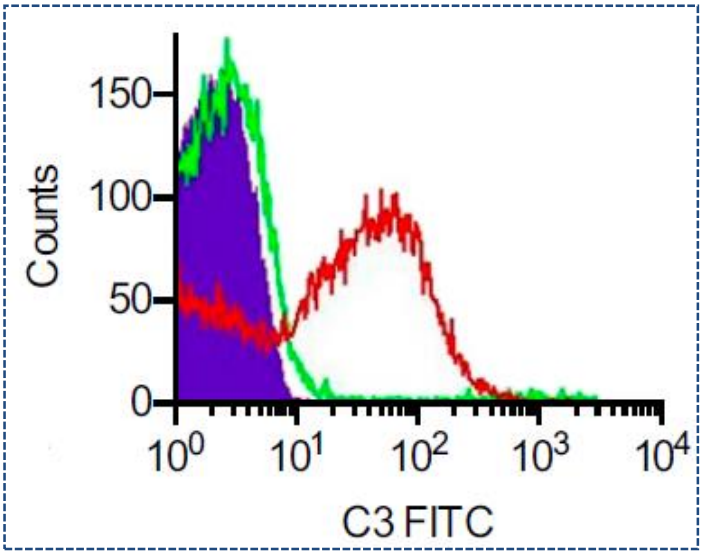

Figure 5.2: Recombinant murine P_n enhances *S. pneumoniae* opsonisation with C3b
1×10^5 *S. pneumoniae* D39 cells were opsonised with 15% wild type mouse serum, with and without the addition of (10 μg/ml) of recombinant murine P_n. The assay was run under alternative pathway specific condition by using AP buffer (BBS/Mg^{+2}/EGTA). FACS analysis was used to assess C3b deposition. Green shows bacteria opsonised with 15% WT mouse serum alone; red, bacteria opsonised with 15% WT mouse serum plus recombinant murine P_n (10 μg/ml); blue, represents unopsonised bacteria. This result represents three independent experiments.

5.1.1.2 S. pneumoniae is resistance to lysis by mouse complement system

In order to test the effectivity of the recombinant murine P_n in enhancing bactericidal activity of the mouse serum, serum bactericidal assay was performed on *S. pneumoniae* (see figure 5.3)**.** Running bactericidal assay by using barbital buffered saline that supports all complement pathways showed no any bactericidal activity of mouse serum on *S. pneumoniae* even after addition of 10 μg/ml of P_n as the bacterial survival count remained similar over 5 time points after incubation and remained unchanged as in heat inactivated mouse serum which was used as a negative control.

