Interactions between Pathways of the Complement System and *Neisseria meningitidis*

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by

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Statement of originality

This accompanying thesis submitted for the degree of PhD entitled (Interactions between Pathways of the Complement System and *Neisseria meningitidis*) is based on work conducted by the author at the University of Leicester mainly during the period between July 2015 and April 2018.

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.

Sign: **Date:**

Abstract

Interactions between Pathways of the Complement System and *Neisseria meningitidis*

Homam H Ageel

Neisseria meningitidis is the main cause of bacterial meningitis among adults. It can also invade the blood stream causing systematic infection sepsis, which can lead to septic shock. The most frequently isolated strain associated with disease in the UK is the genogroup B strain MC58. The complement system is known to be an important part of the immune system. There are many reports of recurrent meningococcal infections in individuals with genetic mutations causing partial or total malfunction of the complement system. Two of the pathways of complement, the Lectin (LP) and Alternative (AP) pathways are activated by recognition molecules binding to components of the bacterial surface. These recognition molecules are collectin 11 (CL-11), Ficolins and Mannan-binding Lectins (MBL), associated with MBL-associated serine proteases (MASPs), which ultimately activate either the LP and/or the AP. It is unclear, however, which recognition molecules interact with *N. meningitidis*. Therefore, *in-vitro* studies of the interaction between different complement components and different clonal complexes of *N. meningitidis* were undertaken, via assays of C3 and C5b-9 deposition. It was found that CL-11 is the major recognition molecule for different clonal complexes of *N. meningitidis* genogroup B, while MBL is the major recognition molecule for genogroup Y. It was found also that the capsule and the phase variation of MC58 have no significant impact on its recognition. *In-vivo*, the effect of inhibition of MASP-2 or MASP-3 on the survival of animals vaccinated against genogroup B and infected with MC58 was also studied. Vaccination overcomes the effect of inhibition of the LP or the AP in infected mice.

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1. Chapter 1: Introduction

1.1. The immune system

Humans are exposed to different microbial organisms and toxins that may interfere with their body's normal physiological function and integrity. This exposure stimulates different defence mechanisms in order to eliminate or neutralize the effects of these stimulants. These defence mechanisms compose of the immune system (Janeway *et al.*, 2001b). The Immune system is composed of cells, proteins and organs that work together to protect the human body (Chaplin, 2010). It consists of two parts: the innate immune system and the adaptive immune system. The innate immune system provides the first line of defence against microorganisms and toxins (Medzhitov, 2007). It mounts a rapid response, which will result in destruction, isolation and neutralization of the stimulant (Borghesi and Milcarek, 2006). It has its anatomical barriers, including biological, chemical and physical barriers. The first line of defence against organisms is the physical barrier, for example, the epithelial surfaces, cilia and peristalsis movements in the respiratory tract and gastrointestinal tract, which support the remove of infectious agents, and mucus, which snares infectious agents. Tears and saliva also prevent infection of the eyes and mouth, respectively by their flushing action and enzymes they contain (Mayer, 2006). The innate immune system is composed of both cellular and humoral elements. Phagocytic cells, mast cells and epithelial cells are of the cellular elements of the innate immune system while coagulation factors, complement components, cytokines and chemokines constitute the humoral elements of the innate immune response. However, the complement system can also be recruited by the adaptive immune system (Takahashi, 2011). The innate immune system provides a rapid response to Pathogen-Associated Molecular Pattern (PAMP) on the surface of a microbe and recognises it through germline encoded receptors and PAMP recognition molecules. After recognition, the innate immunity is then able to eliminate the microbe by phagocytosis utilising various cells, such as macrophages (Goldsby, 2003, Lund-Johansen *et al.*, 1999, Medzhitov, 2007). The pro-inflammatory stimuli, such as cytokines, released during the initial innate immune response to enhance phagocytosis. The presentation of antigens on phagocytes is essential to trigger the adaptive immune response to specific antigens of pathogens (Takahashi, 2011).

Unlike the innate immune system, the adaptive immune system is very specific to a particular antigen and it has memory cells, which will become active after the primary exposure to the microbe leading to rapid response once second exposure to the same antigen. Hence, the adaptive immune system can provide a long-term protection. (Reid, 1983). The adaptive immune system is divided into two parts, cellular and humoral immunity. Cells that perform in the adaptive immune response are lymphocytes, i.e. B cells and T cells. One of the B cells subtypes is plasma cells which are responsible for antibodies secretion (Holmskov *et al.*, 2003, Medzhitov, 2007). Antibodies are ɣ-globulins which can be found in two forms, a soluble form that is secreted from plasma cells to the blood, and attached to the surface of a B cell (Borghesi and Milcarek, 2006). In human, there are five different classes of immunoglobulin (Ig), i.e. IgM, IgA, IgG, IgE, and IgD (Woof and Burton, 2004). During an antibody response, B cells will differentiate either into plasma cells to secrete antibodies or differentiate into memory cells that allow the immune system to remember an antigen and respond faster upon future exposures (Borghesi and Milcarek, 2006). T cells play a vital role in cell-mediated immunity. They are distinguished from other lymphocytes by the presence of a T-cell receptor on their surface (Sigal, 2002). The adaptive immune response usually needs 4-12 days to develop to establish a static, powerful and lifelong acquired immunity (Takahashi, 2011).

1.2. The complement system

The complement system was first described as a system, which is composed of at least two components: heat-stable and heat-labile components. The heat-stable components maintain their activity after heating the serum at 56°C for 30 minutes, while the heat-labile components lose their activity under the same circumstances (Zipfel *et al.,* 2013). In 1890, Paul Ehrlich suggested the name complement since the lost serum killing activity of heated serum can be restored, by complementing this serum with few drops of fresh plasma or serum. Subsequent research showed that the complement system is composed of more than 35 proteins, which include pattern recognition molecules, serine proteases, non-enzymatic components and regulators (Ricklin *et al.,* 2010). The biosynthesis of complement components mainly occurs in hepatocytes. However, some components are synthesized in monocytes, macrophages, alveolar epithelial cells, adipose tissues, hematopoietic cells, osteoblasts and skeletal myoblasts (Laufer *et al.,* 2001). The complement system is activated by three different pathways: the classical pathway (CP), the alternative pathway (AP) and the lectin pathway (LP). This activation leads to the formation of a Membrane Attack Complex (MAC), which can penetrate target cell membrane leading to cell lysis as shown in Figure 1.1 (Fujita *et al.,* 2004, Schwaeble *et al.,* 2011, Sim *et al.,* 1993).

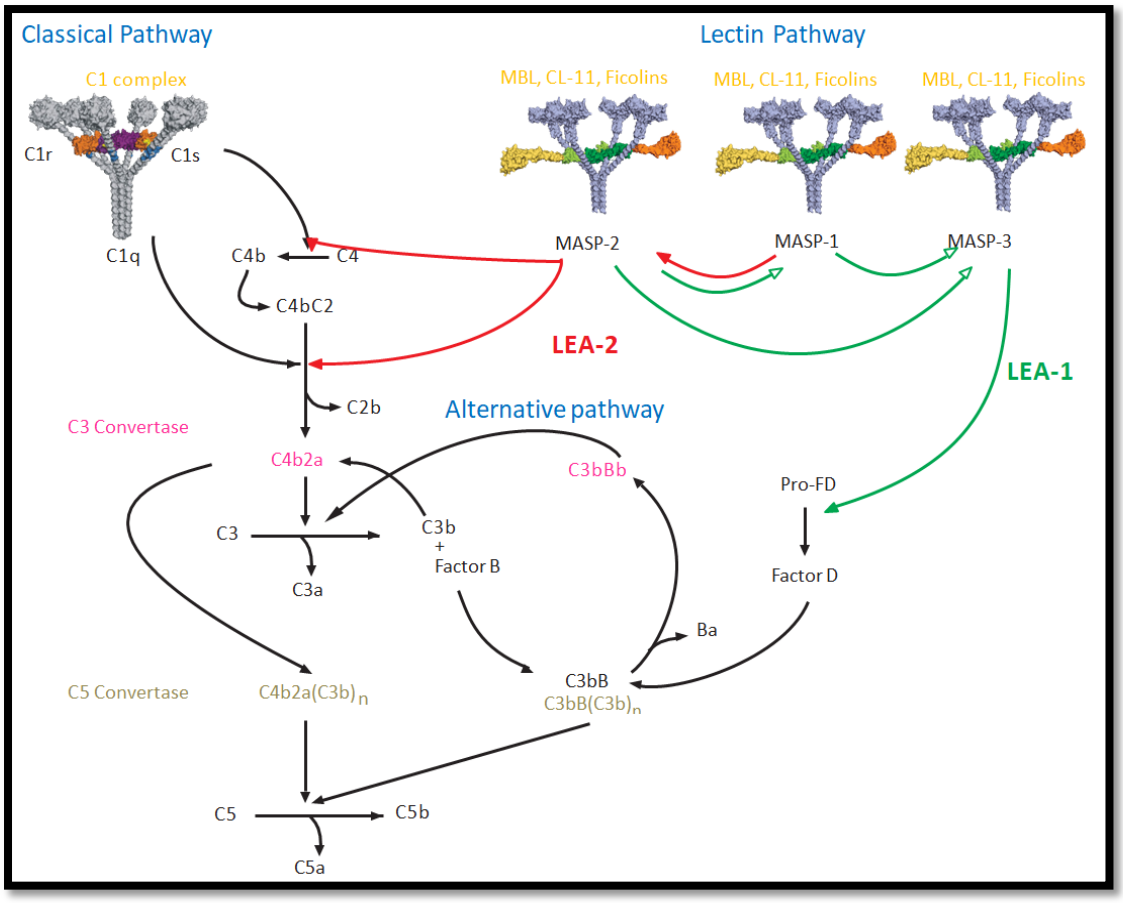


Figure 1.1: The complements activation pathways
The three different pathways of complement activation: alternative, classical and lectin pathways. The CP is activated by C1 complex recognition of immune complex on a target cell surface. The LP recognises pathogen-associated molecular patterns on a target cell leading to activation via, MBL (mannose binding lectin), CL-11 (collectin-11) and/or mainly ficolins, and MASPs (mannan binding lectin associated serine proteases), complex. MASPs activate the LP or the AP via lectin pathway effector arm (LEA). The AP is activated by hydrolysis of C3 into C3(H_2O) and/or by C3b direct binding to a target cell without needing to any recognition molecules (Figure courtesy of Professor W Schwaeble, University of Leicester, UK)

The activation of the CP is initiated when the C1q binds bacterial surface components directly. Another way of binding is indirectly when C1q binds the Fc portion of antigen-bound IgG and IgM antibodies (Boes *et al.*, 1998). C1q is composed of hexamers of the heterotrimeric C1q chains, (C1q-A, C1q-B and C1q-C) and a heterotetramer of the CP specific serine proteases, i.e. C1r and C1s (Wallis *et al.*, 2010). C1q binds to the Fc region of the immunoglobulins IgG and IgM by indirect binding or directly to the surface of target cell. Both IgM and certain subclasses of IgG, i.e. (IgG1, IgG2 and IgG3) can bind to C1q (Carroll and Sim, 2011, Peerschke *et al.*, 2015). This binding leads to an auto-activation of C1r, which activates C1s. C1s sequentially cleaves C4 into C4a, which is an anaphylatoxin, and C4b, which forms the CP C3 convertase, after it binds to C2a, the larger fragment resulted from cleavage of C2 by active C1s (Rawal and Pangburn, 2007). C3 convertase cleaves the most rife third complement component known as C3. The activation of C3 is a major step of complement activation, which acts as opsonin and can lead to the formation of the MAC. C3 cleavage by C3 convertase leads to generate C3a, which will be released as anaphylatoxin to the microenvironment and C3b that binds to the surface of the target. The complex of C3b and C3 convertase is known as C5 convertase (C4b2aC3b) (Rawal and Pangburn, 2007).

Unlike the CP, the LP is incapable of recognising antigen-antibody complexes. Recognition takes place when one or more of the LP carbohydrate-recognition subcomponents, which are: Mannose Binding Lectin (MBL), Collectin-11 (CL-11) and ficolins (M-ficolin, L-ficolin, H-ficolin), bind to pathogen-associated molecular patterns (PAMPs), such as polysaccharides and acetylated sugars, on target cell with the association of the lectin pathway specific serine proteases, known as mannose binding lectin associated serine proteases (MASPs), i.e. MASP-1, MASP-2 and MASP-3 (Schwaeble *et al.*, 2011, Dahl *et al.*, 2001, Matsushita and Fujita, 1992, Thiel *et al.*, 1997). Three forms of MASPs are found in mammals: MASP-1, MASP-2 and MASP-3. In addition to the MASPs, MAP19 and MAP44, which act as LP inhibitors and are of non-enzymatic spliced products of *MASP2* and *MASP1* genes, respectively (Schwaeble *et al.*, 2002, Stover *et al.*, 1999, Wallis *et al.*, 2007). MASP-3 is produced by the liver, spleen, lungs and other tissues. MASP-2 is exclusively produced by the liver, while MASP-1 primarily expressed in the liver (Kuraya *et al.*, 2003, Zhou *et al.*, 2012). As shown in Figure 1.2, all MASPs share the same domain organisation, which is from N-terminal, CUB 1, an epidermal growth factor (EGF)-like domain, CUB 2 domain, two

complement control protein domains (CCP1 and CCP2) and a serine protease (SP) domain (Lynch *et al.,* 2005, Sorensen *et al.,* 2005, Thiel, 2007).

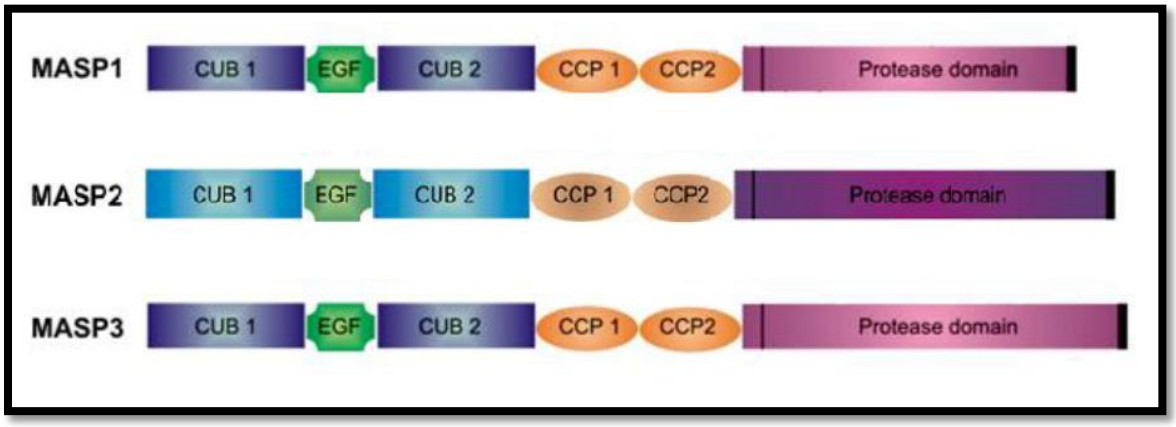


Figure 1.2: MASPs domain organization
All mannan binding lectin associated serine proteases (MASPs), share the same domain organisation, which is N-terminal, CUB 1 (C1r/C1s/Uegf/bone), an EGF (epidermal growth factor-like) domain, CUB 2 domain, two complement control protein domains (CCP1 and CCP2) and a serine protease domain (Yongqing *et al.,* 2012).

MASP-1 cleaves C2, while MASP-2 cleaves both C2 and C4 to form the LP C3 convertase (C4bC2a), which in turn will cleave C3 to form C4b2aC3b, which is the LP C5 convertase (Rawal *et al.,* 2008). It was suggested that MASP-1 cleaves the pro-FD, which in turn cleaves and activates zymogen FB resulting in activation of the alternative pathway. A subsequent paper also suggested that MASP-3 may cleave FB in C3bB complex directly (Iwaki *et al.,* 2011). Recent studies showed that the LP can drive the complement activation by two different effector arms. First arm is the lectin pathway effector arm 1 (LEA-1), which amplifies complement activation through MASP-3 dependent activation of the alternative pathway enzyme factor D (FD) from its zymogen form pro-factor D, which is synthesized and released by adipocytes (Iwaki *et al.,* 2011, Takahashi *et al.,* 2010, Oroszlan *et al.,* 2016). The second effector arm of the lectin pathway (LEA-2) comprises lectin pathway activation by MASP-2 leading to the formation of the lectin pathway specific C3 convertase C4bC2a (Parej *et al.,* 2013).

Unlike the CP and the LP, the AP is initiated when C3b directly binds a target cell. No recognition molecule is involved in this activation. The binding of FB to C3b, which produced by the AP activation or by C3b produced by the LP or the CP, leads to generate C3bB. Activated FD by MASP-3, cleaves FB in this complex generating C3bBb, the AP

C3 convertase (Sim *et al.,* 1993, Oroszlan *et al.,* 2016). As of other C3 convertases, the alternative pathway C3 convertase initiation is by the spontaneous hydrolysis of the thioester bond in C3, which exposes new binding sites for FB. The thioester bond is present in complement components C3, C4 and the protease inhibitor, i.e. α2-macroglobulin. It mediates covalent attachment to target acceptors when cleaved by different C3 convertases (Isaac and Isenman, 1992). FB binds to hydrolysed C3 generating the $C3(H_2O)B$, which is a zymogen complex that enables FB to be cleaved by enzymatically active FD to produce the AP C3 convertase, $C3(H_2O)Bb$ that can cleave native C3 to C3b and C3a (Sim *et al.,* 1993). The AP amplifies rapidly and stabilised by a serum protein called properdin, which is found to regulate the complement activation by the stabilisation of the AP C3 convertase. Properdin stimulates the association between factor B with C3b and provides a major role for the binding of C3bBb on target surface by the assembly with performed C3 convertase giving a stable AP C3 convertase, i.e. C3bBbP (Hourcade, 2006, Chen *et al.,* 2015).

The alternative pathway formed loop supports the CP and the LP functions, by activating C5. The activation of C5 by any of the complement C5 convertases, C4bC2aC3b(n) or C3bBbC3b(n), leads to the complement terminal pathway (TP) (Arlaud *et al.*, 2002, Schwaeble *et al.*, 2011, Vorup-Jensen *et al.*, 2000, Krug *et al.*, 2001, Schneider *et al.*, 2006, Stover *et al.*, 1999). C5 cleavage leads to C5a, which will be released as an anaphylatoxin, and C5b, which has no thioester bond. Therefore, C5b does not bind covalently to the surface but its cleavage exposes a binding site for C6, leading to the formation of C5b-6, C5b-7 and then C5b-8 complexes (Hadders *et al.,* 2012). C5b-8 complex accelerates C9 polymerisation, and that its relative concentration to C9 determines the ultrastructure of the C5b-9 complex as shown in Figure 1.3. In C9 polymerisation, 10 to 16 molecules of C9 will accumulate (Tschopp *et al.,* 1985). This polymerisation forms pores on the target cell surface that allows free diffusion of salts and metabolites in and out. With adequate pores to form, the target cell is unable to survive any longer (Podack *et al.,* 1982). The inability of C5b-7, C5b-8 or C5b-9 complex to insert into the target membrane, leads to binding clusterin and vitronectin, resulting in a soluble complex called sC5b9 or sMAC (Hadders *et al.*, 2012).

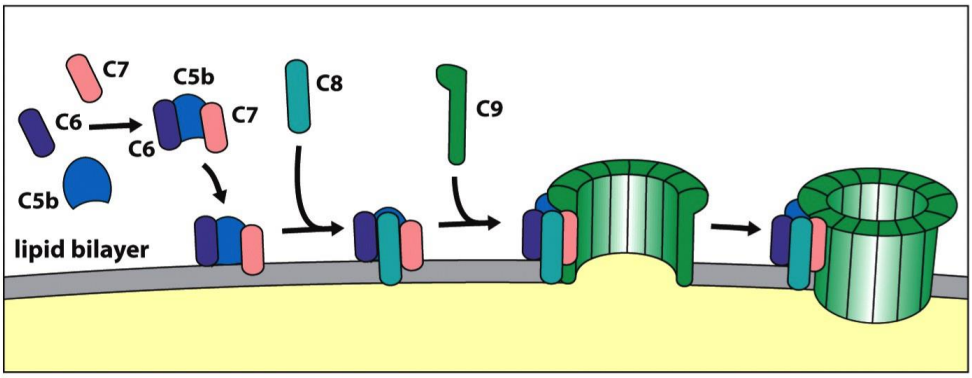


Figure 1.3: MAC formation on target cell surface.
The formation of C5b-8 complex accelerates C9 polymerisation. This polymerisation forms pores on the target cell surface that allows free diffusion of salts and metabolites in and out (Janeway *et al.*, 2001a).