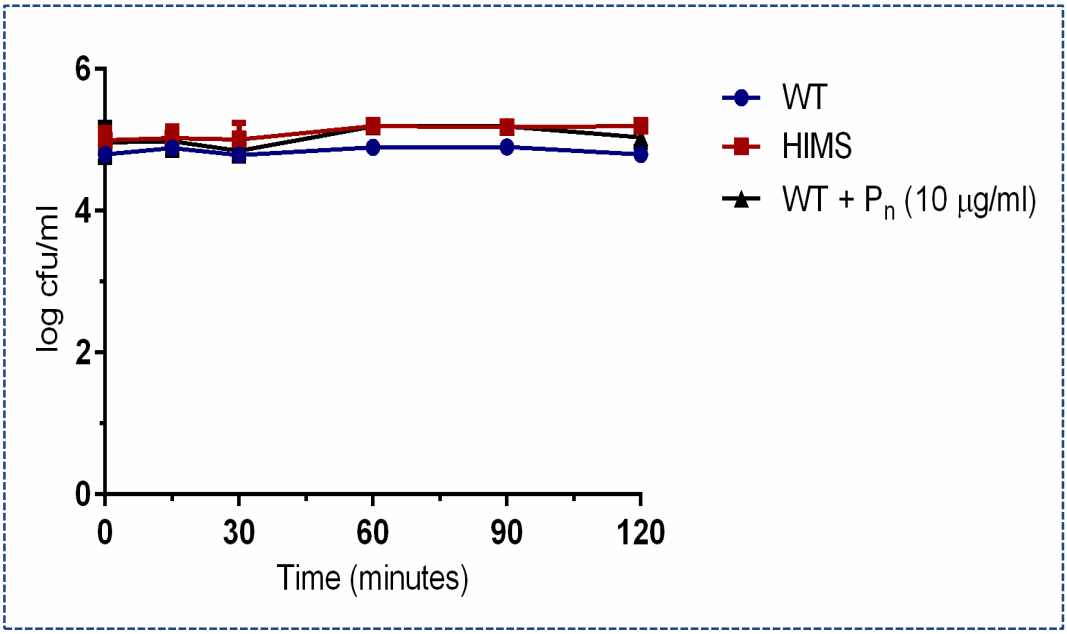

Figure 5.3: *S. pneumoniae* is resistance to complement mediated lysis in mouse serum
Serum bactericidal assay on *S. pneumoniae* by using 40% wild type mouse serum, with and without the addition of (10 μg/ml) of recombinant murine P_n. Barbital buffered saline (BBS/Ca^{+2}/Mg^{+2}) was used to run the assay. Heat inactivated mouse serum was used as a negative control. Student t test was performed and P value between WT and WT with P_n in different time points is > 0.05. Results are mean (±SEM) of three independent experiments.

5.1.2 Results of in vivo study

5.1.2.1 Survival of recombinant Pn treated mice and their littermates control mice after intranasal infection with S. pneumoniae D39

To determine the role of recombinant P_n in fighting against the streptococcus *pneumoniae infection*, 20 wild type female mice (C57BL/6) were challenged with 2.5×10^6 CFU of *S. pneumoniae* D39 by intranasal infection. At the same time, 10 of them injected with 100 μg of recombinant murine Pn intraperitoneally (i.p) and other 10 mice received saline only. The mice monitored for one week post infection. Animals showing severe symptoms were euthanized (see figure 5.4). 34 hours after introducing bacteria to the mice, 7 of the control group mice were showing severe symptoms of infection and were euthanized by cervical dislocation under general anaesthesia. On the other hand, all recombinant murine P_n treated mice were free of symptom at this time point. The remaining three mice from control group were reaching final stage of illness at time points of 40 and 50 hours post infection. While, in the Pn treated group one mouse showed severe symptom at 60 hours and other 3 showed severe symptoms 66 hours after of infection and they were euthanized by cervical dislocation under general anaesthesia. All the mice who survived till 75 hours post infection, survived until the end of experiment. After one week of infection, 40 % of complement factor properdin treated group mice survived (see figure 5.4).

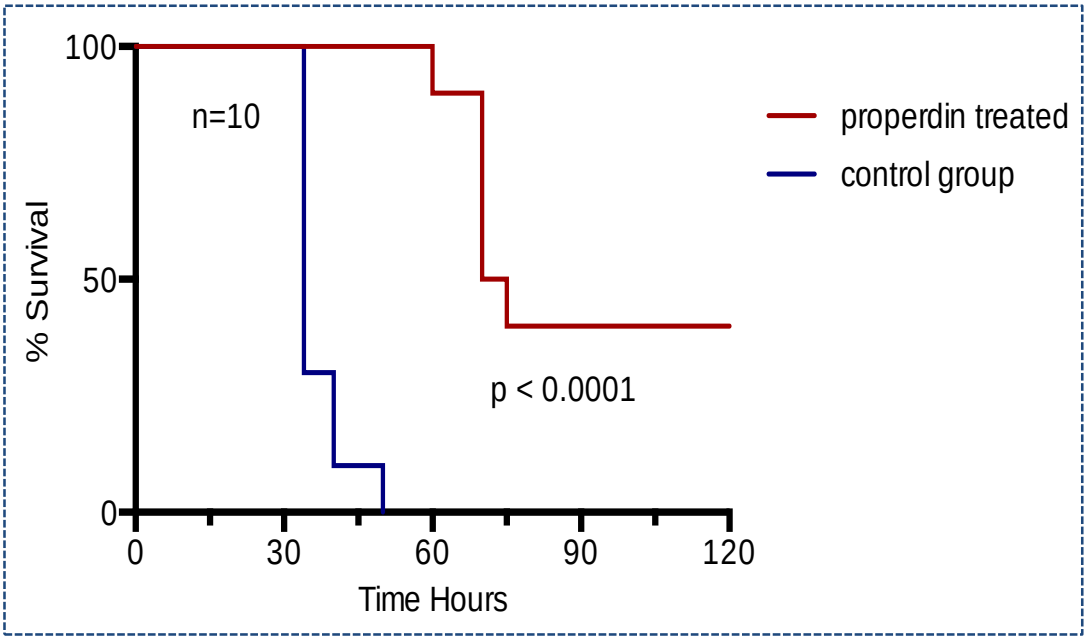

Figure 5.4: Recombinant P_n protects mice against *S. pneumoniae* infection
Survival of mice WT C57BL/6 and C57BL/6 treated with recombinant murine P_n (10/group). Mice were infected intranasally with 2.5 × 10^6 CFU of *S. pneumoniae* D39. Animals in the treated group were injected intraperitoneally with 100 μg of recombinant murine P_n at the time of infection and control group mice injected with saline only. Mantel–Cox log-rank test was performed. Results are representative of two independent experiments with similar outcome.

5.1.2.2 Viable count of S. pneumoniae D39 in peripheral blood of P_n treated mice and their littermates control mice after intranasal infection with Streptococcus pneumoniae D39

During the course of the survival experiment, blood was collected from both recombinant murine P_n treated mice and their mate control mice after 6, 12, and 24 hours post infection via tail bleed. Viable counts of bacteria at selected time points were determined by plating on blood-agar plates. In control group, bacteraemia started after 6 hours of infection. 9 out of 10 mice had bacteraemia after 12 hours of infection (see figure 5.5). Whilst, there was no detection of bacteria in the P_n treated group at 6 or 12 hours post infection. After 24 hours of infection all mice in control group and six out of ten of properdin treated group

had bacteraemia. Although, the CFU count of bacteria in P_n treated mice was significantly lower than control mice.

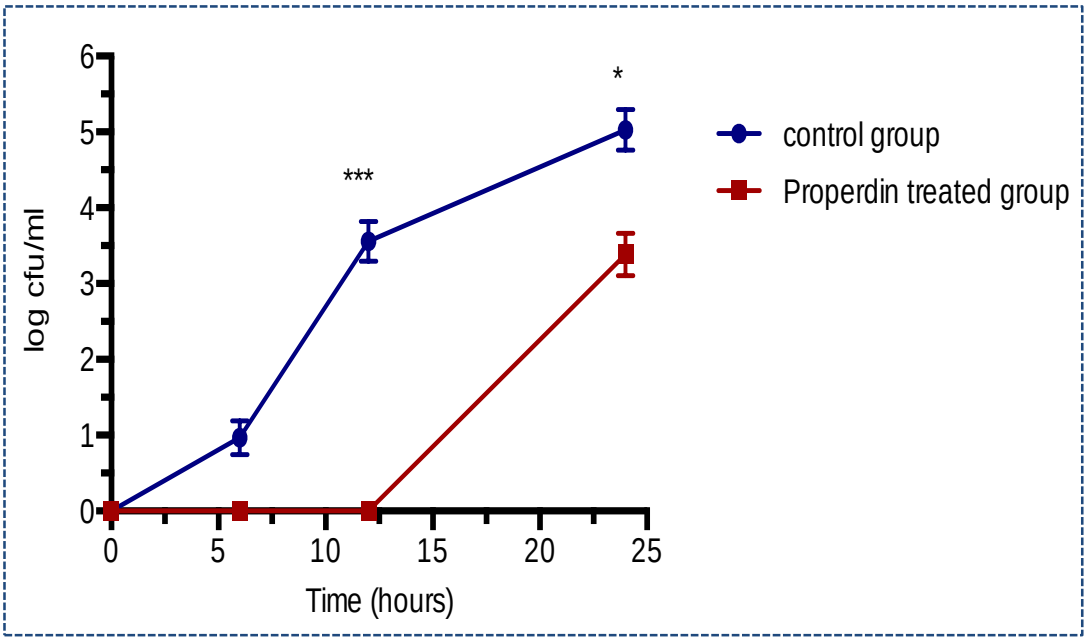

Figure 5.5: Recombinant murine P_n decreases bacteraemia in mouse
Viable *S. pneumoniae* counted in peripheral blood at the indicated time points after intranasal infection with 2.5 × 10^6 CFU of *S. pneumoniae* D39 to WT C57BL/6 and C57BL/6 treated with recombinant murine P_n (10/group). Animals in the treated group were injected intraperitoneally with 100 μg of recombinant murine P_n at the time of infection and control group mice injected with saline only. Data are mean ± SEM; n = 10. *P < 0.05; ***P < 0.001, Student t test at each time point. Results are representative of two independent experiments.