As discussed earlier, the complement system role in the innate immunity is crucial. This role leads to target cell opsonisation and lysis (Dunkelberger and Song, 2010). The inflammatory response, which involves the production of potent anaphylatoxins, is also a part of the complement system participation in the innate immunity (Wetsel, 1995). These anaphylatoxins interacts with cells of myeloid origin through receptors expressed on these cell types, including granulocytes (basophils, eosinophils, and neutrophils), monocytes/macrophages, mast cells and some dendritic cells (Soruri *et al.*, 2003). However, many reports have mentioned the expression of these receptors in a number of non-myeloid cell types, including epithelial, endothelial and smooth muscle cells in the human liver and lung (Zwirner *et al.*, 1999).

Moreover, the complement system has an expanded role in the immune response that links it to the adaptive immunity. As discussed earlier, the activation of the CP can be initiated when the C1q binds the Fc portion of antigen-bound IgG and IgM antibodies (Boes *et al.*, 1998). As the other two pathways activation is antibody-independent, the activation of the different pathways of the complement system represents one of the links between the innate and the adaptive immunity. In regards to adaptive immunity, it was reported that pro-humoral response is mediated by complement receptors (Carroll, 2004). It was also found that one of the major functions of the complement system is to localize foreign antigens for lymphocytes responses (Papamichail *et al.*, 1975). Also, complement acts as a natural instructor of the humoral immune response (Dempsey *et al.*, 1996). When B cell coreceptor complex meets antigen coated with complement opsonins,

complement then enhances B-cell immunity. This enhancement happened mainly through complement receptors expressed on B lymphocytes, causing several orders of magnitude reduction in B-cell activation threshold (Carroll, 2008, Carter and Fearon, 1992, Fang *et al.,* 1998). Complement is also seems to function in the selection and maintenance of B1 cells and helping in the production of natural antibodies (Reid *et al.,* 2002). Moreover, post-germinal centres, where B cell receptor signalling get enhanced by complement receptors on B cells, complement maintains memory B cells affinity, maturation and effective recall responses (Fischer and Hugli, 1997). Thus, the complement system is a robust multifarious effector that links the innate and adaptive immune systems.

1.2.1. The complement control proteins

Complement activation is tightly controlled by fluid phase regulators, which are proteins found in the serum, or surface bound regulators, which are proteins found on the surface of cells (Unsworth *et al.,* 2011). These regulators act to interrupt the formation of complement convertases by shorten the half-life of any complexes that are able to form on the self surfaces (Donoso *et al.,* 2010, Hourcade *et al.,* 1989).

In the fluid phase, the activation of the CP and the LP is regulated by C1 inhibitor (C1-INH). C1-INH attaches to C1 complexes (C1-INH-C1r2-C1s2-C1-INH) to regulate the CP activity. Also, it attaches to the LP complexes (MASP-1-C1-INH and MASP-2-C1-INH) to regulate the LP activity (Ehrnthaller *et al.,* 2011). The most abundant fluid phase mediatory component is factor H. The main role of factor H is to regulate the AP by guaranteeing that the complement system is directed towards infectious agents and does not damage host healthy tissue at the same time. The regulation of the complement activity by factor H is done by having both factor I mediated C3b cleavage and degenerating the amplification activity of the alternative pathway C3-convertase. Factor H binds sialic acid and glycosaminoglycans (GAGs), which are generally present on self-cells but not on microbial surfaces, to apply a protective action on self surfaces but not on the surface of microbes (Pangburn, 2000, Rodriguez de Cordoba *et al.,* 2004). Therefore, mutations and polymorphisms of fH protein structure, will lead to abnormal functional consequences, for example, atypical haemolytic uremic syndrome (aHUS), age-related macular degeneration (AMD) and dense deposit disease (DDD) (Parente *et al.,* 2017). Moreover, Factor H can be recruited by some of the human pathogen such as *N.*

meningitidis. Factor H binding protein (fHbp) of *N. meningitidis* is a major virulence factor since it sequesters fH to the surface of the microbe, increasing the resistance to complement (McNeil *et al.,* 2013). Another crucial liquid phase regulator is C4-binding protein (C4bp). It enhances the production of factor I, which plays a role as a cofactor with factor H in the degradation of C4b to C4dg and C3b to iC3b and C3dg (Ehrnthaller *et al.,* 2011, Jurianz *et al.,* 1999). Also, it reduces C4b-C2 binding to block C3 convertase of the CP and the LP (Jurianz *et al.,* 1999). Moreover, factor J, clusterin and S-protein mediate the terminal pathway by blocking C5b-8 assembly on the cell surface (Ehrnthaller *et al.,* 2011).

All human cells are protected by one or more of the membrane-associated regulator proteins, i.e. CR1 (complement receptor 1), MCP (membrane cofactor protein), DAF (decay accelerating factor) and CD59 (protectin) (Ehrnthaller *et al.,* 2011). CR1 delays the activity of C3 and C5 convertases and works as a cofactor for factor I in C3b and C4b degradation. MCP supports factor I in its deactivation by its attachment to C3b. DAF attaches to C2a in the CP and LP and Bb in the AP to delay the formation of C3 and C5 convertase (Frank, 2010). Finally, host cells are protected by CD59 (protectin) on their surface, which binds to C5b-8 complexes and prevents the polymerisation of C9 (Nesargikar *et al.,* 2012).

1.2.2. The lectin pathway recognition molecules

1.2.2.1. Mannose binding lectin (MBL)

Humans expresses MBL by one *MBL* gene, while rodents MBL expressed by two genes, *MBL-A* and *MBL-C* (Sastry *et al.,* 1995). MBL is a member of the collectin family of proteins and is produced in the liver (Brouwer *et al.,* 2008, McMullen *et al.,* 2006). MBL is a large oligomeric complex (trimers, tetramers and hexamers). Each MBL subunit is composed of three identical polypeptide chains (homotrimers). Each polypeptide chain contains a short N-terminal collagen-like domain, which linked by the neck region to the globular head, which contains a carbohydrates recognition domain (CRD), as shown in Figure 1.4.

MBL binds to microbial polysaccharides or glycoconjugates rich in D-mannose, N-acetyl-d-glucosamine (GlcNAc) or L-fuses (Fuc), in a calcium-dependent medium

(Bartlomiejczyk *et al.*, 2014). A study by McMullen *et al*. (2006), suggested that IgM can activate the LP by binding directly to MBL. Other studies reported that MBL can bind to *N. meningitidis* genogroups B and C (Jack *et al.*, 1998, Jack *et al.*, 2001). Another study reported that MBL binds specifically to genogroups C and Y in two outer membrane proteins, i.e. Opa and PorB (Estabrook *et al.*, 2004). It was shown that MBL works also as an opsonin by enhancing phagocytosis and killing of *N. meningitidis* (Jack *et al.*, 2001, Jack *et al.*, 2005). However, other studies found that MBL cannot enhance phagocytosis individually, and it stimulates phagocytes indirectly by binding to complement receptor 1 (CR1) (Ghiran *et al.*, 2000, Jack *et al.*, 2005). In addition to MBL role in activation and opsonisation, it engages with non-LPS components of *N. meningitidis* to enhance the production of the cytokines IL-1β and IL-10, which have inflammatory properties, by peripheral blood mononuclear cells (Sprong *et al.*, 2004).

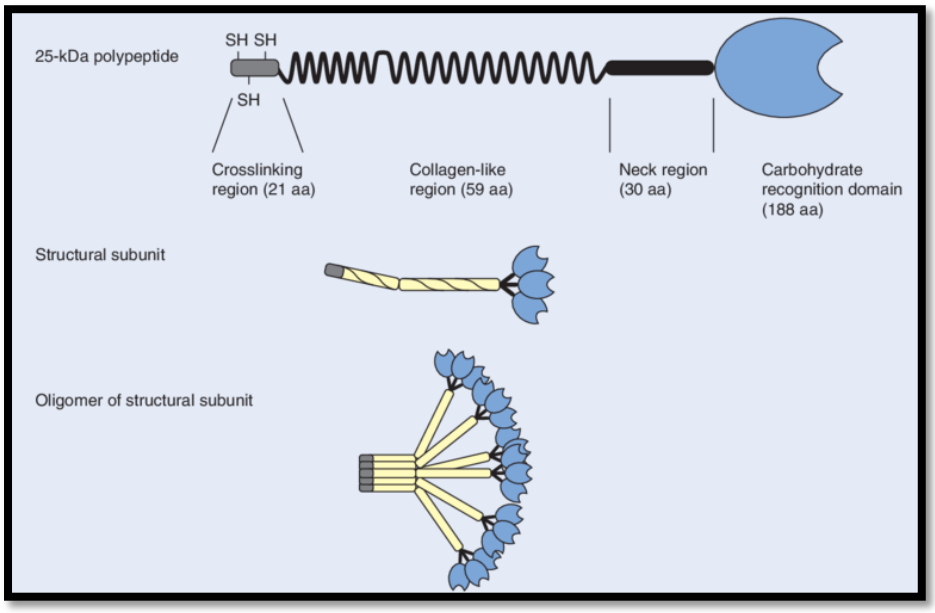


Figure 1.4: Structure of the MBL component
Mannose-binding lectin is composed of multimers of three identical polypeptide chains (homotrimers). Each polypeptide chain contains a short N-terminal collagen-like domain (Nuytinck and Shapiro, 2004).

MBL deficiency is most commonly caused by polymorphism in the *MBL2* gene. This polymorphism is caused by one of three allelic variants with structural single point mutations located in chromosome 10, specifically within exon 1 of the *MBL2* gene (Petersen *et al.*, 2001). Single base mutations will cause replacement at codon 52 (arginine by cysteine), 54 (glycine by aspartic acid) and 57 (glycine by glutamic acid).

These replacements will cause loss of the intact proteins due to interference with the formation of MBL polymers (Lipscombe *et al.,* 1995, Sumiya *et al.,* 1991, Wallis and Cheng, 1999).

In humans, about one third of the population are MBL deficient (Heitzeneder *et al.,* 2012). However, the rates vary according to ethnicity (Heitzeneder *et al.,* 2012). Nevertheless, individuals with inherited MBL deficiency seem to have increased susceptibility to infectious diseases especially when associated with other immunocompromised conditions (Dahl *et al.,* 2004, Heitzeneder *et al.,* 2012). MBL deficiency is also associated with high risk for the development of cardiovascular disorders, arterial thrombosis, rheumatoid arthritis and persistent inflammatory conditions (Garred *et al.,* 2000, Ohlenschlaeger *et al.,* 2004). Surprisingly, low MBL level was found to be beneficial to individuals with MBL deficiency, as it decreases the susceptibility to *Mycobacterium tuberculosis* infections (Soborg *et al.,* 2003).

MBL associated serine proteases (MASPs) interact with MBL through the collagen-like domain as discussed earlier (Roos *et al.,* 2001). Polymorphism of the *MASP2*, which is a very rare condition, was reported. In this polymorphism, aspartic acid is substituted with glycine at the amino acid position 105 (D105G), resulting in the loss of Ca^{2+} binding site that is essential for the binding of MASP-2 to the lectin pathway recognition complexes (Stengaard-Pedersen *et al.,* 2003, Thiel and Gadjeva, 2009). Nevertheless, MASP-2 is still present in serum and still enzymatically active but it cannot bind the lectin pathway activation complexes, although it has a polymorphism (Yaseen *et al.,* 2017).

Other MASPs deficiency is MASP-1/-3 deficiencies, which are also a rare condition. It is caused duo to a mutations in *MASP1* gene that leads to either a MASP-3 or MASP-1/-3 deficiency. This deficiency with or without CL-11 deficiency, which caused by *COLEC11* gene mutation, leads to a syndrome called Carnevale, Mingarelli, Malpuech and Michels syndrome shortly known as 3MC syndrome (Rooryck *et al.,* 2011, Ohtani *et al.,* 2001). The 3MC syndrome is associated with inflammation/chemotaxis cascade in the aetiology of human developmental disorders. The 3MC includes also different symptoms, such as facial cleft, abnormalities of the renal system, male genitalia, skull growth and learning disability (Rooryck *et al.,* 2011). The 3MC patients also show no expression of MASP-3, the known key to activate the AP. This was confirmed by a study, which

showed that MASP-1/3 deficient mouse has shown no functional AP (Sekine *et al.*, 2013).

In mice deficient in *MBL2* gene, it was shown that MBL plays a key role in limiting the complications associated with *S. aureus* infection (Shi *et al.*, 2004). In mice with (*MBL1* and *MBL2*) deficiencies, they were found to be less able to clear apoptotic cells *in-vivo*. Moreover, abnormal B cell homeostasis was found in MBL deficient mice after analysing this phenotype. MBL deficient mice did not develop autoimmunity, lymphoproliferation, or germinal centre expansion, although high numbers of peritoneal B1 cells were detected (Stuart *et al.*, 2005).

1.2.2.2. Collectin-11 (CL-11)

Humans have one CL-11 gene, *COLEC11* that expresses CL-11. CL-11 is produced in kidney, liver and adrenal gland as a member of collectin proteins family (Hansen *et al.*, 2010). It is composed of N-terminal domain, collagen-like domain, neck domain and a carbohydrate recognition domain (CRD) as shown in Figure 1.5. Mouse and human share 90% identity on CL-11 primary amino acid sequence level. However, important amino acid differences exist in their collagen-like and C-type lectin domains (Troegeler *et al.*, 2015). In humans, CL-11 gene is located on chromosome 2 (Ohtani *et al.*, 2012). In mouse, CL-11 gene is located on chromosome 12 (NCBI, 2018). CL-11 binds to L-fucose and D-mannose terminal saccharides on the microbe's surface. A study by Hansen *et al* (2010) showed that CL-11 bound to D-mannose on *E. coli* (O:126, O:60 and HB:101), *C. albicans* and *P. aeruginosa* but not *L. monocytogenes*. This is maybe because the polysaccharide of *L. monocytogenes* is of teichoic acid (Brauge *et al.*, 2016).

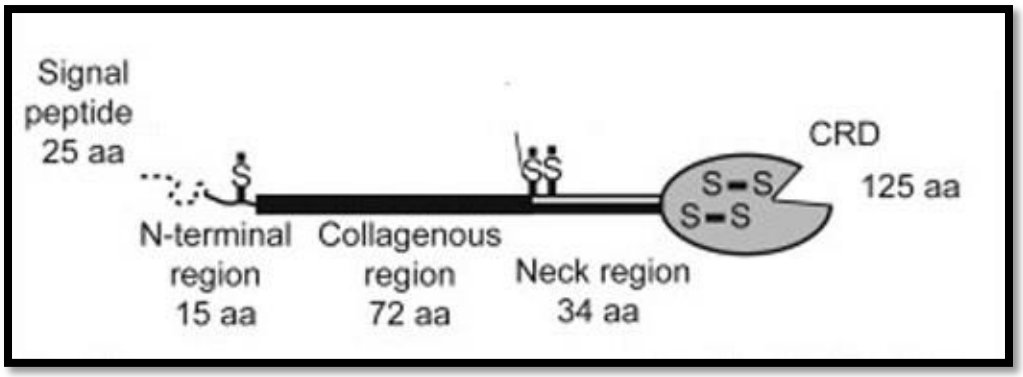


Figure 1.5: Schematic structure of collectin-11
CL-11 monomer composed of four domains, N-terminal region, a collagen-like domain, a neck region and a collagen recognition domain at the C-terminus. Three monomeric subunits assemble to form CL-11 (Selman and Hansen, 2012).

Individuals with mutations in either the *MASP1* gene or the *COLEC11* gene display a wide spectrum of developmental disorders, known as the 3MC syndrome (Carnevale, Mingarelli, Malpuech and Michels syndromes), which is associated with the inflammation/chemotaxis cascade seen in human developmental disorders (Rooryck *et al.,* 2011). In 3MC, there are five mutations in the *COLEC11* gene. Three of them are located within the region coding for the CRD and the other two are in the region coding the collagenous domain (Rooryck *et al.,* 2011). In another disease, high levels of CL-11 were found to be associated with disseminated intravascular coagulopathy (DIC) (Takahashi *et al.,* 2014). High CL-11 levels were also found among patients with schistosomiasis, a parasitic infection caused by *Schistosoma haematobium* (Antony *et al.,* 2015). Conversely, low serum levels of CL-11 were found to be associated with systemic lupus erythematosus (SLE) (Troegeler *et al.,* 2015).

In postischemic renal disease, CL-11 knockout mice showed a significant protection from the loss of renal function and tubule injury compared to wildtype littermates (Farrar *et al.,* 2016). CL-11 knockout mice were also found to have reduced renal tissue inflammation/fibrogenesis and reduced collagen deposition in the kidneys after renal ischemia-reperfusion, which is the leading cause of acute kidney injury (Wu *et al.,* 2018). Because CL-11 binds L-fucose, and therefore, the presentation of a stress-induced L-fucose pattern on the renal tubule cells surfaces increases CL-11 binding, which can trigger LP activation on these cells (Howard *et al.,* 2018, Allison, 2016).

The data on CL-11 involvement in infectious disease are limited. CL-11 knockout mice infected with *M. tuberculosis* did not show increased infection susceptibility or immunopathology alteration compared to wild type controls *in-vivo* (Troegeler *et al.,* 2015). Both groups were similar in organ colonization by the pathogen and inflammatory response in the lung. These data indicate that CL-11 does not play a major role in immune defence nor does it appear to have functional consequence against this pathogen, although other data showed that CL-11 could recognise *M. tuberculosis in-vitro* (Troegeler *et al.,* 2015). In other data, Alkaraawi showed that CL-11 knockout mice were significantly more susceptible to *S. pneumoniae* infections than wildtype littermates. In the CL-11 knockout mice mortality and bacterial burden in the blood and the lungs were significantly higher compared to sex, age and strain matched wildtype control mice (Alkaraawi, 2017). Other study has also showed that CL-11 deficient mice sera formed less C3 deposition on *S. pneumoniae* compared to wildtype littermates *in-vitro*, while CL-11 deficient mice showed severe pulmonary inflammation and weight loss caused by *S. pneumoniae* infection in comparison with WT mice *in-vivo* (Hwang *et al.,* 2017).

1.2.2.3. Ficolins

In humans, ficolins have three different genes, *FCN1*, *FCN2* and *FCN 3*. These genes express ficolins in three forms, i.e. M-ficolin, L-ficolin, and H-ficolin, also known as -1, -2 and ficolin-3, respectively. There are two ficolins in rodents, ficolin-A and -B, which are expressed by *FCNA* and *FCNB* genes, respectively (Endo *et al.,* 2004, Fujita *et al.,* 2004). Ficolins are composed of three identical polypeptide chains, which consist of a short N-terminal cysteine-rich domain, a collagen-like domain, a neck region and a fibrinogen-like carbohydrate recognition domain as shown in Figure 1.6 (Endo *et al.,* 2005, Garred *et al.,* 2010, Lynch *et al.,* 2004, Matsushita *et al.,* 2001).

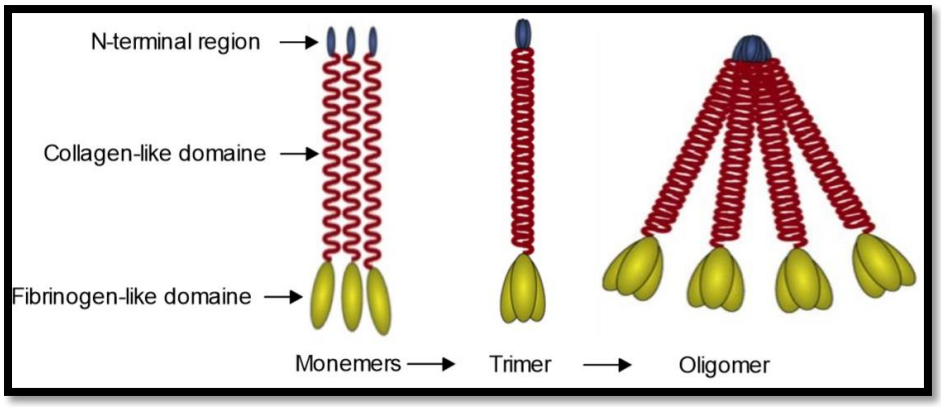


Figure 1.6: Schematic structure of ficolins
The ficolin subunit is composed of an N-terminal region, a collagen-like domain and a C-terminal fibrinogen-like domain. The collagen-like domain is composed of three monomeric subunits to generate trimeric structure and these structures assemble together to form ficolin oligomer (Matsushita *et al.,* 2013).

Humans M-ficolin is the homologue of mouse ficolin-B. The binding ligands of M-ficolin have high specificity for 9-O-acetylated sialic acid in an α2-6 linkage to galactose through its fibrinogen-like domain. M-ficolin constitutively expressed on cells of myeloid origin, i.e. leukocytes surface, neutrophils and monocytes, but not on lymphocytes. It is found also bond to type II alveolar epithelial cells of the lungs. Cell free M-ficolin detection in serum was also reported (Gout *et al.,* 2010, Honore *et al.,* 2010, Honore *et al.,* 2008, Hummelshoj *et al.,* 2008, Liu *et al.,* 2005, Wittenborn *et al.,* 2010). Unlike other ficolins, M-ficolin and ficolin-B, neither bind MASP nor activate complement (Endo *et al.,* 2005). Nevertheless, other studies showed the ability of M-ficolin to form complexes with MASP-1 and MASP-2 (Liu *et al.,* 2005). M-ficolin functions as a phagocytic receptor on the surface of circulating leukocytes (Teh *et al.,* 2000). It was reported that M-ficolin binds strongly to sialic acid on *S. agalactiae* capsule via its fibrinogen-like domain (Kjaer *et al.,* 2011). It was reported also that M-ficolin is capable of binding *S. aureus* and *S. typhimuriu* because the M-ficolin is capable to activate the proMASPs through its binding to GlcNAc (Liu *et al.,* 2005).