Also I assessed the role of P_n when given 6 hours before infection. For that reason, 14 mice were used. Mice in the treated group (7 of them) were injected i.p with 100 μg of recombinant murine P_n 6 hours before infection. Blood was collected from both P_n treated mice and their mate control mice at 6, 12, and 24 hours after infection via tail bleed. Viable counts of bacteria at selected time points were determined by plating on blood-agar plates (see figure 5.6). In control group, bacteraemia started in 3 mice after 6 hours of infection while

none of P_n treated mice developed bacteraemia at this point. After 12 hours bacteraemia started in 5 mice of P_n treated group while all mice in control group had bacteraemia at this stage. The CFU count of bacteria in treated mice was significantly lower than control mice. After 24 hour of infection, 7 out of 10 mice of the properdin treated group had bacteraemia but the CFU count of bacteria was significantly lower than control mice.

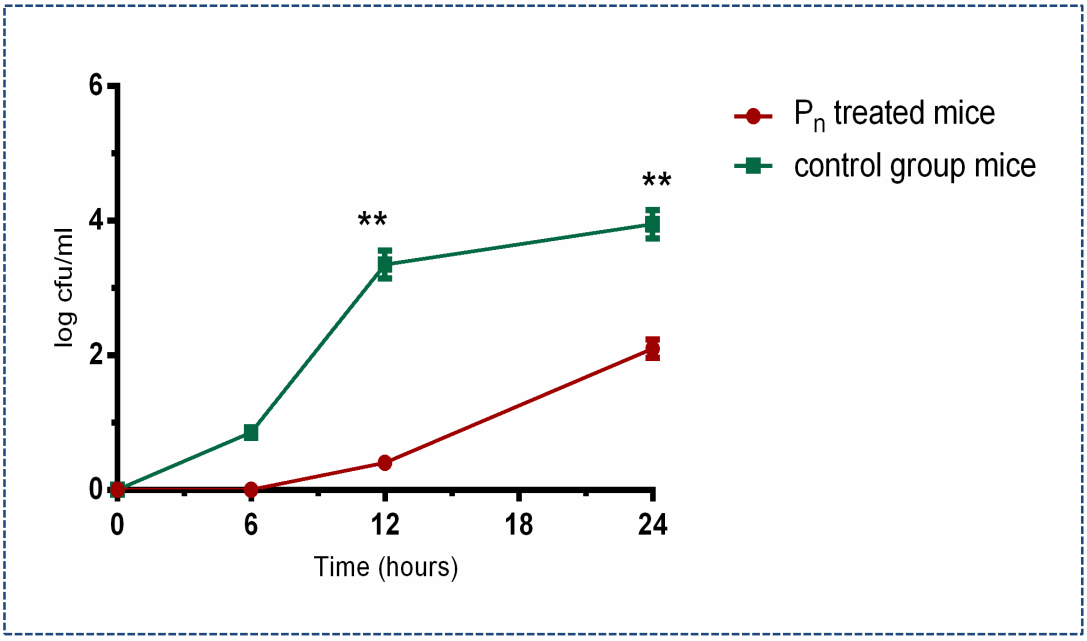

Figure 5.6: Pn treatment reduces bacteraemia in mouse when given 6 hours before intranasal infection with *S. pneumoniae*

Viable *S. pneumoniae* counted in peripheral blood at the indicated time points after intranasal infection with 2.5 × 10^6CFU of S. Pneumonia D39 to WT C57BL/6 and C57BL/6 treated with recombinant murine properdin (7/group). Mice in the treated group were injected intraperitoneally with 100 μg of recombinant murine properdin 6 hours before infection while control group injected with PBS. Data are mean ± SEM; n = 7. **P < 0.01, Student t test at each time point.

5.2 Discussion

In spite of a variety of antibiotics and development of vaccine programme, *Streptococcus pneumoniae* remains the major cause of morbidity in the world and causes more deaths than any other pathogen (Henriques-Normark and Tuomanen, 2013). Causing different types of infections, ranging from mild infections like otitis media to more severe infections like pneumonia, meningitis and septicaemia. The mortality rate of pneumococcal pneumonia in the United State is about 5% while the mortality rate increases to 20% and 30% in septicaemia and meningitis respectively (Henriques-Normark and Tuomanen, 2013). Innate immunity provides a first tool of defence against *S. pneumoniae* infection. Complement dependent phagocytosis is the key feature of innate immunity against *S. pneumoniae* infection (Yuste *et al.,* 2008). Individuals with different complement deficiency are more prone to get *S.pneumoniae* infection e.g. C2 deficiency, C3 deficiency and C4 deficiency (Dieudonne´-Vatran *et al.,* 2009; Picard *et al.,* 2003; Ross and Densen, 1991; Figueroa and Densen, 1991.*)* Different studies show the important role of the complement system in *S. pneumonia* infection. Complement depleted mice and rabbits are more prone to develop severe infection than control groups in different types of experiments (Bogaert *et al.,* 2010; Tuomanen *et al.,* 1987). All complement pathways play a role in the fighting against *S. pneumoniae* infection (Brown *et al.,* 2002; Tong *et al.,* 2010; Ali *et al.,* 2012). My study aims to assess the utility of properdin in fighting *S. pneumoniae.*

Properdin is the only known positive regulator of the complement activation. Its plasma concentration ranges between 5-15 μg/ml (Schwaeble and Reil, 1999). Properdin binds to and stabilizes the C3bBb complex (alternative pathway C3 convertase) and increases its half-life 5-10 folds (Fearon and Austen, 1975). Deficiency of properdin abolishes the serum ability to activate the AP (Schwaeble and Reid, 1999). Some studies show properdin can act as a

recognition molecules and promote complement activation (Spitzer *et al.,* 2007; Cortes *et al*., 2010; Ferreira *et al.,* 2010). However, recent work showed that this function of properdin depends on initial C3b deposition on the structures (Harboe *et al.,* 2012). Properdin deficiency has been recorded to be associated with increasing risk of infections with different bacteria such as *pneumococci (*Singh and Rai, 2009*), meningococci* (Peleg *et al.,* 1992) and *Haemophilus influenza* (Ross and Densen, 1984). Highly polymerized recombinant properdin (P_n) which is formed as a result of freezing and thawing contains high order oligomers as well as native forms of dimer, trimer and tetramer (Agarwal *et al.,* 2010; Farries *et al.,* 1987). P_n is not present in serum (Farries *et al.,* 1987). Recent studies in our lab showed the positive effect of properdin against *Neisseria meninigititis* in an experimental mouse model of infection (Ali *et al.,* 2014). In line with that, I tested the therapeutic utility P_n in fighting *S. pneumoniae* infection.

In our laboratory it has been found that P_n can bind to *S. pneumoniae* and augment complement activation while native properdin lacks this binding ability (Ali *et al.,* 2014). Therefore I tested the effect of recombinant human P_n to opsonise *S. pneumoniae*. P_n enhances C3 opsonisation of *S. pneumoniae* dramatically under alternative pathway specific conditions (see figure 5.1). Similarly, recombinant murine P_n enhances C3 opsonisation of *S. pneumoniae* dramatically (see figure 5.2). These results agree with the previous data from our laboratory that show recombinant unfractionated properdin enhances *Neisseria meningitis* opsonisation with C3b (Ali *et al.,* 2014) and indicates the pivotal role of P_n in the initiation of the process of opsonophagocytosis of the *S. pneumoniae* as C3b on the surface of the pathogen serve as ligands to the complement receptor 1 (CR1, CD35), complement receptor 3 (CD11b/CD18) and complement receptor 4 (CD11c/CD18) which are expressed on macrophages, polymorphonuclear (PMN) cells and monocytes (van Lookeren *et al.,* 2007).

These findings indicate the effect of properdin in augmenting complement function of the normal human and mouse sera.