H-ficolin binds to carbohydrate ligands, specifically D-fucose, galactose and also acetylated albumin, but failed to recognise any of the ligands in carbohydrate arrays (Gout *et al.,* 2010). It was found that H-ficolin binds to the *A. viridans* polysaccharide, which consists of repeating units of D-glucose, D-mannose, N-acetyl-d-glucosamine (GlcNAc) and Dse-xylose, signifying that carbohydrates are not its major target (Garlatti

et al., 2007, Gout *et al.,* 2010, Lacroix *et al.,* 2009, Tsujimura *et al.,* 2001). Both H-ficolin and L-ficolin are produced in the liver (Hummelshoj *et al.,* 2008).

Human L-ficolin has four potential binding sites, which can bind to acetylated carbohydrates, such as N-acetylglucosamine, N-acetylneuraminic acid, N-acetyllactosamine, and neutral carbohydrates, such as β (1→3)-D-glucan. It failed to bind sialylated sugars because of the differences at the binding pocket of S1 (Ali *et al.,* 2013, Girija *et al.,* 2011). S1 pocket is the outer binding site of ficolins, which has different ligand specificity among ficolins. Unlike other ficolins, L-ficolin has three additional binding sites (S2, S3 and S4), which work together to define a variable continuous recognition surface that is able to sense various acetylated and neutral carbohydrates in the context of extended polysaccharides (Garlatti *et al.,* 2007).

Mouse ficolin-A has a narrow spectrum of binding to acetylated carbohydrates and to elastin, although it is considered as the homologue of human L-ficolin (Achtman, 1995, Fujimori *et al.,* 1998, Gout *et al.,* 2010, Matsushita, 2010, Ohashi and Erickson, 1998). It is worth to mention that both mouse ficolins, i.e. ficolin-A and ficolin-B, are bound to *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine when conjugated with BSA. Ficolin-B also binds structures that contain conjugated sialic acid (Endo *et al.,* 2005). Human L-ficolin binds to GlcNAc and other acetylated carbohydrates but not their non-acetylated counterparts (Krarup *et al.,* 2004). It binds also to fungal 1,3-β-D-glucan and lipoteichoic acid purified from Gram-positive bacteria and Gram-positive bacteria in general, for example, *S. aureus*, *S. pneumoniae*, group B streptococci, *B. animalis*, *S. pyogenes*, and *B. subtilis*. Also, it binds to some Gram-negative bacteria, for example, *S. typhimurium* (Achtman, 1995, Krarup *et al.,* 2005, Lynch *et al.,* 2004, Ma *et al.,* 2004). L-ficolin was found as critical LP recognition molecule that drives LP activation on pneumolysin (PLY) of *S. pneumoniae* while all of the murine LP recognition components failed to bind and activate complement on PLY.

No L-ficolin value much below 1000 ng/mL has been detected in a healthy adult; therefore, absolute L-ficolin deficiency has not been shown to exist (Herpers *et al.,* 2006). Recent studies suggested that the insufficiency of L-ficolin might increase susceptibility to respiratory infections. In particular, protection from *Pseudomonas aeruginosa* (Kilpatrick and Chalmers, 2012). A polymorphism in the *FCN2* gene causes change in the fibrinogen-like binding domain of Ficolin-2, which affects the binding to N-

acetylglucosamine, reduced L-ficolin binding to multifunctional soluble pattern recognition molecule (Ma *et al.*, 2009).

In mice, it has been reported that there is no abnormality in ficolin-deficient mouse lineages, i.e. FCN-a/KO, FCN-b/KO. The appearance, body weights, reproductive fitness, liver, spleen, lung and peripheral blood cell counts were all normal and have no significant difference to wild type mice (Endo *et al.*, 2012).

1.3. *Neisseria meningitidis*

Neisseria meningitidis is a fastidious, encapsulated, aerobic Gram-negative diplococcus. Colonies are positive by the oxidase test and most strains utilize maltose. It is the main cause of bacterial meningitis, which is an acute inflammation of the meninges, a protective membrane around the brain and the spinal cord (Saez-Llorens and McCracken, 2003). Meningitis is considered as medical emergency because it is a life-threatening disease. This is because of the proximity of the disease to the brain and spinal cord (Saez-Llorens and McCracken, 2003, Tunkel *et al.,* 2004). Infectious meningitis is caused by microorganisms, i.e. viruses, bacteria, fungi, and protozoa (Ginsberg, 2004). Most common cause is viral meningitis which can be caused by enteroviruses, herpes simplex, varicella zoster virus, mumps virus or HIV (Logan and MacMahon, 2008). In bacterial meningitis, the type of bacteria that cause meningitis is age dependant. In new-borns, up to three months old, the most causative agent is group B streptococci, *E. coli* and *L. monocytogenes* (Buchs, 1983). Children under five years old are commonly infected by *H. influenzae* type B. Older children are usually infected by *N. meningitidis* and serotypes, 6, 9, 14, 18 and 23 of *S. pneumoniae* (Saez-Llorens and McCracken, 2003, Tunkel *et al.,* 2004). Around 80% of bacterial meningitis in adults is caused by *N. meningitidis* and *S. pneumoniae* (Tunkel *et al.,* 2004, van de Beek *et al.,* 2006). *N. meningitidis* can also invade the blood stream causing systematic infection sepsis which can lead to septic shock. *N. meningitidis* constitute up to 5–15% of the adult human nasopharynx normal microbiota (Epstein, 2015).

The phenotypic classification of meningococci, is based on genome sequence typing (ST), serological differences in capsular polysaccharide, lipooligosaccharide (LOS) and outer membrane proteins (OMPs) (Rouphael and Stephens, 2012). As shown in Table 1.1, on the basis of serological differences of the polysaccharide capsule, *N. meningitidis* is divided into thirteen serogroups. Each group, is defined by its specific polysaccharides designated A, B, C, D, H, I, K, L, M, X, Y, Z, 29E and W135 (Manchanda *et al.,* 2006). Among these serogroups, serogroup D is no longer recognised (Frasch *et al.,* 1985). Out of these thirteen, only six serogroups appear to cause invasive disease, i.e. serogroup A, B, C, W135, X, and Y (Jafri *et al.,* 2013, Panatto *et al.,* 2013). Further classification into sero-subtype, serotype, and immuno-type is based on PorA, PorB, and LOS structure, respectively (Rosenstein *et al.,* 2001). Porins are the most immunogenic and are therefore can also be used for a serological classification of meningococci. The PorB protein is

responsible for serotype specificity while PorA is responsible for sero-subtype specificity (Vermont *et al.,* 2003, Massari *et al.,* 2003). Most *N.meningitidis* strains show more than one immuno-type specific epitope on their LOS, which will make the classification of the strains as L3,7,9, L2,4, or L1,8 (Tsai *et al.,* 1983). The most common LOS immuno-types that are associated with meningococcal disease are L3, 7, 9 (McLeod Griffiss *et al.,* 2000). The function of these proteins will be discussed later.

Table 1.1: Different classification systems of *N. meningitidis* (Manchanda *et al.,* 2006)

Bases	**Classification System**	**Number of groups**	**Names**
Capsule	Serogroups	13	A, B, C, E-29, H, I, K, L, M, W-135, X, Y, Z
PorA	Sub-serotypes	12	P1.1, P1.2, P1.4, P1.5, P1.6, P1.7, P1.9, P1.10, P1.12, P1.14, P1.15, P1.16
PorB	Serotypes	20	1, 2a, 2b…21
Lipooligosaccharide	Immuno-types	13	L1, L2,…L13

The preferred and most recent approach for identifying clonal complexes is genogrouping. Currently, multilocus sequence typing (MLST) is the gold standard for genogrouping and classifies meningococcal strains into different sequence types (STs). Sequence types are grouped into clonal complexes by their similarity to a central allelic profile (genotype). Clonal complexes are defined as including any sequence type that matches the central genotype at four or more loci (Maiden *et al.,* 1998). Meningococcal STs are different from the serogroups. Serogrouping is based on the immunological specificities of *N. meningitidis* capsule, while MILST is based on genetic characteristics of different strains and according to variations in enzymes with metabolic functions (Choudhury *et al.,* 2008, Maiden *et al.,* 1998, Stephens *et al.,* 2007). MLST is exploits the clear nature and electronic portability nucleotide sequence data needed for the characterization of microorganisms (Maiden *et al.,* 1998). MLST is used for genogrouping characterization to reveal the clonal relationships of organisms (Ramsay *et al.,* 1997). Although, *N. meningitidis* genetic diversity is extensive, it is highly structured.

Genetically closely related strains are grouped into clonal complexes (Caugant, 2008). MLST for a number of *N. meningitidis* strains, for example, MC58 of genogroup B (ST-32), Z2491 of genogroup A (ST-4), FAM18 of genogroup C (ST-11) and NMB-CDC of genogroup B (ST-8) have been reported (Parkhill *et al.*, 2000, Tettelin *et al.*, 2000). Of these groups, genogroup A (ST-5, ST-7), B (ST-41/44, ST-32, ST-18, ST-269, ST-8, ST-35), C (ST-11), Y (ST-23, ST-167), W-135 (ST-11) and X (ST-181) are causing almost all meningococcal invasive disease (Chang *et al.*, 2012). A study was done in Cuba to figure out the clonal distribution of disease-associated and healthy carrier isolates of *N. meningitidis* between 1983 and 2005 as shown in Figure 1.7 (Climent *et al.*, 2010).

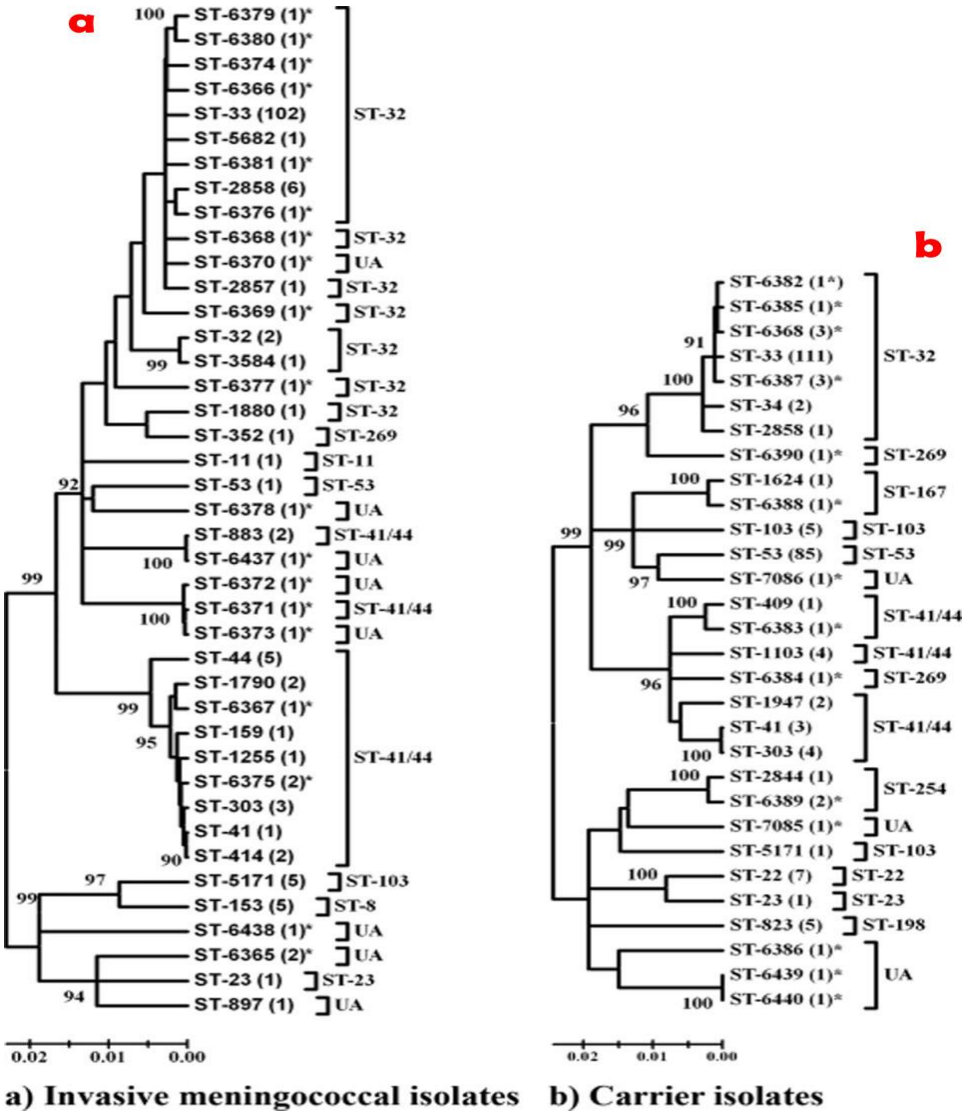


Figure 1.7: Genetic relationships among STs of Cuban isolates of meningococci
(a) Phylogenetic relationships of invasive meningococcal isolates. (b) Phylogenetic relationships of carrier isolates. The numbers of strains are given in parentheses. The clonal complexes are indicated on the right of each dendrogram (Climent *et al.*, 2010).

For the majority of the cases, serogroups A and C are responsible for meningococcal infections in third-world countries, while serogroups B and C cause meningococcal infections in developed countries (Achtman, 1995, Caugant, 1998, Takahashi *et al.*, 2008a). In Asia, serogroup A which originally derived from north China was the cause of meningococcal disease outbreak (Wang *et al.*, 1992). In the United States, serogroup Y is the most causative agent for meningococcal disease (Racoosin *et al.*, 1998). In north-western Europe, serogroups B has shown a high degree of virulence since the middle of the 1970s (Caugant, 1998, van Deuren *et al.*, 2000). In the UK, *N. meningitidis* causes disease to over than 2,000 people each year causing a mortality rate of 10%. Recovered patients, suffered from partial paralysis or deafness and mental retardation (Baraff *et al.*, 1993).

Neisseria meningitidis can be transmitted through saliva and other respiratory secretions droplet during coughing, sneezing and/or kissing (MacLennan *et al.*, 2006). Close contact with a carrier of *N. meningitidis* is the main risk factor (Orr *et al.*, 2003). Meningococcal carriage and transmission rate increases in closed populations as it can be as high as 40% (Ala'Aldeen *et al.*, 2000, Caugant *et al.*, 1992).

Colonisation of *N. meningitidis* takes place in both the sub-epithelial and the exterior surface of the mucosal cells. Rarely, it can penetrate mucosal membrane to the blood stream via endocytosis, causing sepsis, systemic meningococcal infection, and meningitis (van Deuren *et al.*, 2000). Low grade bacteraemia can lead to flu-like symptoms and usually is self-limited by the immune system (Gedde-Dahl *et al.*, 1990). *N. meningitidis* can cause disseminated intravascular coagulation leading to ischemic tissue damage when thrombi obstruct blood flow, which leads to haemorrhage, because coagulation factors have been consumed. A rash can develop under the skin because of blood leakage that may leave red or brownish or even purple spots. This rash does not fade under pressure by a test known as, glass test (Connett *et al.*, 1996). In meningococcal meningitis, small children often do not show sever symptoms, and may only be irritable and look unwell (Riedo *et al.*, 1995). The fontanelle can swell in infants aged up to 6 months and leg pain may occur (AmericanAcademyofPediatrics, 1996, Bilukha *et al.*, 2005). The beginning of the disease can be shown as acute fever, chills, back pain and muscle aches (Louria *et al.*, 1985). The consequences of the disease might be conjunctivitis, otitis, sinusitis, pneumonia, septic arthritis, purulent pericarditis, urethritis, organ failure and death (Ross

and Densen, 1984, Tzeng and Stephens, 2000). In acute meningitis, signs and symptoms may include, fever, stiff neck, nausea, sudden onset of headache, vomiting, photophobia and an altered mental status (Rosenstein *et al.,* 2001). *N. meningitidis* can cause disease, for example, meningococcal pneumonia which can appear during influenza pandemics and it might associated with septic shock (Llorens-Terol *et al.,* 1984). Pericarditis can also appear, either as a septic pericarditis or as a reactive pericarditis. Myocarditis can be a result of meningococcemia. Pharyngitis, conjunctivitis and septic arthritis caused by *N. meningitidis* were also reported (Gundersen, 1977). If the immune system was unable to clear the bacteria and pathogen replication occurred, clinically uncontrolled disease will develop (Brandtzaeg *et al.,* 1995). Even with antibiotic treatment, around 1 in 10 infected patients by meningococcal meningitis will die. Furthermore, survivors of the disease usually lose a limb or hearing, or suffer permanent brain damage (MMWR, 2000). Patients are treated usually with high dose of antibiotic but as the bacteria are killed, even more toxin is released. It takes long time for the toxin to be cleared from the body (Roback *et al.,* 1998).

The best way to prevent meningococcal infections is to be vaccinated. A vaccine is a biological immunogen that provides active acquired immunity against a particular antigen. A vaccine typically contains an antigen that resembles a disease-causing microorganism and is often made from life attenuated or killed forms of the microbe, its toxins, or its surface components (WHO, 2011). Capsular polysaccharide based vaccine contains purified capsular polysaccharides from serogroups A, C, Y and W-135 (ACWY). Nevertheless, similar approaches to develop a serogroup B vaccine have failed because of its capsule structural issue that will be discussed later (Finne *et al.,* 1983, Zimmer and Stephens, 2006). The successful approach to develop a serogroup B (MenB) vaccine was done by producing a vaccine based on recombinant outer membrane proteins (OMPs), including PorA (Porin-A), NHBA (Neisseria Heparin Binding Antigen), NadA (Neisserial adhesin A) and fHbp (factor H binding protein) (Zlotnick *et al.,* 2015). The *nhba* gene is abundant in all serogroup of meningococcal strains. This gene is also found in *N. gonorrhoeae*, *N. lactamica*, *N. polysaccharea* and *N. flavescens.* Therefore, the combination of proteins in *N. meningitidis* serogroup B vaccine is expected to protect against other types of pathogenic meningococcal bacteria (Bambini *et al.,* 2009, Jacobsson *et al.,* 2006). Both ACWY and MenB vaccines were proved to be effective

with acceptable safety profile in reported clinical trials (Baxter *et al.*, 2011, Watson and Turner, 2016).

Neisseria meningitidis is enclosed by three layers: outer membrane (OM), peptidoglycan layer and inner membrane. The OM is composed of lipooligosaccharide and proteins, while the inner membrane layer is composed of phospholipids. Phosphatidylethanolamine (PE), phosphatidylglycerol (PG), cardiolipin (CL), and phosphatidate (PA), are the major phospholipids in the inner membrane. They are responsible for regulating the flow of nutrients and metabolic products (Nikaido, 1999, Rahman *et al.*, 2000). Moreover, there is a different degree of cross-linking and O-acetylation in the peptidoglycan layer, which causes strain to strain variation in the layer structure (Antignac *et al.*, 2003). In addition, some *N. meningitidis* strains have expressed polysaccharide capsule. These layers in addition to other proteins are responsible for the virulence of *N. meningitidis* as shown in Figure 1.8 (van Deuren *et al.*, 2000).

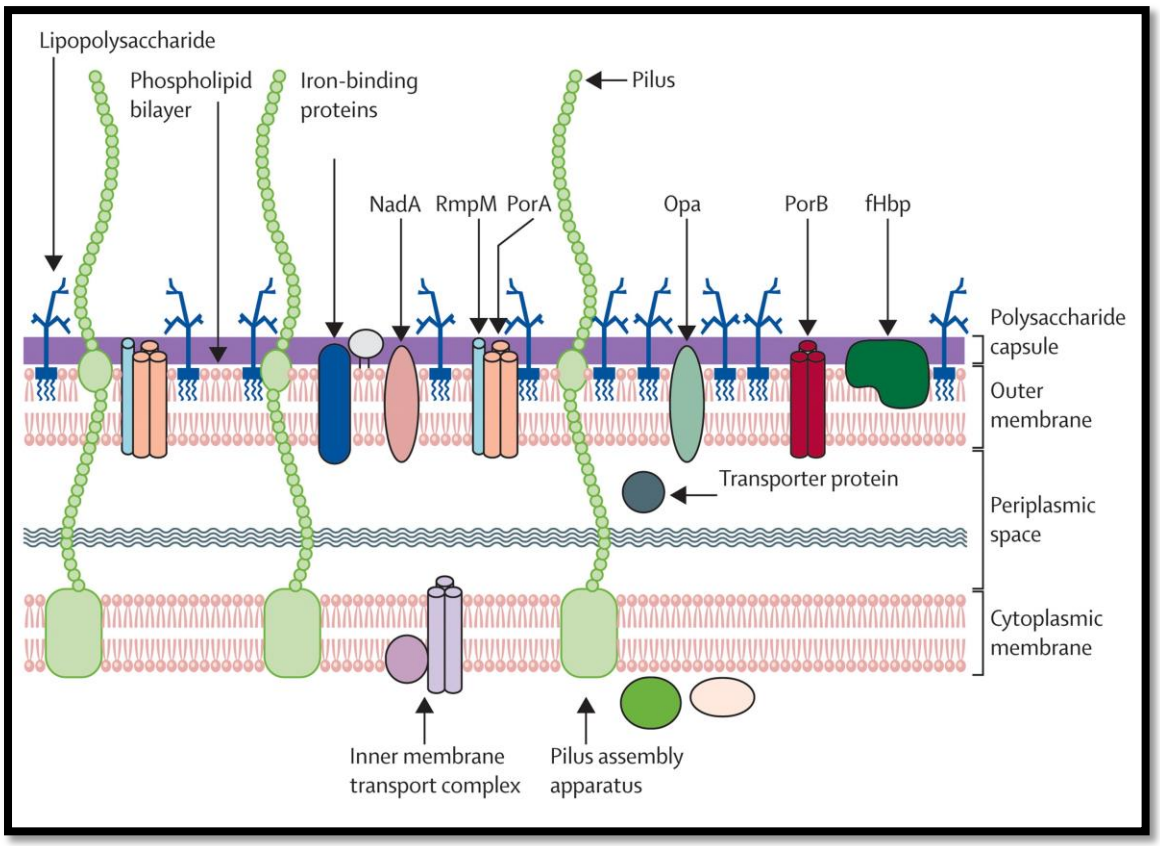


Figure 1.8: *Neisseria meningitidis* cell membrane proteins
PorA (porin A), PorB (Porin B), fHbp (factor H binding protein), NadA (neisserial adhesin A), Opa (opacity-associated) and RmpM (Outer membrane protein class 4) (Sadarangani and Pollard, 2010).

1.3.1. Virulence factors

1.3.1.1. Capsule

Neisseria meningitidis can be either encapsulated or non-capsulated. Isolates of *N. meningitidis*, which cause invasive disease, are almost always encapsulated (Rouphael and Stephens, 2012). Meningococcal capsular is mostly expressed upon invasion of bloodstream or CSF but the non-capsulated strains are capable of adhering to the mucosal epithelium of human (Dolan-Livengood *et al.,* 2003). The capsule is crucial for the survival of the bacteria in the blood as it inhibits phagocytosis and provides resistance to the CP attack (Uria *et al.,* 2008, Agarwal *et al.,* 2014). The capsule of serogroup A is composed of repeating units of N-acetyl-mannosamine-1-phosphate. Capsules of serogroups B, C, W, and Y are composed of sialic acid derivatives (N-acetylneuraminic acid) Neu5Ac. Neu5Ac is synthesized from N-acetylmannosamine (ManNAc) and phosphoenolpyruvate without phosphorylated intermediates (Blacklow and Warren, 1962, Harrison *et al.,* 2013). As Neu5Ac is the most common form of sialic acid in humans, and because of molecular mimicry, the integration of Neu5Ac into meningococcal capsules allows the meningococcus to become less visible to the host immune system (Estabrook *et al.*, 1997, Kahler *et al.,* 1998, Varki, 1997). Serogroups W and Y capsules are of α2-6-N-acetylneuraminic acid. Serogroup C is of α2-9-N-acetylneuraminic acid. However, serogroup B capsule is of α2-8-N-acetylneuraminic acid, which shares part of the molecular structure of human neural cell adhesion molecule (N-CAM) (Hill *et al.,* 2010). N-CAM is a specific neural cell surface glycoprotein that mediates neural cell adhesion. Each N-CAM molecule is heavily glycosylated by units of 10–12 of α2-8-N-acetylneuraminic acid residues (Weledji and Assob, 2014). This will lead to poor immune response against serogroup B as the immune system will recognise it as a self-antigen (Zimmer and Stephens, 2006).

1.3.1.2. Outer membrane

Lipopolysaccharide (LPS) is a surface component of the outer membrane of *N. meningitidis* (Verheul *et al.,* 1993). In addition to LPS, *N. meningitidis* virulence is affected by outer membrane proteins (OMPs), including Lipooligosaccharide (LOS), porins (PorA and B), adhesion molecules (pili, Opa and Opc), iron sequestration mechanisms and other proteins (Stephens, 2009).

Meningococcal LOS binds to host transfer molecules and receptors on the innate immune system cells, for example, monocyte and dendritic cells. Also, it binds to LPS-binding protein and myeloid differentiation protein 2 (Zughaier *et al.,* 2007, Zughaier *et al.,* 2004). LOS is anchored to the outer membrane via an amphipathic molecule that consists of a hydrophilic carbohydrate portion and a hydrophobic lipid A. Lipid A is responsible for the biological activity and toxicity of meningococcal endotoxin, as it activates macrophages and stimulates the production of various cytokines and chemokines by the innate immune system. The severity of the disease correlates with the concentrations of circulating LOS and proinflammatory cytokines (Albiger *et al.,* 2003, Rosenstein *et al.,* 2001). Overstimulation of the inflammatory response causes meningococcal septic shock (Albiger *et al.,* 2003).

Meningococcal porins (PorA and PorB) are the most abundant proteins present on the bacterial surface. Porins act as membrane pores through which small hydrophilic nutrients can enter the bacterial cell, via either anion or cation selection (Tommassen *et al.,* 1990). PorB protein inserts into membranes, induces Ca^{2+} influx and activates Toll-like receptor 2, which recognises foreign substances and passes an appropriate signal to the cells of the immune system, and for cell apoptosis (Massari *et al.,* 2003). PorB is used to define specific serotypes. PorA is vesicle-based vaccines major component of the outer membrane and a target for bactericidal antibodies (Rouphael and Stephens, 2012). PorA can be used to definer sero-subtype (Vermont *et al.,* 2003, Massari *et al.,* 2003).

In *N. meningitidis*, among many factors that play an important role in the ability of meningococci to interact with host cells are Type IV pili (Tfp) and pili subunits (Ieva *et al.,* 2005). Tfp are long hair-like surface-associated structures, which are mainly involved in launching the infection after attachment to membrane cofactor protein or CD46 receptors, which is expressed on all human cells except erythrocytes (Johansson *et al.,* 2003). Tfp of *N. meningitidis* are composed of thousands of subunits of pilin and a few copies of pilus-associated proteins, including PilC and PilV (Albiger *et al.,* 2003). When *N. meningitidis* attach to the host cells, subsequent close attachment is induced by PilT, which is another important pilus component that promotes pilus retraction needed for the motility (Pujol *et al.,* 1999).

Neisseria meningitidis strains express two types of opacity proteins, Opa and Opc. During inflammation, high levels of carcinoembryonic antigen-related cell-adhesion molecule

family (CEACAM), which interact with Opa, are expressed, assisting Opa interactions, which lead to cellular attachment and invasion (Vermont *et al.,* 2003, Virji *et al.,* 1992). Both Opa and Opc are also interacting with cell-surface associated Heparan Sulfate Proteoglycans (HSPGs), which involve in cell adhesion and motility (Virji *et al.,* 1999).

Neisseria meningitidis exclusively infects humans and its natural habitat in the human nasopharynx (Pollard and Frasch, 2001). This is believed to be because of the inability to obtain iron from hosts other than humans, as *N. meningitidis* iron uptake systems depend on high-affinity receptors that bind to host iron sources, including transferrin, lactoferrin, and haemoglobin, and it can manage to overcome free iron limitation by the evolution of iron acquisition systems, which enable it to use transferrin, lactoferrin, haemoglobin, and haptoglobin-haemoglobin as iron sources (Mickelsen *et al.,* 1982, Mickelsen and Sparling, 1981, Dyer *et al.,* 1987, Schryvers and Stojiljkovic, 1999, Pollard and Frasch, 2001, Perkins-Balding *et al.,* 2004). In humans, the two major sources of intracellular iron are ferritin and haemoglobin (Bullen and Griffiths, 1999), while extracellular iron is attached to transferrin in serum and lymph and lactoferrin in milk and secretions. *N. meningitidis* can cause disease by surviving in various environments within the host, which contain different forms and concentrations of free and complexed iron. The cerebrospinal fluid (CSF) has the highest content of free iron of these environments (Bullen and Griffiths, 1999).

Factor H is a regulator of the complement AP, which will work as a cofactor for factor I mediating the cleavage of C3b to iC3b, which is an enzymatically inactive decay fragment (Pangburn, 2000). Factor H binding protein (fHbp) is an important OMP of *N. meningitidis* since it sequesters fH to the surface of the pathogen, providing much resistance to complement attack (McNeil *et al.,* 2013, Vu *et al.,* 2012). However, Recent studies have shown that factor H binds to *N. meningitidis* via the neisserial surface protein A (NspA) and PorB which have the ability to provide serum resistance to *N. meningitidis* even without presence of fHbp (Lewis *et al.,* 2010, Lewis *et al.,* 2012).

The expression of several genes involved in host adaptation by bacteria, including *N. meningitidis*, is a process known as phase variation (PV) (Richardson and Stojiljkovic, 2001). As some bacteria species facing unforeseeable challenges in the host environment, it has to evolve different strategies for optimising the adaptation, which is especially important for *N. meningitidis* since it is usually carried asymptomatically for up to several

months in the nasopharynx (Stern *et al.,* 1986). *N. meningitidis* phase varies the expression of more than 30 surface-exposed molecules, including capsule, lipooligosaccharide, iron receptors, adhesins and pili, allowing the pathogen to avoid host immune responses more efficiently (Maskell *et al.,* 1993, Parkhill *et al.,* 2000, Tettelin *et al.,* 2000). The instability of repeated nucleotide tracts within or near coding regions during replication of *N. meningitidis* can affect the gene expression by shifting reading frames or altering the strength of promoters (Moxon *et al.,* 2006). The fitness advantages associated with PV in the levels of expression of an OMP of meningococci can facilitate the resistance to the bactericidal activities of human serum, including antigen-specific bactericidal antibody responses, adhesion, iron acquisition, and other phenotypes critical for host adaptation (Bayliss *et al.,* 2001, Moxon *et al.,* 2006, Razin *et al.,* 1998, Tauseef *et al.,* 2013, van der Woude and Baumler, 2004). However, in *N. meningitidis* PV, the escape from specific antibodies has not been substantially documented even though antibodies specific for phase-variable surface structures are present in sera from patients and carriers and bactericidal antibodies are associated with protection against this pathogen (Borrow *et al.,* 2005, Heyderman *et al.,* 2006, Jordens *et al.,* 2004, Litt *et al.,* 2004, Plested *et al.,* 2000).

1.4. Mechanisms of *N. meningitidis* to escape complement system

Neisseria meningitidis has a many surface-exposed antigens which are able to inhibit the complement system attack as shown in Figure 1.9. As mentioned earlier, fHbp, NspA and PorB2 can bind to human fH (Lewis *et al.,* 2012, Lewis *et al.,* 2010). When C3 deposited on the bacterial surface, sialylated LOS works to enhance the binding of fH to bacteria and factor H will inactivate the AP C3b (Lewis *et al.,* 2012).

Moreover, neisserial heparin binding antigen (NHBA) is able to bind heparin through an Arg-rich region and this property associates with increased survival of *N. meningitidis* in human blood (Serruto *et al.,* 2010). Heparin can also interact with other complement regulatory proteins, such as, factor H and C4b-binding protein. Hence, NHBA–heparin complex on the cell surface of *N. meningitidis* can recruit complement system inhibitors (Agarwal *et al.,* 2014). In serogroups B, C, W, and Y, the capsular polysaccharide prevents the CP activation by bringing less C4b deposition via capsule-mediated

inhibition of C1q, which in turn will limit the antibodies ability to mediate bacterial killing (Agarwal *et al.*, 2014). PorA has also been shown to bind to the major CP inhibitor, i.e. C4b-binding protein (C4bp). Studies showed that strains which are capable to express PorA were more resistant to complement serum bactericidal assay (Jarva *et al.*, 2005).

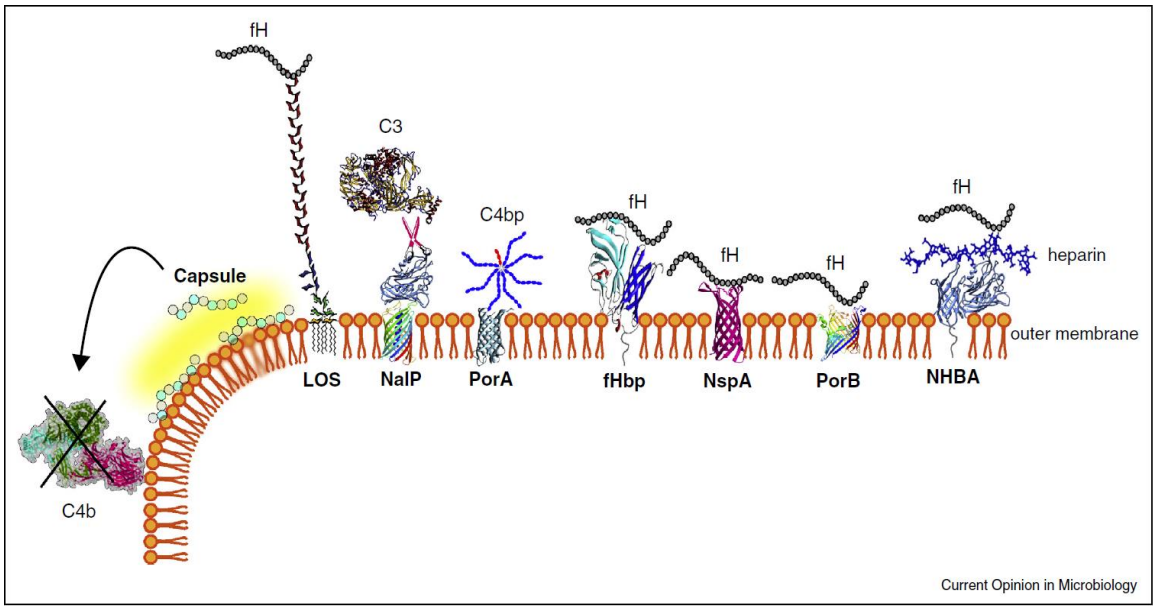


Figure 1.9: Schematic illustration of *Neisseria meningitidis* outer membrane proteins interacting with complement components
LOS (lipooligosaccharide), NalP (Neisseria autotransporter serine protease), PorA (porin A), PorB (Porin B), fHbp (factor H binding protein), fH (factor H), NspA (Neisseria surface protein A) and NHBA (Neisserial heparin binding antigen) (Pizza and Rappuoli, 2015).

1.5. The impact of complement component deficiency in *N. meningitidis* infections

The complement system role in fighting meningococcal infection is crucial, so any complement component deficiency might increase the susceptibility to meningococcal infection (Fine *et al.*, 1983, Rossi *et al.*, 2001, Spath *et al.*, 1999).

Additional to what have discussed earlier, in humans, malfunction of the AP is usually associated with deficiency of factor D, factor B MASP-3 and properdin. Factor D deficiency increases susceptibility to invasive meningococcal disease because factor D is important to cleave factor B in C3bB complex leading to reduced alternative pathway activation (Sprong *et al.*, 2006, Hiemstra *et al.*, 1989). The deficiency of factor B also leads to recurrent meningococcal infection (Slade *et al.*, 2013, Sprong *et al.*, 2006). Alshamrani have shown that Factor B knockout mice were more susceptible to *N. meningitidis* infection with 100 % mortality compared to a 100% survival rate of the Factor B sufficient mice (Alshamrani, 2016). The most common deficiency of the AP is the lack of properdin, which was shown to cause an increase in the risk of meningococcal diseases (Sprong *et al.*, 2006). Properdin-KO mice also showed significant decrease in survival when infected with meningococci, compared to wild type littermates (Fijen *et al.*, 1999, Schejbel *et al.*, 2009, Stover *et al.*, 2008). More recent work has shown an enhancement in the killing of *N. meningitidis* B-MC58 when WT mouse serum was supplemented with recombinant properdin (Ali *et al.*, 2014). Another study by Alshamrani has assessed the role of the AP in fighting *N. meningitidis* infection. Using MASP-1/3 deficient mice, data have shown that MASP-1/3 deficient mice were significantly more susceptible to meningococcal infection compared to wild type littermates (Alshamrani, 2016).

In the lectin pathway deficiencies, low MBL serum levels do not appear to increase susceptibility to serogroup B or C meningococcal infection, although other studies suggested that children with genetic variants MBL are more susceptible to meningococcal disease (Hibberd *et al.*, 1999). L-ficolin deficiency was found to be associated with recurrent respiratory infection, but no data on meningococcal infections (Atkinson *et al.*, 2004). In MASP-2 deficiency, a study by Hayat has compared between MASP-2 knockout mice with wildtype littermates. Data have shown that wildtype mice were significantly more susceptible to *N. meningitidis* serogroup A strain Z2491 infection

compared to MASP-2 KO mice. The study also showed no significant differences in survival rate between same groups when they were challenged with *N. meningitidis* serogroup C strain 6414 (Hayat, 2012).

C3 is the point at which all complement pathways join where it plays critical roles as discussed earlier. Therefore, C3 deficiency leads to severe infections, including meningococcal infection (Ram *et al.,* 2010). Individuals with C3 deficiency were found to have a high risk of meningococcal disease (Figueroa and Densen, 1991). Studies in C3 knockout mice have shown reduced responses of T helper cell-dependent IgG. The lack of C3 prevents C3a and C5a generation, which are anaphylatoxins important for immune response to combat different pathogens (Kopf *et al.,* 2002).

In terminal pathway components (C5, C6, C7, C8 and C9), the deficiency of one or more of these components increased the susceptibility to recurrent meningococcal meningitis (Figueroa and Densen, 1991). In C5- and C6-deficient mice, poor MAC formation was correlated to multiple diseases, including infectious diseases (Buckingham *et al.,* 2014). However, C5a deficient mice were more resistant to invasive meningococcal infection compared to wildtype littermates because of the reduction in the inflammatory cytokine response to *N. meningitidis* (Herrmann *et al.,* 2018).

The deficiency of C7 and C9 is associated with increased risk of meningococcal infection (Nagata *et al.,* 1989). However, the deficiency in C9 alone was found to increase the risk of meningococcal infection to 1000 fold (Nagata *et al.,* 1989). There were no available C9 knockout mice to assess the role of C5b-9 in the pathogenesis of human diseases. Nevertheless, a successful generation of C9 knockout has recently reported. This will provide a new tool to assess the *in-vivo* function of the terminal complement pathway in fighting infectious diseases (Fu *et al.,* 2016).

1.6. Aims of the study

It is clear that the complement system has a crucial role in fighting infection and disease. However, the capabilities of the individual pathways and components of the complement system vary but the details of the variations in the interactions in the complement components with *N. meningitidis* are unclear. Likewise, the effects of differences in the components of the bacterium on interaction with complement components are unclear. Both of these aspects were studied. Additionally, *in-vivo* experiments were undertaken to investigate if vaccination could overcome the absence of functional LP and/or AP. Therefore, these studies were conducted in order to achieve the following goals:

- Assess the role of each of the LP recognition molecules in fighting different clonal complexes of *N. meningitidis*.
- Assess the role of different *N. meningitidis* outer membrane molecules in escaping complement attack.
- Evaluate if and to what extent vaccination can overcome the inhibition of either the lectin pathway or the alternative pathway of complement activation to combat *N. meningitidis* infection in experimental models of meningococcal infection.

2. Chapter 2: Material and Methods

2.1. Materials

2.1.1. Proteins and antibodies

Unless mentioned in the text, all antibodies are supplied by: Sigma-Aldrich, Hycult, Immunsystem AB, Dako, Biorbyt, Santa Cruz and Abcam.

2.1.2. Buffers and media preparation

Buffers and media were prepared as shown in Table 2.1.

Table 2.1: Summary of media and buffers preparation
All the preparation protocols are provided by Dr. Nicholas Lynch from his personal communications

Buffer/media	Preparation
Coating buffer	15mM Na_2CO_3, 35mM $NaHCO_3$ (pH 9.6)
Tris buffer saline (TBS)	10mM Tris-HCL, 140mM NaCl (pH 7.4)
BSA-TBS blocking buffer	TBS with 1% (w/v) BSA (pH 7.4)
Washing buffer	TBS with 0.05% (v/v) tween-20, 5mM $CaCl_2$ (pH 7.40)
Barbital buffer saline (BBS)	4mM barbital, 145mM NaCl, 1mM $MgCl_2$, 2mM $CaCl_2$ (pH7.4)
Binding buffer	20mM Tris-HCl, 10mM $CaCl_2$, 1M NaCl (pH 7.4)
Levinthal's supplement	400mL of brain-heart infusion was mixed with 200mL defibrinated horse blood and heated up to 90°C for 50 minutes. Then mixtures was cooled for 15 minutes at room temperature and centrifuged at 5350 xg for 25 minutes at 4°C. Then the supernatant was aliquoted and kept at -20°C.
Chocolate Blood Agar	In 400mL bottle, 16g of blood agar base was added and distilled water added to 400mL. The bottle then autoclaved and allowed to cool to 40°C. 28 mL of defibrinated horse blood was added and mixed. The bottle then placed in 60-65°C water bath for 20 min. The bottle content then were poured into sterile Petri dish and allowed to solidify and stored at 4°C.