To assess the therapeutic value of properdin against the *S. pneumoniae in vivo*, wild type mice injected with recombinant murine P_n at the time of introducing *S. pneumoniae* intranasally. After 34 hours of infection, only 30 % of the control group mice survived the infection compared to 100 % of properdin treated group mice. The P_n treated mice had significantly longer survival times in comparison to the control group ($P < 0.0001$) (see figure 5.4). Moreover, the P_n treated group mice exhibited no bacteraemia after 6 hours of infection while in control group mice bacteraemia started at this time and continues to rise. Bacteraemia in treated group mice started after 12 hours of infection in a significantly less than in the untreated group mice (see figure 5.5). Injecting recombinant murine P_n 6 hours before introducing *S. pneumoniae* intranasally reduces bacteraemia significantly at 12 and 24 hours after infection (see figure 5.6). These results come with the previous finding in our laboratory that shows P_n possess antimicrobial activity against *Neisseria meningitis*. Furthermore, shows the important role of properdin in the fighting *S.pneumoniae* infection. These results show that exogenous properdin can be used as a therapeutic tool against the pneumococcal infection.

The complement system mediates its function against pathogens most prominently through enhancing opsonophagocytosis of the pathogen by phagocytic cells and/or through the lysis of the pathogen by forming pores through membrane attack complex. To identify which of these mechanisms is enhanced by murine P_n, mouse serum bactericidal assay was performed on *S. pneumonia*e and result showed that *S. pneumoniae* is resistance to lysis by the mouse serum. Furthermore, results in this thesis show that P_n enhances opsonisation of the *S. pneumoniae* by C3b. So, the fighting mechanism of P_n against *S. pneumoniae* is by enhancing phagocytosis of the *S.pneumoniae* by

phagocytic cells. This result comes with the line of the previous data that shows complement dependent phagocytosis is the key feature of innate immunity against *S. pneumoniae* infection (Yuste *et al.,* 2008). These results show P_n may be a useful therapeutic tool to be used in adjunct to antibiotic in treating infection caused by *S. pneumoniae*, and perhaps other different pathogens, especially in the treatment of the multidrug resistance pathogens. As mentioned earlier properdin deficiency is associated with increasing risk of getting frequent and severe infections by different pathogens including *S. pneumoniae* (Densen *et al*., 1987; Singh and Rai, 2009). In these patients using P_n prophylactically might decrease the risk of these infections.

Chapter six: Conclusion and future direction

6.1 Conclusion

This thesis has assessed the role of mannose binding lectin associated serine protease MASP-3 in complement mediated haemolysis and the utility of recombinant properdin in fighting *Streptococcus pneumoniae* infection. The experimental findings described in this thesis revealed the following:

1. Evaluation of the complement system mediated haemolytic activity

a. The alternative pathway of the complement system plays a key role in the complement mediated haemolytic activity in physiological conditions in human, mouse and chicken.

b. MASP-3 deficiency abolishes complement mediated haemolytic activity in human and mouse sera. By using 3MC sera from two patients (which lack MASP-3) and MASP-1/3 deficient mouse serum on erythrocytes of three different species (rabbit, guinea pig and chicken), I showed these sera lack complement mediated haemolytic activity. Furthermore, reconstitution of 3MC sera and MASP-1/3 deficient mouse serum with recombinant MASP-3 restores complement mediated haemolytic activity in a concentration dependent manner.

c. MASP-3 deficiency significantly decreased C3 deposition ability of human and mouse sera. Under alternative pathway specific conditions, 3MC sera and MASP-1/3 deficient mouse serum are significantly compromised in the ability of C3 deposition. Moreover, reconstitution of 3MC sera and MASP-1/3 deficient mouse serum with recombinant MASP-3 restores this ability in a concentration dependent manner.

d. 3MC serum lacks ability to lyse PNH clone erythrocytes while normal human serum lyse these erythrocytes. Furthermore, reconstitution of 3MC

serum with recombinant MASP-3 restores 3MC haemolytic activity towards PNH clone erythrocytes.

These results show that targeting MASP-3 by monoclonal antibody may be a therapeutic tool in PNH disease. Furthermore, as MASP-3 deficiency abolishes AP functional activity, anti-MASP-3 antibody may have therapeutic benefit in the treatment of the other AP mediated diseases as well.

2. The utility of recombinant properdin (P_n) in fighting *Streptococcus pneumoniae*

a. Addition of recombinant P_n to human and mouse sera leads to increased opsonisation of *S. pneumonia*e with C3b, potentially enhancing phagocytosis.

b. Injection of recombinant P_n to mice at a time of introducing *S. pneumoniae* protects these mice as they had significantly longer survival times and less bacteraemia in comparison to the control group mice.

6.2 Future direction

1. My results showed MASP-3 plays important role in the complement mediated haemolytic activity. Furthermore, my results with results from our laboratory showed that MASP-3 is an essential component of the alternative pathway. Therapeutic effect of MASP-3 inhibition (by anti MASP-3 monoclonal antibody) in experimental models of disease where alternative pathway functional activity significantly contributes to pathology will be analysed. This includes models of haemolytic disease, such as Paroxysmal Nocturnal Haemoglobinuria (PNH).
2. My results and results of previous work from my laboratory show recombinant properdin has therapeutic utility in fighting *Streptococcus pneumoniae* and *Neisseria meningitidis.* A beneficial therapeutic application administering recombinant properdin against other pathogens will be analysed.

Chapter seven: Appendices

7.1 Buffers and solutions used in this thesis

- Barbital buffered saline (BBS) i.e. (pH 7.4)
    - 4 mM barbital
    - 145 mM NaCl
    - 1 mM $MgCl_2$
    - 2 mM $CaCl_2$
- Alternative pathway specific buffer i.e. AP Buffer, (pH 7.4)
    - 4 mM barbital
    - 145 mM NaCl
    - 5 mM $MgCl_2$
    - 10 mM EGTA
- BBS + gelatin
    - BBS with 0.1% (w/v) gelatine
- AP buffer + gelatin
    - AP buffer with 0.1% (w/v) gelatine
- Coating buffer (pH 9.6)
    - 15 mM Na_2CO_3
    - 35 mM $NaHCO_3$
- Tris buffered saline (TBS), (pH 7.4)
    - 10 mM Tris-HCl
    - 140 mM NaCl
- BSA-TBS blocking buffer (pH 7.4)
    - TBS with 1% (w/v) BSA

7.2 *Genotyping of MASP1/3 deficient mouse*

MASP-1/3 mouse lines with gene-targeted deficiencies in MASP-1/3 were genotyped by PCR to confirm the homozygous presence of the gene disruption in the targeted allele.

MASP1 gene is located on chromosome 16 (B2-B3) in mouse. Three different proteins MASP-1, MASP-3 and a truncated gene product of 44 kDa MAp44 are encoded by this gene. The exon 2 is targeted in *MASP1* gene so that, MASP-1 and MASP-3 as well as MAp44 are absent since the disruption of exon 2 terminates the translation of all gene products. The size of the PCR product amplified in a multiplex PCR from the wild type allele is about 539bp and that amplified from the gene targeted allele about 639bp.

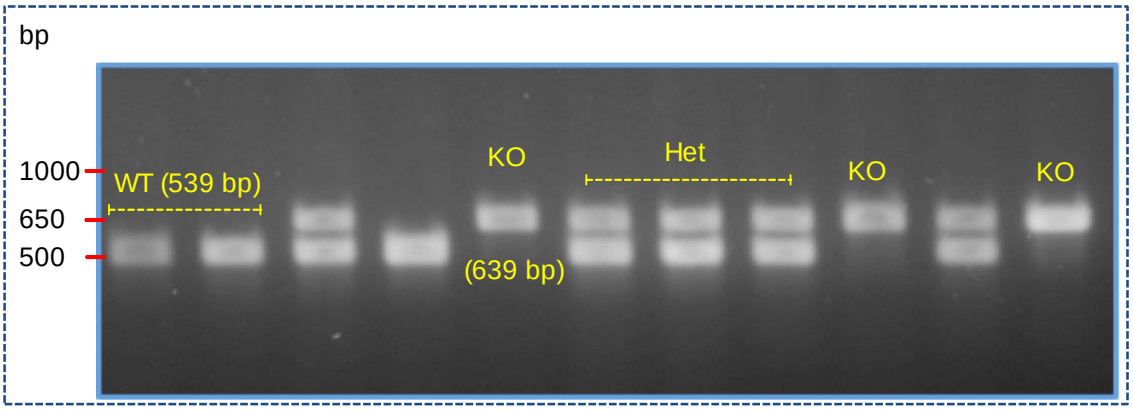

Figure 7.1 Screening for complement *MASP1* gene targeted in mice
Screening for *MASP1* gene. The MASP-1/3$^{-/-}$ mice show a band of approximately 639bp and the wild type band is 539bp.

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