2.1.3. *Neisseria meningitidis* strains

A list of strains used and their details is shown in Table 2.2.

Table 2.2: *Neisseria meningitidis* strains used in the study
Strains were provided by Dr. Christopher D. Bayliss and Dr. Luke Green from the Department of Genetics and Genome Biology at the University of Leicester

Strain	Genogroup	Porin A	Sequence Type (Clonal Complex)	Epidemiology
MC58	B	P1.7,16-2	ST-74 (cc32)	Epidemic
MC58-capsule/knock-out	B	P1.7,16-2	ST-74 (cc32)	Lab strain
H44/76	B	P1.7,16	ST-32 (cc32)	Epidemic
N176.1	B	P1.19, 15	ST-5682 (cc32)	Carrier
22401	B	P1.22,14	ST-213 (cc213)	Carrier
N121.1	B	P1.22,14	ST-213 (cc213)	Carrier
N132.1	B	P1.22,14	ST-213 (cc213)	Carrier
20342	B	P1.7-38,1	ST-1433 (cc41/44)	Carrier
N122.1	B	P1.18-1, 30	ST-8511 (cc41/44)	Carrier
22812	B	P1.5-1,2-2	ST-269 (cc269)	Carrier
N207.1	B	P1.19-1, 15-11	ST-269 (cc269)	Carrier
N417.1	Y	P1.5-1, 10-62	ST-767 (cc167)	Carrier
N59.1	Y	P1.21,16	ST-1466 (cc174)	Carrier
N222.1	Y	P1.5-1, 10-1	ST-1655 (cc23)	Carrier
N117.1	Y	P1.5-1, 10-1	ST-767 (cc167)	Carrier
N222.2	Y	P1.5-1,10-1	ST-1655 (cc23)	Carrier
N459.5	Y	P1.5-1, 10-1	ST-1655 (cc23)	Carrier
N459.6	Y	P1.5-1, 10-1	ST-1655 (cc23)	Carrier
N199.1	B	P1.22, 9-10	ST-283 (cc269)	Carrier
N199.2	B	P1.22, 9-10	ST-283 (cc269)	Carrier
N378.1	B	P1.22, 9-10	ST-283 (cc269)	Carrier
N378.2	B	P1.22, 9-10	ST-283 (cc269)	Carrier

2.2. Methods

2.2.1. *In-vitro* experiments

2.2.1.1. Bacteria stock identification

Neisseria meningitidis was streaked on a chocolate blood agar (CBA) plate and the plate was incubated for 16-18 hours at 37^{o}C. The next day, a few colonies were picked and re-streaked onto a fresh CBA plate and incubated overnight at 37°C. From the overnight culture, a loopful of bacteria was picked and suspended in 1mL of phosphate-buffered saline (PBS) supplemented with 0.5mM $MgCl_2$ and 0.9mM $CaCl_2$ (pH 7.4). 500µL from the suspension were inoculated into two tubes containing 10mL of brain-heart infusion (BHI) with 5% (v/v) Levinthal's supplement. Then, the bacterial suspension was incubated at 37°C with shaking for 300 minutes to reach mid-log phase. Tubes were centrifuged at 3000 xg for 10 minutes and the supernatant was discarded and the pellet was re-suspended in 2mL 15% (v/v) glycerol in BHI. The mixture was then distributed into cryotubes and stored at -80^{o}C.

2.2.1.1.1. Gram stain

The Gram stain was used to differentiate bacteria based on their cell wall structure. *N. meningitidis* is Gram negative. For staining, bacteria were spread on a slide and then the slide was heated for fixation. Using Gram Staining Kit (Sigma-Aldrich) and following the kit's protocol, a few drops of crystal violet stain were added to the slide and left for 1 minute. Then the slide was rinsed with distilled water and few drops of iodine solution were added and incubated for 1 minute. The slide was then rinsed with distilled water followed by addition of a few drops of ethanol for 5 seconds to decolourise the smear before the slide was immediately rinsed with distilled water and few drops of safranin were added and incubated for 1 minute. Then the slide was rinsed with distilled water and left to dry and examined by bright-field microscopy.

2.2.1.1.2. Oxidase test

The oxidase test is an important differential procedure to identify aerobic bacteria that produce cytochrome c oxidase, an enzyme of the aerobic bacterial electron transport chain. Using Oxidase Test discs (Sigma-Aldrich), and following the manufacturer's protocol, a toothpick was used to pick a colony of bacteria to spread it on an oxidase disc at room temperature. In the presence of the enzyme cytochrome oxidase, the N,N-dimethyl-p-phenylenediamine oxalate and α-naphthol react to form indophenol blue. Neisseria stocks were confirmed as oxidase positive.

2.2.1.1.3. Serogrouping of *N. meningitidis*

Neisseria meningitidis B and $ACYW_{135}$ rapid latex tests kit (Wellcogen) were used for serogrouping. Based on the kit's protocol, a drop of *N. meningitidis* culture was mixed with a drop of polystyrene latex particles, which have been coated with antibodies specific to meningococcus antigens. These latex particles agglutinate in the presence of homologous antigen.

2.2.1.1.4. Qualitative detection of β-Galactosidase

β-Galactosidase is an enzyme that is produced by *N. lactmica* but not by *N. meningitidis* bacteria. Therefore, to identify β-Galactosidase negative, o-nitrophenyl-D-galactopyranoside (ONPG) discs (Sigma-Aldrich) were placed into sterile test tubes and 0.1mL of sterile 0.85% (w/v) sodium chloride solution was added. A colony of bacteria under test was emulsified in the solution and incubated at 35°C and incubated for 3 hours in room temperature. A tube containing *N. lactamica* was used as a positive control.

2.2.1.1.5. Genogrouping of strains of *N. meningitidis*

Sequence types are grouped into clonal complexes by their similarity to a central allelic profile (genotype). Clonal complexes are defined as including any sequence type that matches the central genotype at four or more loci. Genotyping and sequencing of different strains tested of *N. meningitidis* were performed to identify strains in the department of Genetics and Genome Biology, University of Leicester, by Dr. Christopher Bayliss and his group. The genogrouping results are detailed in Table 2.3.

Table 2.3: Genogrouping, sequence type and capsule state of different strains of *N. meningitidis*

Isolate	Genogroup	Sequence type (Clonal complex)	Capsule
MC58	B	ST-74 (cc32)	B
MC58 (capsule-KO)	B	ST-74 (cc32)	Non-capsulated
H44/76	B	ST-32 (cc32)	B
N176.1	B	ST-5682 (cc32)	Non-capsulated
22401	B	ST-213 (cc213)	B
N121.1	B	ST-213 (cc213)	B
N132.1	B	ST-213 (cc213)	Non-capsulated
20342	B	ST-1433 (cc41/44)	B
N122.1	B	ST-8511 (cc41/44)	Non-capsulated
22812	B	ST-269 (cc269)	B
N199.1	B	ST-283 (cc269)	Non-capsulated
N199.2	B	ST-283 (cc269)	Non-capsulated
N378.1	B	ST-283 (cc269)	Non-capsulated
N378.2	B	ST-283 (cc269)	Non-capsulated
N207.1	B	ST-269 (cc269)	B
N117.1	Y	ST-767 (cc167)	Y
N417.1	Y	ST-767 (cc167)	Non-capsulated
N59.1	Y	ST-1466 (cc174)	Y
N222.1	Y	ST-1655 (cc23)	Y
N222.2	Y	ST-1655 (cc23)	Y
N459.5	Y	ST-1655 (cc23)	Y
N459.6	Y	ST-1655 (cc23)	Y

2.2.1.2. Preparation of *N. meningitidis* for Enzyme Linked Immunosorbent Assay (ELISA)

Neisseria meningitidis strains were grown overnight on BHI supplemented with 5% (v/v) Levanthal's supplement in 5% (v/v) CO_2 and at 37ºC. Then the culture was centrifuged at 4000 ×g for 10 minutes. The pellets were washed three times with phosphate buffer saline (PBS), and 8mL of 4% (v/v) formaldehyde in PBS was added to resuspend the pellets, which then have incubated for 40 minutes at room temperature and the suspensions were washed by centrifugation, three times, with PBS. A loopful of each culture was grown overnight on BHI agar supplemented with 5% (v/v) Levanthal's supplement at 37ºC and in 5% (v/v) CO_2 to ensure the formalin fixation. Finally the pellets were resuspended in coating buffer for later use, after adjusting the optical density (OD) to 0.6 with spectrophotometer.

2.2.1.3. C3 deposition assay

C3 deposition assays were performed to assess the ability of *N. meningitidis* to activate complement by measuring the amount of C3b that was deposited on the surface of different strains of *N. meningitidis.* Microtitre ELISA plates were coated with 100µL of formalin-fixed *N. meningitidis* strains in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6). As positive controls, plates were coated with 100µL of 10µg/mL zymosan (Sigma-Aldrich) in coating buffer. As negative controls, plates received 100µL of 1% (w/v) BSA in coating buffer. Then, plates were incubated overnight at 4°C. The next day, plates were washed three times, with washing buffer (TBS with 0.05% (v/v) tween-20, 5mM $CaCl_2$, pH 7.4). Then, plates were blocked with 1% (w/v) bovine serum albumin (BSA) in TBS buffer (10mM Tris-HCl, 140mM NaCl, pH 7.4) and incubated at room temperature for 2 hours. Six folds serial dilutions of serum were prepared in BBS Ca^{2+} Mg^{2+} (4mM barbital, 145mM NaCl, 1mM $MgCl_2$, 2mM $CaCl_2$, pH 7.4) starting from 1/20. Plates were washed three times with washing buffer, before adding serum dilutions, in duplicate, into wells and incubating at 37°C for 1 hour. Washing buffer was used to repeat washing, followed by the addition of 100µL of rabbit anti-human C3c antibodies (DAKO) diluted 1/5000 in washing buffer. Plates were incubated at 37°C for 90 minutes before repeating the washing. 100µL of goat anti-rabbit IgG-alkaline phosphatase conjugated antibody (Sigma-Aldrich) diluted 1/10000 in washing buffer was added to

each well and incubated at RT for 90 minutes. Plates were washed again, followed by the addition of 100µL of Fast p-Nitrophenyl Phosphate substrate solution (Sigma-Aldrich). Hydrolysis of substrates was measured using a BioRad Microtitre ELISA plate reader at an absorbance at 415nm.

2.2.1.4. C5b-9 deposition assay

To assess the ability of complement system to drive MAC formation on *N. meningitidis* different strains, C5b-9 deposition assays were performed. Microtitre ELISA plates were coated with 100µL of formalin-fixed *N. meningitidis* strains in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6). As positive controls, plates were coated with 100µL of (0.1 OD) of *N. meningitidis* genogroup B (H44/76) in coating buffer. As negative controls, plates received 100µL of 1% (w/v) BSA in coating buffer. Then, plates were incubated overnight at 4°C. The next day, plates were washed three times, with washing buffer (TBS with 0.05% (v/v) tween-20, 5mM $CaCl_2$, pH 7.4). Then, plates were blocked with 1% (w/v) bovine serum albumin (BSA) in TBS buffer (10mM Tris-HCl, 140mM NaCl, pH 7.4) and incubated at room temperature for 2 hours. Six folds serial dilutions of serum were prepared in BBS Ca^{2+} Mg^{2+} (4mM barbital, 145mM NaCl, 1mM $MgCl_2$, 2mM $CaCl_2$, pH 7.4) starting from 1/5. Plates were washed three times with washing buffer, before adding serum dilutions, in duplicate, into wells and incubating at 37°C for 1 hour. Washing buffer was used to repeat washing, followed by the addition of 100µL of mouse anti-human Anti-C5b-9 antibodies (abcam) diluted 1/5000 in washing buffer. Plates were incubated at 37°C for 90 minutes before repeating the washing. 100µL of rabbit anti-mouse IgG-alkaline phosphatase conjugate (Sigma-Aldrich) diluted 1/10000 in washing buffer was added to each well and incubated at room temperature for 90 minutes. Plates were washed again, followed by the addition of 100µL of Fast p-Nitrophenyl Phosphate substrate solution (Sigma-Aldrich). Hydrolysis of substrates was measured using a BioRad Microtitre ELISA plate reader at an absorbance at 415nm.

2.2.1.5. Sandwich ELISA to bind human L-ficolin

To test L-ficolin binding ability, a sandwich ELISA was done. Microtitre ELISA plates were coated with 100µL of 1 µg/well of rabbit Ficolin 2 polyclonal antibody (pAb) (biorbyte) in coating buffer (15mM Na2CO3, 35mM NaHCO3, pH 9.6). Then, plates were incubated overnight at 4°C. The next day, plates were washed three times, with washing buffer (TBS with 0.05% (v/v) tween-20, 5mM CaCl2, pH 7.4). Then, plates were blocked with 1% (w/v) bovine serum albumin (BSA) in TBS buffer (10mM Tris-HCl, 140mM NaCl, pH 7.4) and incubated at room temperature for 2 hours. Six folds serial dilutions of serum were prepared in BBS Ca2+ Mg2+ (4mM barbital, 145mM NaCl, 1mM MgCl2, 2mM CaCl2, pH 7.4) starting from 1/5. Plates were washed three times with washing buffer, before adding serum dilutions, in duplicate, into wells and incubating at 37°C for 1 hour. Washing buffer was used to repeat washing, followed by the addition of 100µL mouse anti-human L-ficolin monoclonal antibody (mAb) (Santa Cruz) diluted 1/1000 in washing buffer. Plates were incubated at 37°C for 90 minutes before repeating the washing. 100µL of rabbit anti-mouse IgG-alkaline phosphatase conjugated antibody (Sigma-Aldrich) diluted 1/10000 in washing buffer was added to each well and incubated at RT for 90 minutes. Plates were washed again, followed by the addition of 100µL of Fast p-Nitrophenyl Phosphate substrate solution (Sigma-Aldrich). Hydrolysis of substrates was measured using a BioRad Microtitre ELISA plate reader at an absorbance at 415nm.

2.2.1.6. Serum bactericidal activity

Serum bactericidal assay was used to assess the sensitivity of *N. meningitidis* toward different types of serum. *N. meningitidis* was grown in 10mL BHI supplemented with 5% (v/v) Levanthal's and incubated 3 hours at 37ºC in 5% CO_2. Then aliquots of 500µL were made of the growth and kept at -80ºC. Viable counts were done to confirm the bacterial count. An aliquot was resuspended in phosphate buffered saline (PBS) to get the desired count of bacteria ($1X10^5$ CFU/mL). A suspension of a known concentration of *N. meningitidis* was mixed with a desired concentration of serum samples on BBS and incubated at 37^oC, with shaking at 120 rpm. Samples of reaction were taken at 0, 30, 60, 90, and 120 min, and serially diluted in PBS before plating onto BHI agar with 5% (v/v) Levanthal's supplement and incubated overnight at 37^oC in 5% CO_2.

2.2.1.7. Characterisation of the lectin pathway components in volunteer sera

In order to understand the nature of the sera to be used in subsequent assays, binding assays were used to measure MBL, CL-11 and L-ficolin in volunteer blood that will be used in the study. Microtitre ELISA plates were coated with 100µL of 1µg/mL mannan in MBL assays, 10µg/mL zymosan in CL-11 assays and 10µg/mL N-acetylated BSA in L-ficolin assays in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6). As negative controls, plates received 100µL of 1% (w/v) BSA in coating buffer. Then, plates were incubated overnight at 4°C. The next day, plates were washed three times, with washing buffer (TBS with 0.05% (v/v) tween-20, 5mM $CaCl_2$, pH 7.4). Then, plates were blocked with 1% (w/v) bovine serum albumin (BSA) in TBS buffer (10mM Tris-HCl, 140mM NaCl, pH 7.4) and incubated at room temperature for 2 hours. Six folds serial dilutions of serum were prepared in BBS Ca^{2+} Mg^{2+} (4mM barbital, 145mM NaCl, 1mM $MgCl_2$, 2mM $CaCl_2$, pH 7.4) starting from 1/5. Plates were washed three times with washing buffer, before adding serum dilutions, in duplicate, into wells and incubating at 37°C for 1 hour. Washing buffer was used to repeat washing, followed by the addition of 100µL of primary antibodies, mouse anti-human MBL antibody (Santa Cruz Biotechnology), goat anti-human CL-11 antibody (Santa Cruz Biotechnology), rabbit anti-human L-ficolin antibody (Biorbyt). All antibodies were diluted 1/1000 in washing buffer. Plates were incubated at 37°C for 90 minutes before repeating the washing. 100µL of rabbit anti-mouse IgG-alkaline phosphatase conjugated antibody (Sigma-Aldrich) for MBL assays, rabbit anti-goat IgG-alkaline phosphatase conjugated antibody (Sigma-Aldrich) for CL-11 assays and goat anti rabbit IgG-alkaline phosphatase conjugated antibody (Sigma-Aldrich) were diluted 1/10000 in washing buffer and added to the corresponding wells. Plates were incubated at room temperature for 90 minutes before washing again, followed by the addition of 100µL of Fast p-Nitrophenyl Phosphate substrate solution (Sigma-Aldrich). Hydrolysis of substrates was measured using a BioRad Microtitre ELISA plate reader at an absorbance at 415nm.

2.2.1.8. Human sera BEXSERO antibody levels

The presence of specific antibodies against N.meningitidis will decrease the relatability of the results interpretation. Therefore, volunteer's antibody levels against BEXSERO vaccine were determined in their serum to eliminate the immune volunteers against BEXSERO vaccine components. Active ingredients of BEXSERO vaccine are: Recombinant *N. meningitidis* group B (NHBA fusion protein, NadA protein, fHbp fusion

protein) and Outer membrane vesicles (OMV) from *N. meningitidis* group B strain NZ98/254, which contains PorA P1.4 (GlaxoSmithKlineUK, 2014). Microtitre ELISA plates were coated with 100µL of 1% (v/v) BEXSERO vaccine in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6). As negative controls, plates received 100µL of 1% (w/v) bovine serum albumin (BSA) in TBS buffer (10mM Tris-HCl, 140mM NaCl, pH 7.4). Then, plates were incubated overnight at 4°C. The next day, plates were washed three times, with washing buffer (TBS with 0.05% (v/v) tween-20, 5mM $CaCl_2$, pH 7.4). Then, plates were blocked with 1% (w/v) bovine serum albumin (BSA) in TBS buffer and incubated at room temperature for 2 hours. Five folds serial dilutions of serum were prepared in BBS Ca^{2+} Mg^{2+} (4mM barbital, 145mM NaCl, 1mM $MgCl_2$, 2mM $CaCl_2$, pH 7.4) starting from 1/10. Plates were washed three times with washing buffer, before adding serum dilutions, in duplicate, into wells and incubating at 37°C for 1 hour. Washing buffer was used to repeat washing, followed by the addition of 100µL of goat anti-human IgG-alkaline phosphatase conjugate (Sigma-Aldrich) diluted 1/10000 in washing buffer. Plates were incubated at 37°C for 90 minutes before repeating the washing. 100µL of Fast p-Nitrophenyl Phosphate substrate solution (Sigma-Aldrich) was added to the plates. Hydrolysis of substrates was measured using a BioRad Microtitre ELISA plate reader at an absorbance at 415nm.

2.2.1.9. Minimum serum concentration to activate the alternative pathway

To distinguish the role of the LP from the AP in fighting different strains of *N. meningitidis*, minimum concentration of serum needed to activate the AP was measured. Microtitre ELISA plates were coated with 100µL of 10µg/mL zymosan in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6) and incubated overnight at 4°C. Then, plates were blocked with 1% (w/v) bovine serum albumin (BSA) in TBS buffer (10mM Tris-HCl, 140mM NaCl, pH 7.4) and incubated at room temperature for 2 hours. Seven folds serial dilutions of serum were prepared in GVB-EGTA buffer (10mM EGTA, 5mM $MgCl_2$, 5mM barbital, 145mM NaCl, 0.1% (w/v) gelatine, pH 7.3) starting from 13% (v/v) wild type mouse serum and 12% (v/v) normal human serum. Plates were washed three times with washing buffer, before adding serum dilutions, in duplicate, into wells and incubating at 37°C for 1 hour. Washing buffer was used to repeat washing, followed by the addition of 100µL of rabbit anti human C3c (Dako) diluted 1/5000 in washing buffer. Plates were incubated at 37°C for 90 minutes before repeating the washing. 100µL of goat anti rabbit IgG-alkaline phosphatase conjugate (Sigma-Aldrich) diluted 1/10000

in washing buffer was added to each well and incubated at room temperature for 90 minutes. Plates were washed again, followed by the addition of 100µL of Fast p-Nitrophenyl Phosphate substrate solution (Sigma-Aldrich). Hydrolysis of substrates was measured using a BioRad Microtitre ELISA plate reader at an absorbance at 415nm.

2.2.1.10. Genomic DNA isolation from mouse ear snipes

To confirm and identify the MBL/KO, CL-11/KO and FCNa/KO, mice DNA should be isolated and analysed. Following the protocol of QIAGEN MagAttract kit, pieces of mice ear snips around 4mm were mixed in tubes with master mix (Thermo Fisher), which contain 250µL of 0.5M EDTA solution, 60µL of Nuclei Lysis Solution and 10µL of Proteinase K (Qiagen). Tubes were incubated overnight at 55°C. Next day, 1.5µL of RNase solution (4mg/mL) was mixed with ear snipes, and incubated at 37°C for 30 mins. Samples were cooled to room temperature before 100µL of protein precipitation was added to the tubes and vortexed for 20 seconds. Ice was used to cool the samples before centrifuged them for four minutes at 7500 xg. Then, 300µL of isopropanol was added to the supernatant and then centrifuged for five minutes at 7500 xg. Supernatants were discarded and 300µL of 70% (v/v) ethanol was added. Then tubes were centrifuged for 1 minute at 7500 xg. The tubes were kept to dry from ethanol for 30 minutes. Finally, 100µL of DNA re-hydration solution was added and tubes were stored at 4°C overnight.

2.2.1.11. Polymerase chain reaction (PCR) for mice purified DNA

PCR was done to determine the genomic DNA (gDNA) and therefore identify the MBL/KO, CL-11/KO and FCNa/KO. PCR amplifies a specific region of DNA that lies between DNA known sequences. The reaction of PCR is consisting of three main steps (denaturation, annealing and synthesis). In denaturation step, using heat, DNA template is denatured, followed by a cooling step which allows the primer to link to a certain target. Then the amplification allows synthesis of the DNA by optimal temperature. Using an automated thermal cycler, these steps are repeated 35 times. PCR was done to determine the genomic DNA (gDNA) and therefore identifying the deficiency of lectin pathway recognition molecules.

The primers used for genotyping of MBL are:

Primer name	**Primer sequence**
oIMR5044	AGTGAAGGCCCTGTGCTCCGAATTC
oIMR5045	CCCACAGAGCACAAGAGTCATAAATG
Ne	GCGCATCGCCTTCTATCGCCTTC

The primers used for genotyping of CL-11 are:

Primer name	**Primer sequence**
CL-11_wto_F1	CAGATTCTTGTCCCTGGCCTCA
CL-11_scr_R1	CTCAGTGTCAGCTGAATAAATGCCA
Neo3a	GCAGCGCATCGCCTTCTATC

The primers used for genotyping of ficolin-A are:

Primer name	**Primer sequence**
FcnA_F1	GTTAGAGAGCTGGCTACTCCGATGA
L3865	TCTCCACCTTCCTCTTCCTCCTCTA
Ne	CATCGCCTTCTATCGCCTTCTTGA

Each PCR reaction tube contains:

Genomic DNA (200ng/µL)	1µL
Reaction buffer (10x)	1.5µL
MgCl2 (2.5mM)	1.5µL
dNTP mix (10mM)	0.3µL
scr_F1	1.5µL
wto_R1	1.5µL
Neo	1.5µL
Taq-DNA polymerase	0.12µL
Nanopure distilled water	6.08µL

Programme used for PCR

Step	Temperature (°C)	Time (s)	Cycles
Initiation	98	90	
Denaturation	98	15	
Annealing	60	30	
Elongation	72	30	35
Final elongation	72	300	
Hold	4	∞	

Images of the agarose gel were missed in the lab fire, but data were registered in the university data base before the fire incident.

2.2.1.12. Testing the antigen binding of anti-MASP-2 and anti-MASP-3

To validate the efficacy of the inhibitory antibodies, anti-MASP-2 (OMS 721-HG4) and anti-MASP-3 (13b1) monoclonal antibodies (OMEROS) specificity was tested. Microtitre ELISA plates were coated with 100µL of 0.5µg/mL of MASP-2 (CCP1-SP) in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6) or with 0.5µg/mL of full length MASP-3 in coating buffer. Then, plates were incubated overnight at 4°C. The next day, plates were washed three times, with washing buffer (TBS with 0.05% (v/v) tween-20, 5mM $CaCl_2$, pH 7.4). Then, plates were blocked with 1% (w/v) bovine serum albumin (BSA) in TBS buffer (10mM Tris-HCl, 140mM NaCl, pH 7.4) and incubated at room temperature for 2 hours. Six folds serial dilutions of the antibodies were prepared in BBS Ca^{2+} Mg^{2+} (4mM barbital, 145mM NaCl, 1mM $MgCl_2$, 2mM $CaCl_2$, pH 7.4) starting from 5µg/mL. As negative controls, plates received 100µL of 5µg/mL isotype control (ITC) antibody in BBS Ca^{2+} Mg^{2+}. Plates were washed three times with washing buffer, before adding the antibodies in duplicate into wells, and incubated at room temperature for 2 hours. Washing buffer was used to repeat washing, followed by the addition of 100µL of goat anti-human IgG-alkaline phosphatase conjugate (Sigma-Aldrich) diluted 1/10000 in washing buffer. Plates were incubated at 37°C for 90 minutes before repeating the washing. 100µL of Fast p-Nitrophenyl Phosphate substrate solution (Sigma-Aldrich) was added to the plates. Hydrolysis of substrates was measured using a BioRad Microtitre ELISA plate reader at an absorbance of 415nm.

2.2.2. *In-vivo* experiments

2.2.2.1. Infection studies

All mice were provided by Charles River UK. Used strain was C57BL/6. The age of the mice was 8 weeks at the beginning of the study. All infection studies were run under the project license (PPL 60/4327). I am legally authorised to work on animal under home office animal scientific procedure act with personal license number (PIL BE2618). Maintenance and breeding were handled by the University of Leicester Preclinical Research Facility (PRF). I would like to thank Dr. Vitor Fernandes, Dr. Sarah Glenn and Dr. Nicholas Lynch for helping me in my animal studies.

2.2.2.2. Virulence testing of *N. meningitidis*

To assess the virulence of *N. meningitidis*, mice were injected i.p. with 80µL of (400mg/kg body weight) iron dextran 12 hours prior to infection. An aliquot of *N. meningitidis* (MC58) was diluted in PBS to obtain the required infection dose. Mice were divided into three groups based on the infection dose, i.e. ($1.6X10^7$, $1.6X10^6$ and $4X10^5$ CFU/mouse). Mice were injected i.p. with a mixture of 100µL bacteria and 80µL of iron dextran. Following infection, mice were regularly monitored for the illness signs. At the endpoint of the experiments, either by signs of illness (very lethargic) or time (72 hours), survival rates were scored.

2.2.2.3. Vaccinating mice with Bexsero (MenB)

To confirm immunization, antibody levels against BEXSERO vaccine were measured. C57BL/6 wild-type mice were treated with two intraperitoneal (i.p.) doses of 0.1 mL Bexsero MenB vaccine (GlaxoSmithKline) per mouse, one on day 0 and the second dose a week later. Two weeks later, blood was drawn by cardiac puncture from terminally anaesthetized mice. Antibody levels of vaccinated mice was measured by the same method used to measure human antibody absorbance, except the antibody used, which was rabbit anti-mouse IgG-alkaline phosphatase conjugate (Sigma-Aldrich). As shown in Figure 3.4, antibody absorbance for all vaccinated mice was significantly higher than control.

2.2.2.4. Infection of mice pre-treated with complement inhibitory antibodies with *N. meningitidis*

Sixty C57BL/6 wild type mice were divided into two groups based on their vaccination status. Each group was divided into 3 subgroups based on the type of antibody that these groups will be treated with. These antibodies are anti-MASP-2 (OMS 721-HG4), anti-MASP-3 (13b1) or isotype control (OMEROS). Each group was treated i.p. with 100µL of 22.5µg/mL antibody on 7 days (13b1 and control), 3 days (OMS 721-HG4) and 1 day (all antibodies) before the infection. Following infection, mice were regularly monitored for the illness signs. At the endpoint of the experiments, either by signs of illness (very lethargic) or time (72 hours), survival rates were scored.

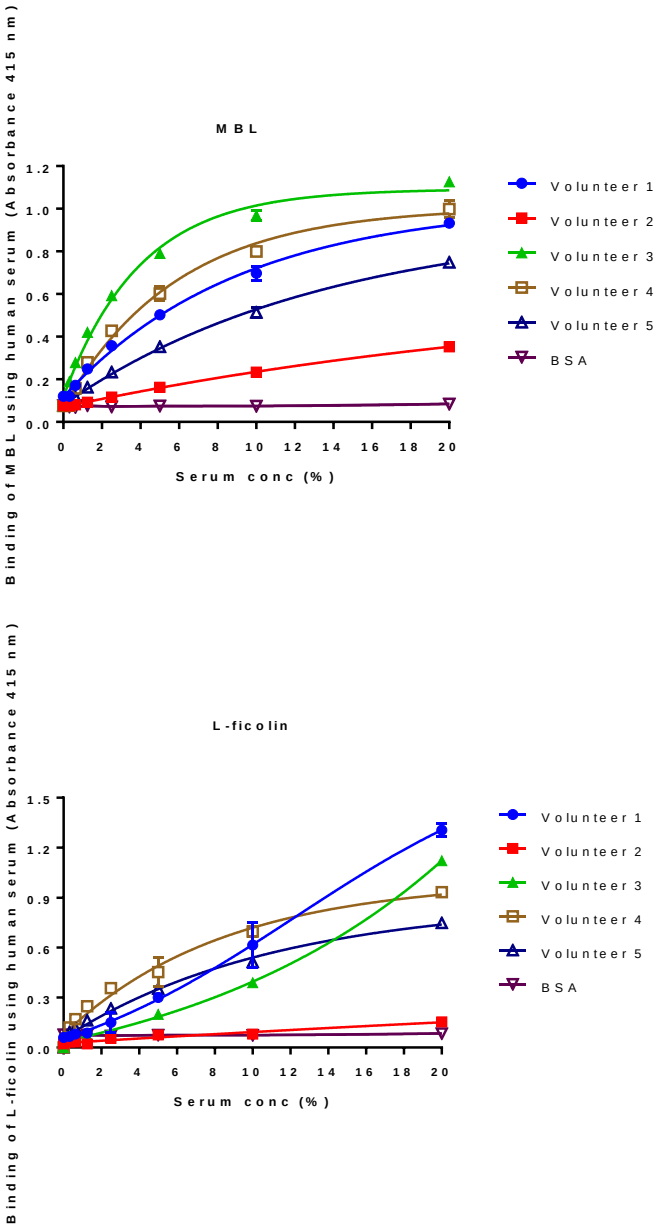
2.3. Statistical analysis

GraphPad prism version 7 was used to preform statistical analysis by using 2 way ANOVA combined with Tukey's multiple comparisons test. Agonist vs response sigmoidal non-linear regression was used to plot all the curves in the *in-vitro* studies. Survival data was analysed by Kaplan-Meier plots and compared by log rank test/Gehan-Wilcoxon method.

3. Chapter 3: Characterisation of Samples

3.1. Characterisation of human sera

In order to understand the nature of the sera to be used in subsequent assays, binding assays were used to measure MBL, CL-11 and L-ficolin in volunteer blood samples that were to be used in the study. As shown in Figure 3.1, all volunteers are of normal function of LP recognition molecule, except volunteer 2 who has non-functional L-ficolin and MBL. Therefore, any volunteer serum could be used in subsequent assays, except volunteer 2.



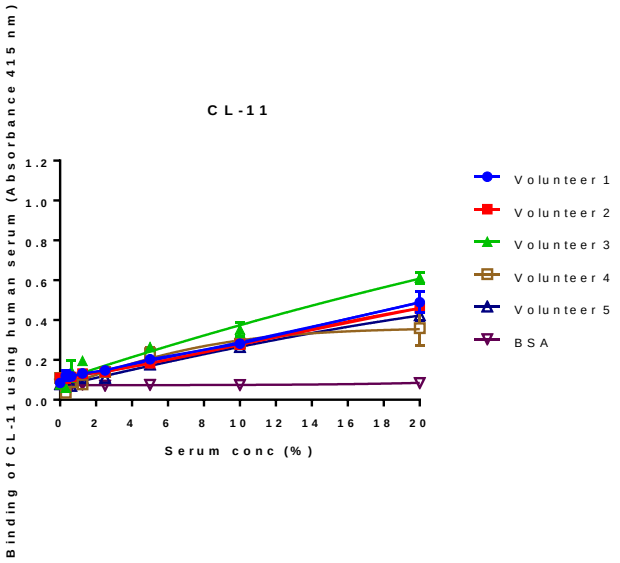


Figure 3.1: Binding of human LP recognition molecules to their appropriate ligands positive control
The LP recognition molecules binding were testing in the sera of volunteers 1 to 5. The ligands for MBL, CL-11 and L-ficolin were mannan, zymosan and N-acetylated BSA respectively. BSA was used as the negative control. The data are presented as a mean of technical duplicates +/- SEM. Curves were plotted using sigmoidal non-linear regression.

Moreover, volunteer's serum levels of antibody against BEXSERO vaccine components, were tested. As shown in Figure 3.2, volunteer 1 and 2 antibody levels are significantly lower ($P<0.05$) than other volunteers. Therefore, Volunteer 1 serum was used in subsequent assays as normal human serum.

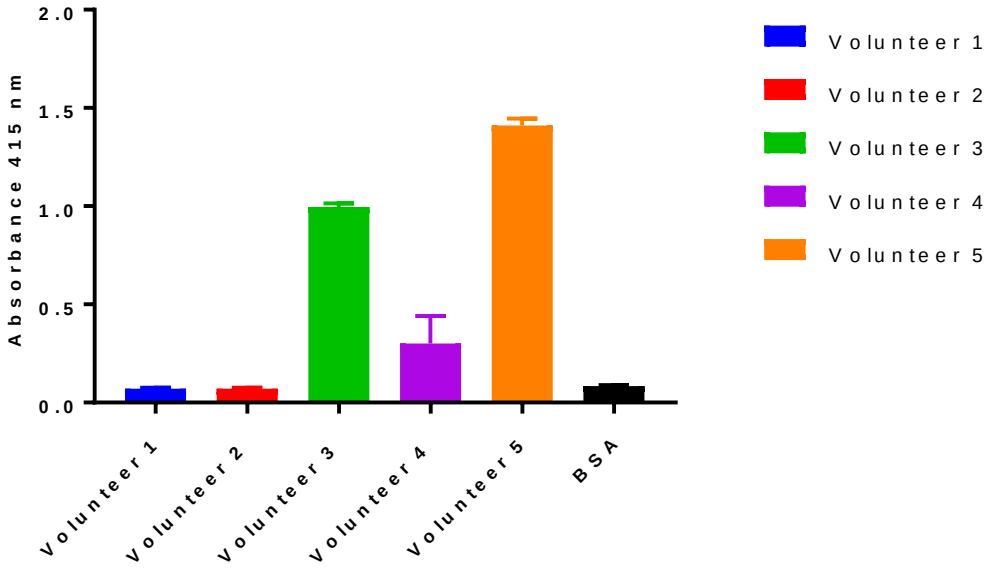


Figure 3.2: Antibody levels against BEXSERO vaccine
Unlike other volunteers, volunteer 1 and 2 antibody levels are significantly lower ($P<0.05$) than other volunteers. (The data are presented as a mean of technical duplicates +/- SEM).

3.2. Minimum serum concentration to activate the alternative pathway

To distinguish the role of the LP from the AP in fighting different strains of *N. meningitidis*, minimum concentration of serum needed to activate the AP was measured. The alternative pathway half maximal effective concentration (EC_{50}) using mouse serum was equal to 5.2 as shown in Figure 3.3A. The alternative pathway EC_{50} using normal human serum was equal to 3.9 as shown in Figure 3.3B.

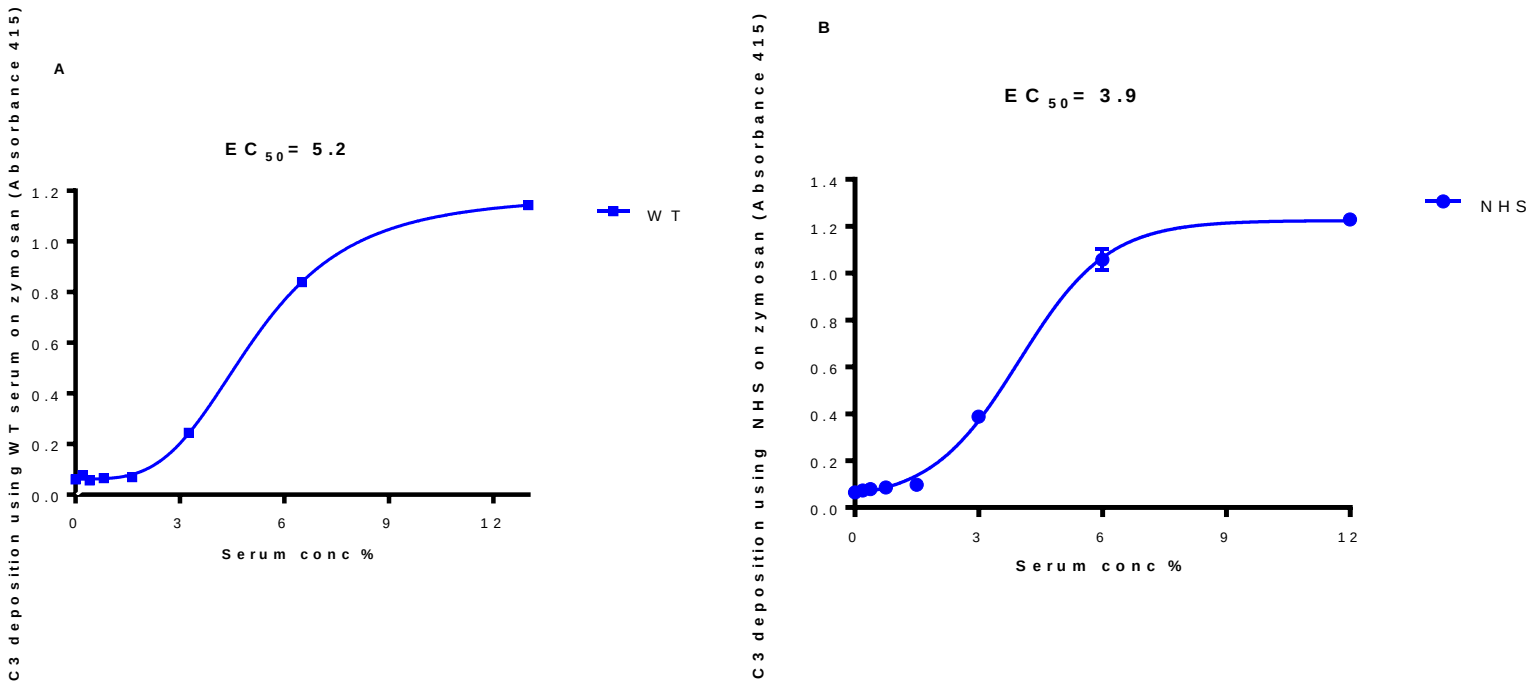


Figure 3.3: C3 deposition on zymosan under alternative pathway permissive conditions (high serum concentration in EGTA buffer) using wild type mouse serum and normal human serum.
EGTA buffer contains Mg^{2+} and lacks Ca^{2+}, as in the absence of Ca^{2+} complement can only be activated via the alternative pathway. Figure 3.3A represents WT mouse serum. Figure 3.3B represents normal human serum. The EC50 data are presented as a mean of biological and technical duplicates +/- SEM. Curves were plotted using sigmoidal non-linear regression.

3.3. Vaccinating mice with Bexsero (MenB)

To confirm immunization, antibody levels against BEXSERO vaccine were measured. As shown in Figure 3.4, antibody absorbance for all vaccinated mice was significantly higher than negative control confirming antibody production against BEXSERO vaccine.

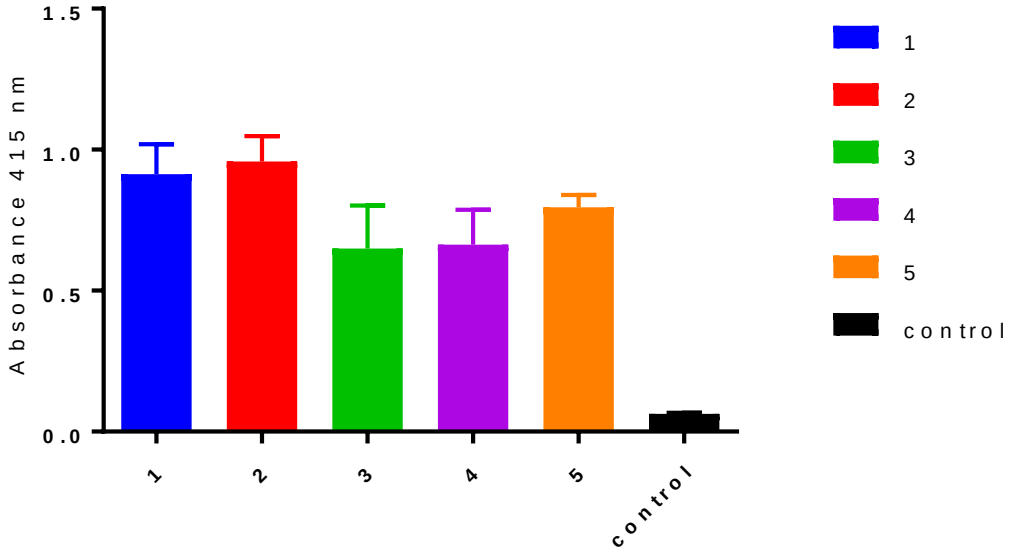


Figure 3.4: Vaccinated mice antibody absorbance
Five samples represent 40 vaccinated mice absorbance average compared to the negative control. The data are presented as a mean of technical duplicates +/- SEM.

3.4. Testing the antigen binding of anti-MASP-2 and anti-MASP-3

To validate the efficacy of the inhibitory antibodies, anti-MASP-2 (OMS 721-HG4) and anti-MASP-3 (13b1) monoclonal antibodies (OMEROS) specificity was tested. Anti-MASP-2 (OMS 721-HG4) and anti-MASP-3 (13b1) antibodies were bound to their corresponding proteins significantly more that the isotype control as shown in Figure 3.5A and Figure 3.5B.

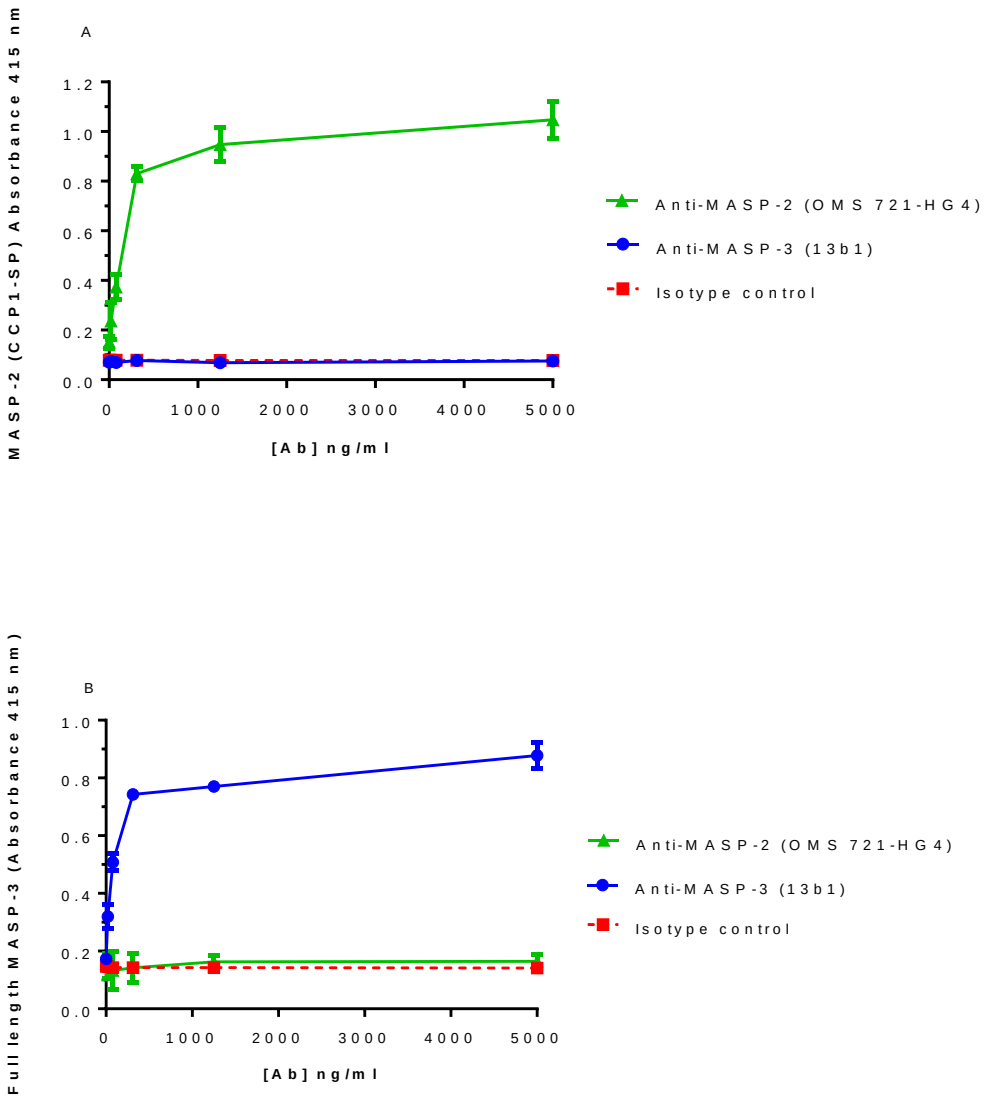


Figure 3.5: The validation of anti-MASP-2 and anti-MASP-3 antibodies
Figure 3.5A represent anti-MASP-2 (OMS 721-HG4) antibody binding to MASP-2 protein significantly higher than isotype control antibodies (ITC). Likewise, Figure 3.5B represent binding of anti-MASP-3 (13b1) to MASP-3 protein significantly higher than the isotype controls. The data are presented as a mean of technical duplicates +/- SEM.

4. Chapter 4: *In-vitro* studies

The complement system plays a crucial role in fighting against invading microbes, including *N. meningitidis* and is a vital part of the immune system. Nevertheless, the role of the LP recognition molecules and their ability to drive C3 activation on *N. meningitidis*, which in turn leads to MAC formation, needs more clarification. To achieve this, C3 deposition, MAC formation on different strains of *N. meningitidis* and the impact of the deficiency of any of the LP pathway recognition molecules on binding outer membrane ligands, were studied.

4.1. C3 deposition assays

C3 deposition assays were performed to assess the ability of *N. meningitidis* to activate complement by measuring the amount of C3b that was deposited on the surface of different strains of *N. meningitidis*. C3b ability to deposit on *N. meningitidis* reflects the ability of the complement system to recognise different ligands on the surface of the pathogen, which ultimately lead to bacteria lysis by MAC formation. In these assays, rabbit anti-human C3c antibody (SIGMA) was used to detect the deposition of C3b as described in section 2.2.1.3. For these assays, different strains of formalin-fixed *N. meningitidis* were coated on ELISA microtitre plates. Naive mouse sera and human sera were tested to confirm the absence of detectable anti-BEXSERO antibody. Also, low concentration of sera was used to retain LP functionality while disabling AP functioning, as shown in Figure 3.3.

All data were used to calculate an EC50 of C3 deposition and a semi-quantitative deposition score relative to the positive control. The score is based on the extent of the deposition at highest serum concentration used and ranges from - (no deposition), ± (>0<10% of the positive control), + (>10<50% of the positive control), ++ (>50<80% of the positive control) and +++ (>80% of the positive control).

4.1.1. C3 deposition using human and mouse sera on different clonal complexes of *N. meningitidis*

Starting with 5% (v/v) normal human serum and *N. meningitidis* strains, which were grouped based on their clonal complexes and genogroups, C3 deposition assays were performed to define a specific pattern within and/or between clonal complexes and genogroups. Clonal complex (cc32) showed no significant differences in C3 deposition between MC58 and N176.1 ($P>0.05$) but both strains were significantly different from H44/76 ($P<0.05$) as shown in Figure 4.1 and Table 4.1.

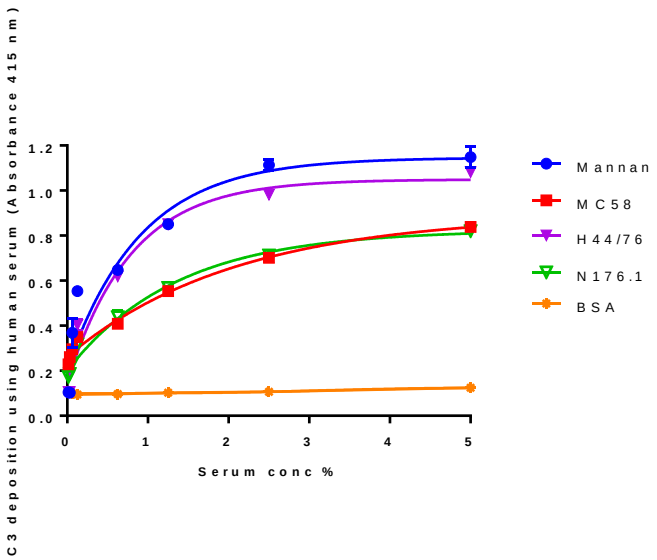


Figure 4.1: C3 deposition on clonal complex (cc32) of *N. meningitidis* genogroup B using different normal human serum concentrations up to 5% (v/v)
Microtitre plates were coated with formalin-fixed *N. meningitidis* (or mannan as a positive control) then blocked with BSA that was used as the negative control. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. The binding of the C3 cleavage product (C3c) was detected by using rabbit anti-human C3c polyclonal antibody. Only H44/76 strain is significantly higher ($P<0.05$) in C3 deposition than the other strains in the same clonal complex which have no significant difference between them ($P= 0.47$). The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

Table 4.1: Semi-quantitative scoring for the highest serum concentration and EC50 of C3 deposition on clonal complex (cc32) of *N. meningitidis* genogroup B using normal human serum.
The score is based on the extent of the deposition at highest serum concentration used and ranges from - (no deposition), ± (>0<10% of the positive control), + (>10<50% of the positive control), ++ (>50<80% of the positive control) and +++ (>80% of the positive control).

Isolate	Genogroup	Sequence type (Clonal complex)	% to the positive control	score	EC50 Control/strain ± SEM
MC58	B	ST-74 (cc32)	78	++	0.45/2.51 ± 0.51
H44/76	B	ST-32 (cc32)	91	+++	0.45/0.52 ± 0.11
N176.1	B	ST-5682 (cc32)	71	++	0.45/1.26 ± 0.22

In clonal complex (cc213), no significant differences (P>0.05) were shown in C3 deposition between strains tested as shown in Figure 4.2 and Table 4.2.

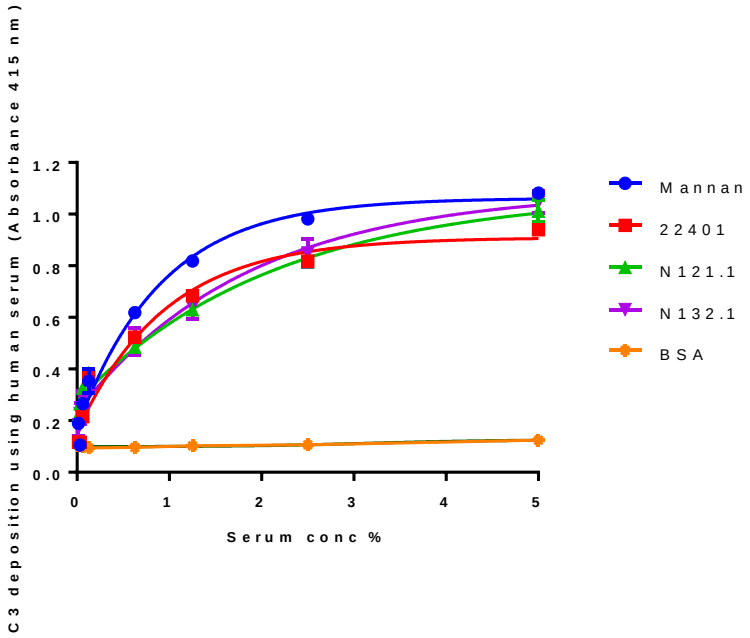


Figure 4.2: C3 deposition on clonal complex (cc213) of *N. meningitidis* genogroup B using different normal human serum concentrations up to 5% (v/v)
Microtitre plates were coated with formalin-fixed *N. meningitidis* (or mannan as a positive control) then blocked with BSA that was used as the negative control. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. The binding of the C3 cleavage product (C3c) was detected by using rabbit anti-human C3c polyclonal antibody. No significant difference between all the strains tested in this group (P>0.05). The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

Table 4.2: Semi-quantitative scoring for the highest serum concentration and EC50 of C3 deposition on clonal complex (cc213) of *N. meningitidis* genogroup B using normal human serum
The score is based on the extent of the deposition at highest serum concentration used and ranges from - (no deposition), ± (>0<10% of the positive control), + (>10<50% of the positive control), ++ (>50<80% of the positive control) and +++ (>80% of the positive control).

Isolate	Genogroup	Sequence type (Clonal complex)	% to the positive control	score	EC50 Control/strain ± SEM
22401	B	ST-213 (cc213)	85	+++	0.71/0.68 ± 0.16
N121.1	B	ST-213 (cc213)	102	+++	0.71/2.53 ± 0.56
N132.1	B	ST-213 (cc213)	103	+++	0.71/1.99 ± 0.47

Strains under clonal complex (cc41/44) show no significant differences (P>0.05) in C3 deposition on as shown in Figure 4.3 and Table 4.3.

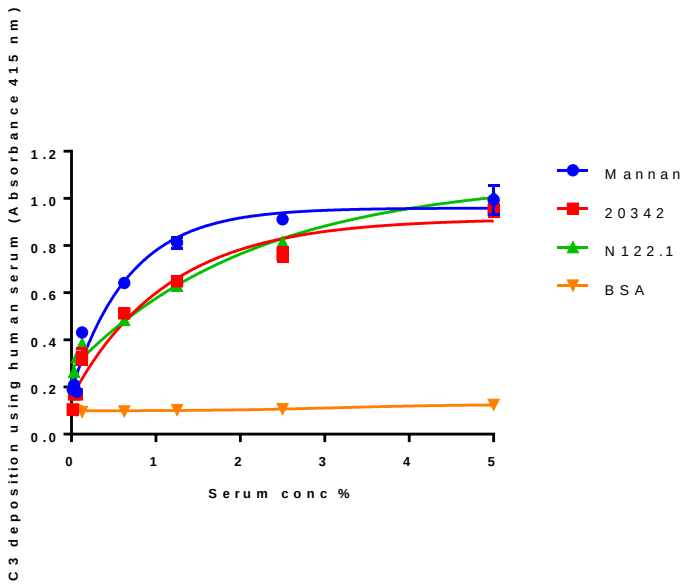


Figure 4.3: C3 deposition on clonal complex (cc41/44) of *N. meningitidis* genogroup B using different normal human serum concentrations up to 5% (v/v)
Microtitre plates were coated with formalin-fixed *N. meningitidis* (or mannan as a positive control) then blocked with BSA that was used as the negative control. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. The binding of the C3 cleavage product (C3c) was detected by using rabbit anti-human C3c polyclonal antibody. No significant difference between both strains tested in this group (P=0.07). The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

Table 4.3: Semi-quantitative scoring for the highest serum concentration and EC50 of C3 deposition on clonal complex (cc41/44) of *N. meningitidis* genogroup B using normal human serum.
The score is based on the extent of the deposition at highest serum concentration used and ranges from - (no deposition), ± (>0<10% of the positive control), + (>10<50% of the positive control), ++ (>50<80% of the positive control) and +++ (>80% of the positive control).

Isolate	Genogroup	Sequence type (Clonal complex)	% to the positive control	score	EC50 Control/strain ± SEM
20342	B	ST-1433 (cc41/44)	95	+++	0.46/0.91 ± 0.19
N122.1	B	ST-8511 (cc41/44)	113	+++	0.46/2.53 ± 0.56

As shown in Figure 4.4 and Table 4.4, the differences between strains in this group are significant (P<0.05) although they are of the same clonal complex (cc269).

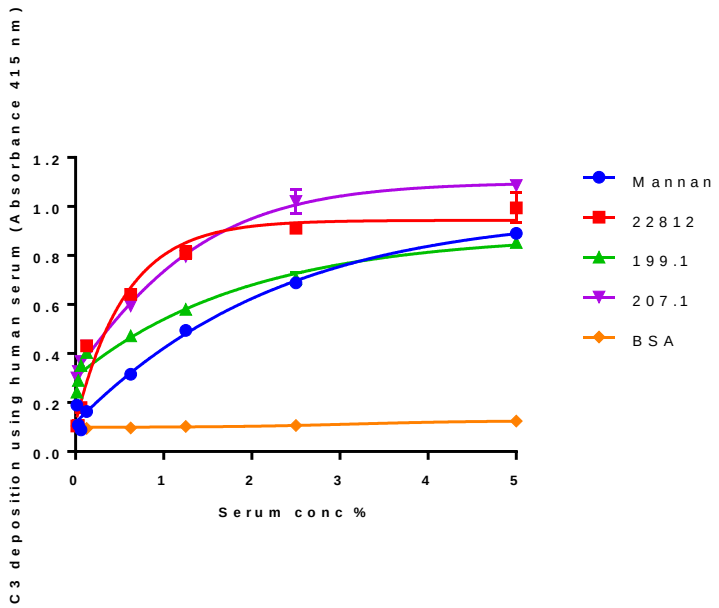


Figure 4.4: C3 deposition on clonal complex (cc269) of *N. meningitidis* genogroup B using different normal human serum concentrations up to 5% (v/v)
Microtitre plates were coated with formalin-fixed *N. meningitidis* (or mannan as a positive control) then blocked with BSA that was used as the negative control. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. The binding of the C3 cleavage product (C3c) was detected by using rabbit anti-human C3c polyclonal antibody. All the strains in this group show different C3 deposition (P<0.05). The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

Table 4.4: Semi-quantitative scoring for the highest serum concentration and EC50 of C3 deposition on clonal complex (cc269) of *N. meningitidis* genogroup B using normal human serum.
The score is based on the extent of the deposition at highest serum concentration used and ranges from - (no deposition), ± (>0<10% of the positive control), + (>10<50% of the positive control), ++ (>50<80% of the positive control) and +++ (>80% of the positive control).

Isolate	Genogroup	Sequence type (Clonal complex)	% to the positive control	score	EC50 Control/strain ± SEM
22812	B	ST-269 (cc269)	97	+++	2.97/0.30 ± 0.30
N199.1	B	ST-283 (cc269)	91	+++	2.97/2.08 ± 2.08
N207.1	B	ST-269 (cc269)	113	+++	2.97/1.33 ± 1.33

To summarise, using normal human serum on genogroup B of *N. meningitidis*, clonal complexes (cc213) and (cc41/44) show consistent amount of C3 deposition between strains within each group. With an exception of H44/76 in clonal complex (cc32) and N199.1 in clonal complex (cc269), all other strains showed same amount of C3 deposition within their groups.

Also, using normal human serum, on clonal complexes (cc167) of *N. meningitidis* genogroup Y, there is no significant difference (P>0.05) between both strains in the same group as shown in Figure 4.5 and Table 4.5.

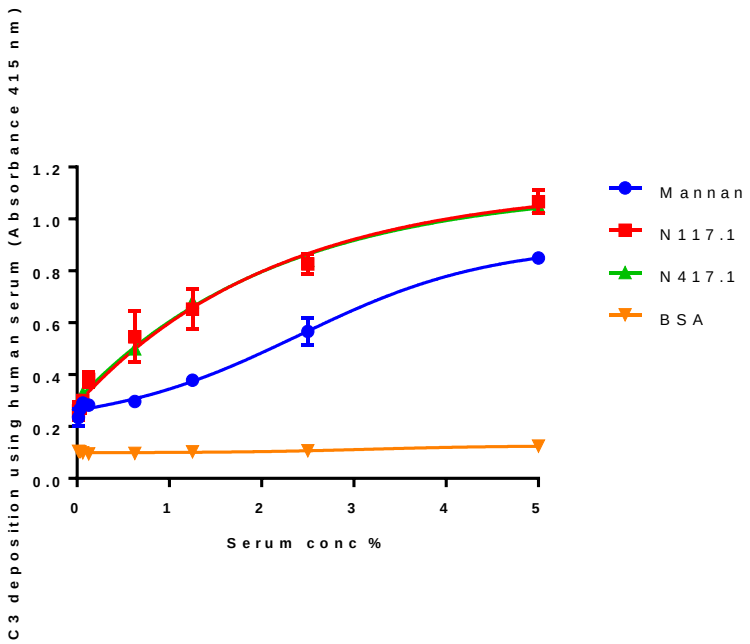


Figure 4.5: C3 deposition on clonal complex (cc167) of *N. meningitidis* genogroup Y using different normal human serum concentrations up to 5% (v/v)
Microtitre plates were coated with formalin-fixed *N. meningitidis* (or mannan as a positive control) then blocked with BSA that was used as the negative control. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. The binding of the C3 cleavage product (C3c) was detected by using rabbit anti-human C3c polyclonal antibody. No significant difference between both strains of same clonal complex (P>0.05). The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

Table 4.5: Semi-quantitative scoring for the highest serum concentration and EC50 of C3 deposition on clonal complex (cc167) of *N. meningitidis* genogroup Y using normal human serum.
The score is based on the extent of the deposition at highest serum concentration used and ranges from - (no deposition), ± (>0<10% of the positive control), + (>10<50% of the positive control), ++ (>50<80% of the positive control) and +++ (>80% of the positive control).

Isolate	Genogroup	Sequence type (Clonal complex)	% to the positive control	score	EC50 Control/strain ± SEM
N117.1	Y	ST-767 (cc167)	125	+++	3.71/2.43 ± 0.65
N417.1	Y	ST-767 (cc167)	125	+++	3.71/2.62 ± 0.28

Other two strains of different clonal complexes but of the same genogroup Y were tested. Although they are not of the same clonal complex, they showed no significant difference (P>0.05) in the amount of C3 deposition as shown in Figure 4.6 and Table 4.6.

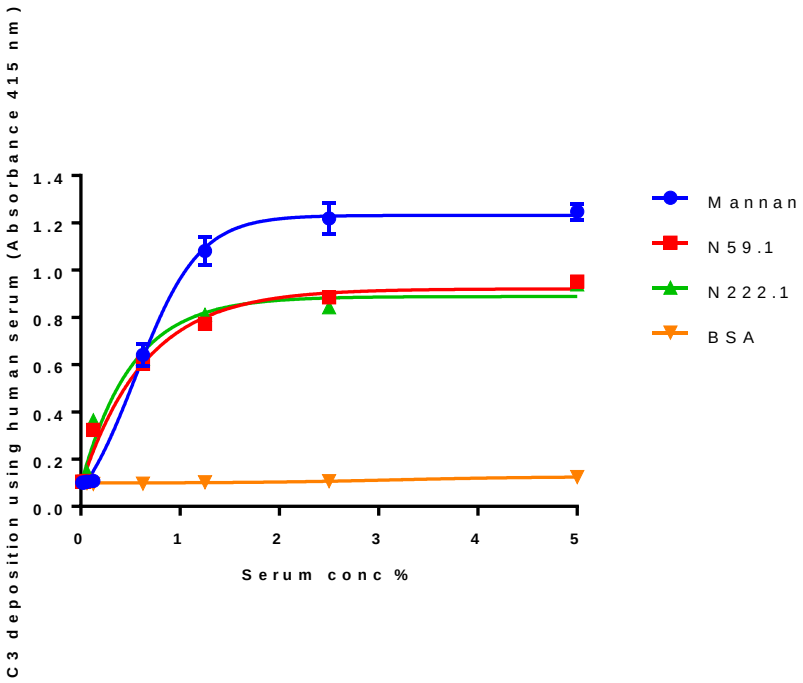


Figure 4.6: C3 deposition on different clonal complexes of *N. meningitidis* genogroup Y using different normal human serum concentrations up to 5% (v/v)
Microtitre plates were coated with formalin-fixed *N. meningitidis* (or mannan as a positive control) then blocked with BSA that was used as the negative control. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. The binding of the C3 cleavage product (C3c) was detected by using rabbit anti-human C3c polyclonal antibody. Strain N59.1 is of clonal complex (cc174) while strain N222.1 is of clonal complex (cc23). C3 deposition was the same on both strains (P>0.05). The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

Table 4.6: Semi-quantitative scoring for the highest serum concentration and EC50 of C3 deposition on clonal complex (cc174) and (cc23) of *N. meningitidis* genogroup Y using normal human serum.
The score is based on the extent of the deposition at highest serum concentration used and ranges from - (no deposition), ± (>0<10% of the positive control), + (>10<50% of the positive control), ++ (>50<80% of the positive control) and +++ (>80% of the positive control).

Isolate	Genogroup	Sequence type (Clonal complex)	% to the positive control	score	EC50 Control/strain ± SEM
N59.1	Y	ST-1466 (cc174)	75	++	0.73/0.44 ± 0.44
N222.1	Y	ST-1655 (cc23)	72	++	0.73/0.32 ± 0.32

To summarise, using normal human serum on genogroup Y, clonal complex (cc167) show consistent amount of C3 deposition between strains within the group. Strains N59.1 and N222.1 are of different clonal complexes. However, they showed same amount of C3 deposition and no significant difference was found between them.

Based on the previous findings, using human serum, there is consistent C3 deposition amount within all groups, except H44/76 in clonal complex (cc32) and N199.1 in clonal complex (cc269). Although the experiments were done under specific conditions, the natural antibodies occurrence and/or cross reactivity between antibodies of different genogroups or even different species, might have an effect on the reliability of the results. Therefore, wild type (WT) mouse serum was used on same clonal complexes. Unlike human serum, mouse sera are immunologically naive. Hence, repeating same experiments under same conditions with changing the negative control to C3/KO mouse serum, will increase the reliability of the findings and would allow comparing the differences between human and mouse sera in regards to C3 deposition.

Using 5% (v/v) WT mouse serum, there was no significant differences in C3 deposition ($P>0.05$) between strains within the clonal complexes (cc23), (cc213) and (cc41/44) as shown in Figure 4.7 and Table 4.7.

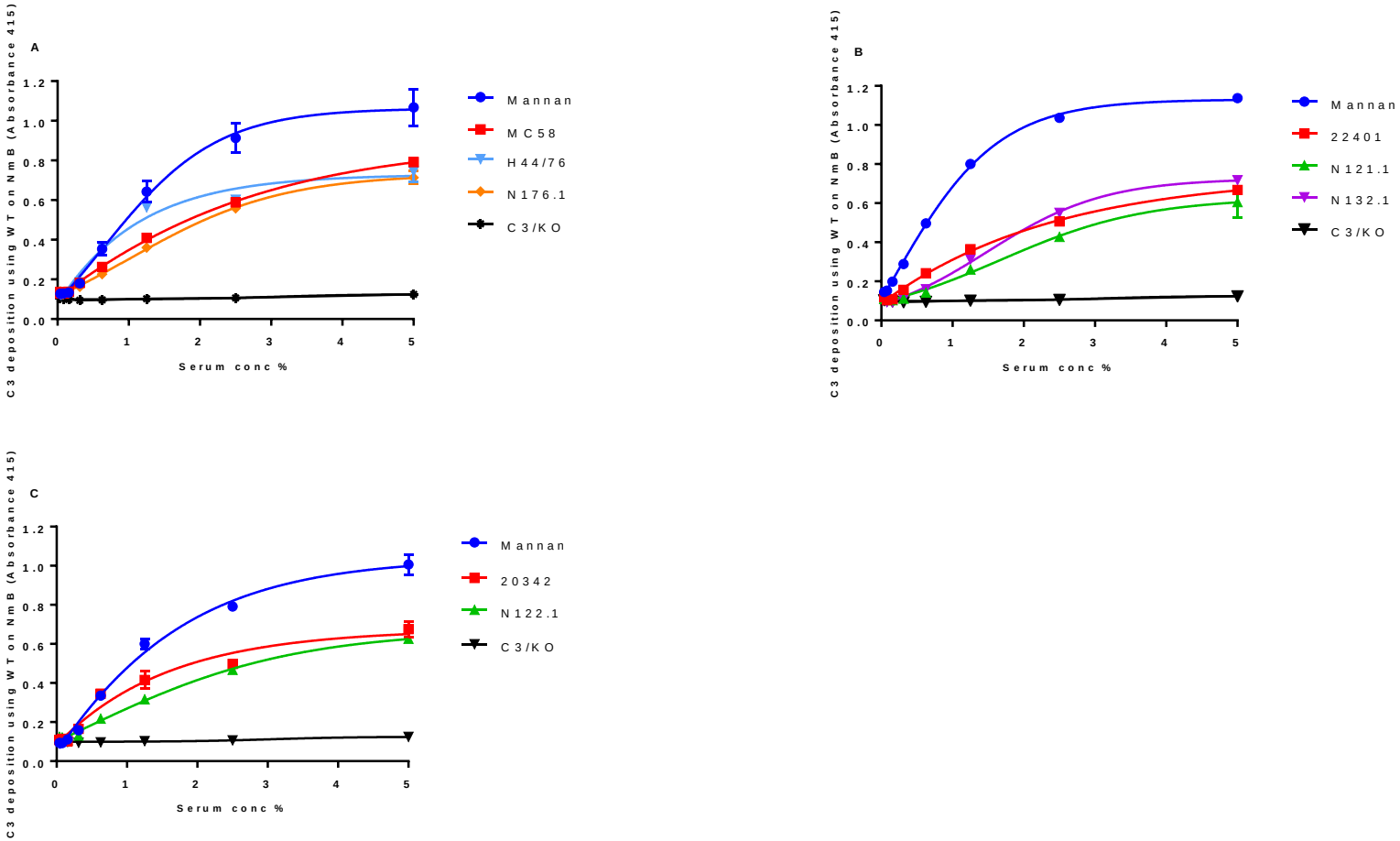


Figure 4.7: C3 deposition on different clonal complexes of *N. meningitidis* genogroup B using wild type serum concentrations up to 5% (v/v)
Microtitre plates were coated with formalin-fixed *N. meningitidis* (or mannan as a positive control) then blocked with BSA. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. C3/KO mouse sera were used as the negative control, the binding of the C3 cleavage product (C3c) was detected by using rabbit anti-human C3c polyclonal antibody. No significant difference in C3 deposition among all strains within each clonal complex (P>0.05). Figure 4.7A shows clonal complex (cc32), Figure 4.7B shows clonal complex (cc213) and Figure 4.7C shows clonal complex (cc41/44). The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

Table 4.7: Semi-quantitative scoring for the highest serum concentration and EC50 of C3 deposition on clonal complex of *N. meningitidis* genogroup B using wild type mouse serum.
The score is based on the extent of the deposition at highest serum concentration used and ranges from - (no deposition), ± (>0<10% of the positive control), + (>10<50% of the positive control), ++ (>50<80% of the positive control) and +++ (>80% of the positive control).

Isolate	Genogroup	Sequence type (Clonal complex)	% to the positive control	score	EC50 Control/strain ± SEM
MC58	B	ST-74 (cc32)	84	+++	2.10/3.92 ± 0.42
H44/76	B	ST-32 (cc32)	69	++	2.10/1.16 ± 0.27
N176.1	B	ST-5682 (cc32)	69	++	2.10/4.22 ± 0.83
22401	B	ST-213 (cc213)	66	++	1.27/3.14 ± 0.42
N121.1	B	ST-213 (cc213)	55	++	1.27/9.43 ± 4.43
N132.1	B	ST-213 (cc213)	64	++	1.27/5.61 ± 1.69
20342	B	ST-1433 (cc41/44)	65	++	2.09/1.58 ± 0.07
N122.1	B	ST-8511 (cc41/44)	64	++	2.09/4.55 ± 0.11

In clonal complex (cc269), strain 22812 was significantly higher (P<0.05) in C3 deposition than other strains of the same group, while no significant difference (P>0.05) was found between strain N199.1 and N207.1 as shown in Figure 4.8 and Table 4.8.

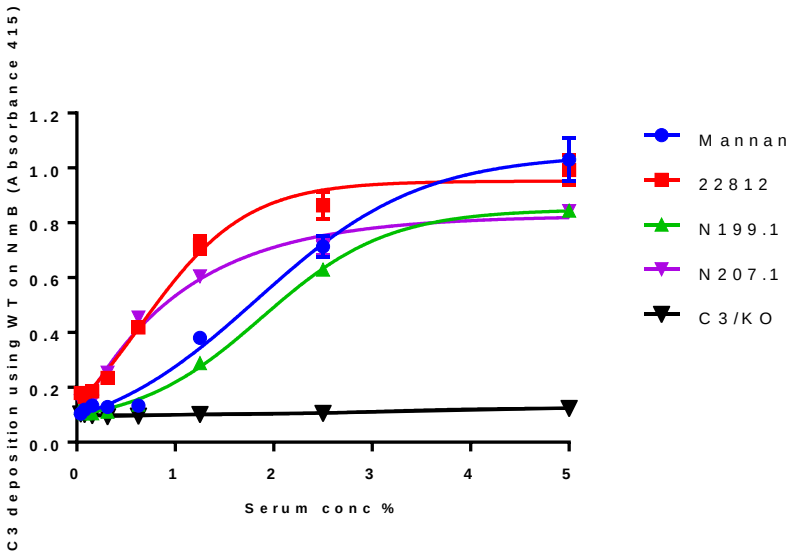


Figure 4.8: C3 deposition on clonal complex (cc269) of *N. meningitidis* genogroup B using different wild type mouse serum concentrations up to 5% (v/v)
Microtitre plates were coated with formalin-fixed *N. meningitidis* (or mannan as a positive control) then blocked with BSA. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. C3/KO mouse sera were used as the negative control, the binding of the C3 cleavage product (C3c) was detected by using rabbit anti-human C3c polyclonal antibody. C3 deposition was significantly higher on strain 22812 compared with other strains of the same group (P<0.05). There was no significant difference between N199.1 and 207.1 (P= 0.63). The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

Table 4.8: Semi-quantitative scoring for the highest serum concentration and EC50 of C3 deposition on clonal complex (cc269) of *N. meningitidis* genogroup B using wild type mouse serum.
The score is based on the extent of the deposition at highest serum concentration used and ranges from - (no deposition), ± (>0<10% of the positive control), + (>10<50% of the positive control), ++ (>50<80% of the positive control) and +++ (>80% of the positive control).

Isolate	Genogroup	Sequence type (Clonal complex)	% to the positive control	score	EC50 Control/strain ± SEM
22812	B	ST-269 (cc269)	91	+++	10.88/1.47 ± 0.33
N199.1	B	ST-283 (cc269)	81	+++	10.88/10.3 ± 5.92
N207.1	B	ST-269 (cc269)	79	++	10.88/1.03 ± 0.17

In genogroup Y, strains within clonal complex (cc167) and strains of different clonal complexes (cc223) and (cc174), have showed significant differences ($P<0.05$) in C3 deposition as shown in Figure 4.9 and Table 3.9.

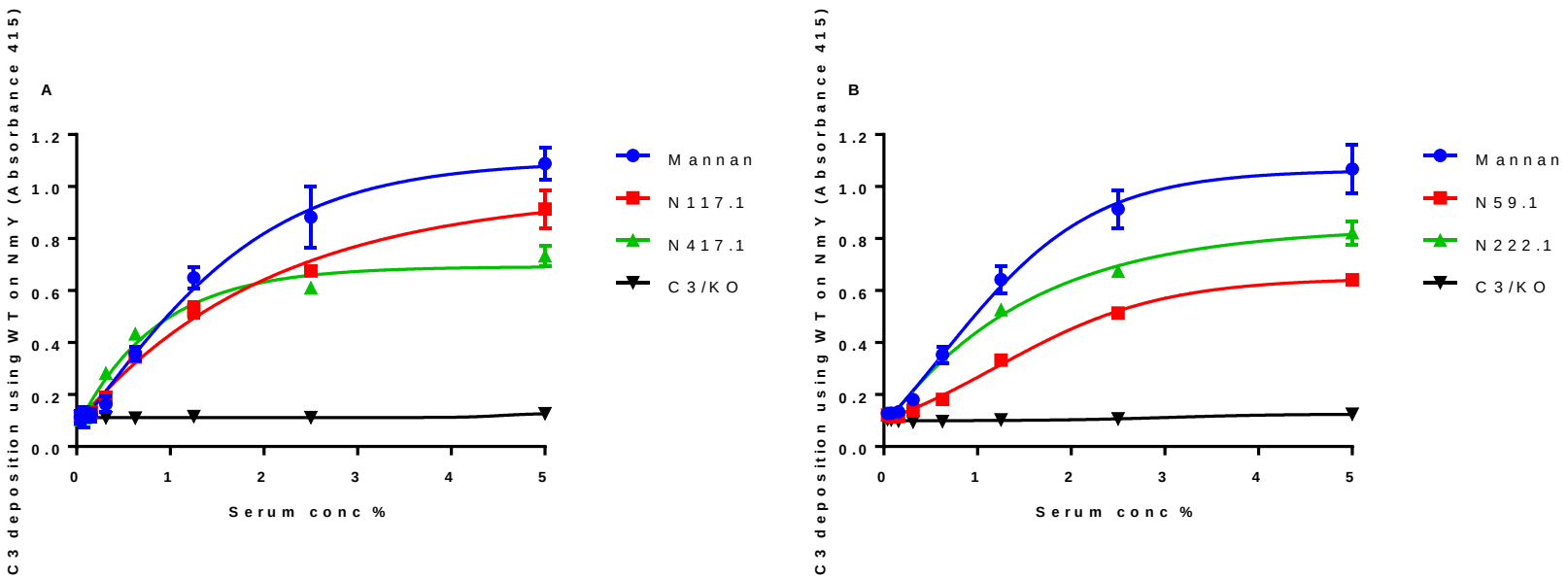


Figure 4.9: C3 deposition on different clonal complexes of *N. meningitidis* genogroup Y using different wild type mouse serum concentrations up to 5% (v/v)

Microtitre plates were coated with formalin-fixed *N. meningitidis* (or mannan as a positive control) then blocked with BSA. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. C3/KO mouse sera were used as the negative control, the binding of the C3 cleavage product (C3c) was detected by using rabbit anti-human C3c polyclonal antibody. Figure 4.9A shows clonal complex (cc167) and Figure 4.9B shows clonal complexes (cc23 and cc174). Strains within same and different clonal complexes on genogroup Y showed significant variations in C3 deposition ($P<0.05$). The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

Table 4.9: Semi-quantitative scoring for the highest serum concentration and EC50 of C3 deposition on clonal complex of *N. meningitidis* genogroup Y using wild type mouse serum.
The score is based on the extent of the deposition at highest serum concentration used and ranges from - (no deposition), ± (>0<10% of the positive control), + (>10<50% of the positive control), ++ (>50<80% of the positive control) and +++ (>80% of the positive control).

Isolate	Genogroup	Sequence type (Clonal complex)	% to the positive control	score	EC50 Control/strain ± SEM
N117.1	Y	ST-767 (cc167)	89	+++	2.14/2.45 ± 0.42
N417.1	Y	ST-767 (cc167)	63	++	2.14/0.71 ± 0.10
N59.1	Y	ST-1466 (cc174)	61	++	2.10/4.10 ± 0.99
N222.1	Y	ST-1655 (cc23)	79	++	2.10/1.69 ± 0.25

Previous results showed similarities in driving C3 using mouse serum on different strains and differences on others. Each clonal complex showed same amount of C3 deposition, except, for strain 22812 in (cc269) and N117.1 in (cc167).

Overall, with some exceptions, there are consistency between human and mouse sera versus different *N. meningitidis* clonal complexes. The C3 scoring summery of mouse serum compared to human serum is shown in Table 4.10.

Table 4.10: Semi-quantitative scoring of C3 deposition on different clonal complexes of *N. meningitidis* genogroups B and Y using wild type mouse serum compared to human serum.
The score is based on the extent of the deposition at highest serum concentration used and ranges from - (no deposition), ± (>0<10% of the positive control), + (>10<50% of the positive control), ++ (>50<80% of the positive control) and +++ (>80% of the positive control).

Isolate	Genogroup	Sequence type (Clonal Complex)	C3 deposition using wild type mouse serum	C3 deposition using normal human serum
MC58	B	ST-74 (cc32)	+++	++
H44/76	B	ST-32 (cc32)	++	+++
N176.1	B	ST-5682 (cc32)	++	++
22401	B	ST-213 (cc213)	++	+++
N121.1	B	ST-213 (cc213)	++	+++
N132.1	B	ST-213 (cc213)	++	+++
20342	B	ST-1433 (cc41/44)	++	+++
N122.1	B	ST-8511 (cc41/44)	++	+++
22812	B	ST-269 (cc269)	+++	+++
N199.1	B	ST-283 (cc269)	+++	+++
N207.1	B	ST-269 (cc269)	++	+++
N117.1	Y	ST-767 (cc167)	+++	+++
N417.1	Y	ST-767 (cc167)	++	+++
N59.1	Y	ST-1466 (cc174)	++	++
N222.1	Y	ST-1655 (cc23)	++	++

4.1.2. Defining the lectin pathway recognition molecules that contribute to complement activation on *N. meningitidis*

To determine which LP recognition molecules drive C3 deposition on different strains of *N. meningitidis*, sera from MBL/KO, CL-11/KO, FCNa/KO were used. Wildtype serum was used as positive control and C3/KO serum as negative control.

All data were used to calculate an EC50 of C3 deposition and a semi-quantitative deposition score relative to the positive control. The score is based on the extent of the complement component deficiency impact on C3 deposition at highest serum concentration used and ranges +++ (<20% of the positive control as high impact), ++ (>20<50% of the positive control as medium impact), + (>50<80% of the positive control as low impact) and ± (>80<90% of the positive control as very low impact) and - (>90% of the positive control as no impact).

In the clonal complex (cc32), results show that CL-11 is essential to drive the deposition of C3 on *N. meningitidis* B (MC58). In the absence of CL-11, C3 deposition values dropped significantly ($P<0.05$). Ficolin-a and MBL are less important. Sera from FCNa/KO and MBL/KO show induced C3 deposition as shown in Figure 4.10A. Unlike (MC58), both MBL and CL-11 share the role of activating C3 on *N. meningitidis* B (H44/76) as shown in Figure 4.10B. Although they are of the same clonal complex, unlike the previous strains, CL-11 and FCNa are important to drive C3 cleavage on *N. meningitidis* B (N176.1) as shown in Figure 4.10C. Overall, CL-11 seems to plays the major role in driving C3 deposition on strains of clonal complex (cc32), more so than the other recognition molecules of the LP as shown in Table 4.11.

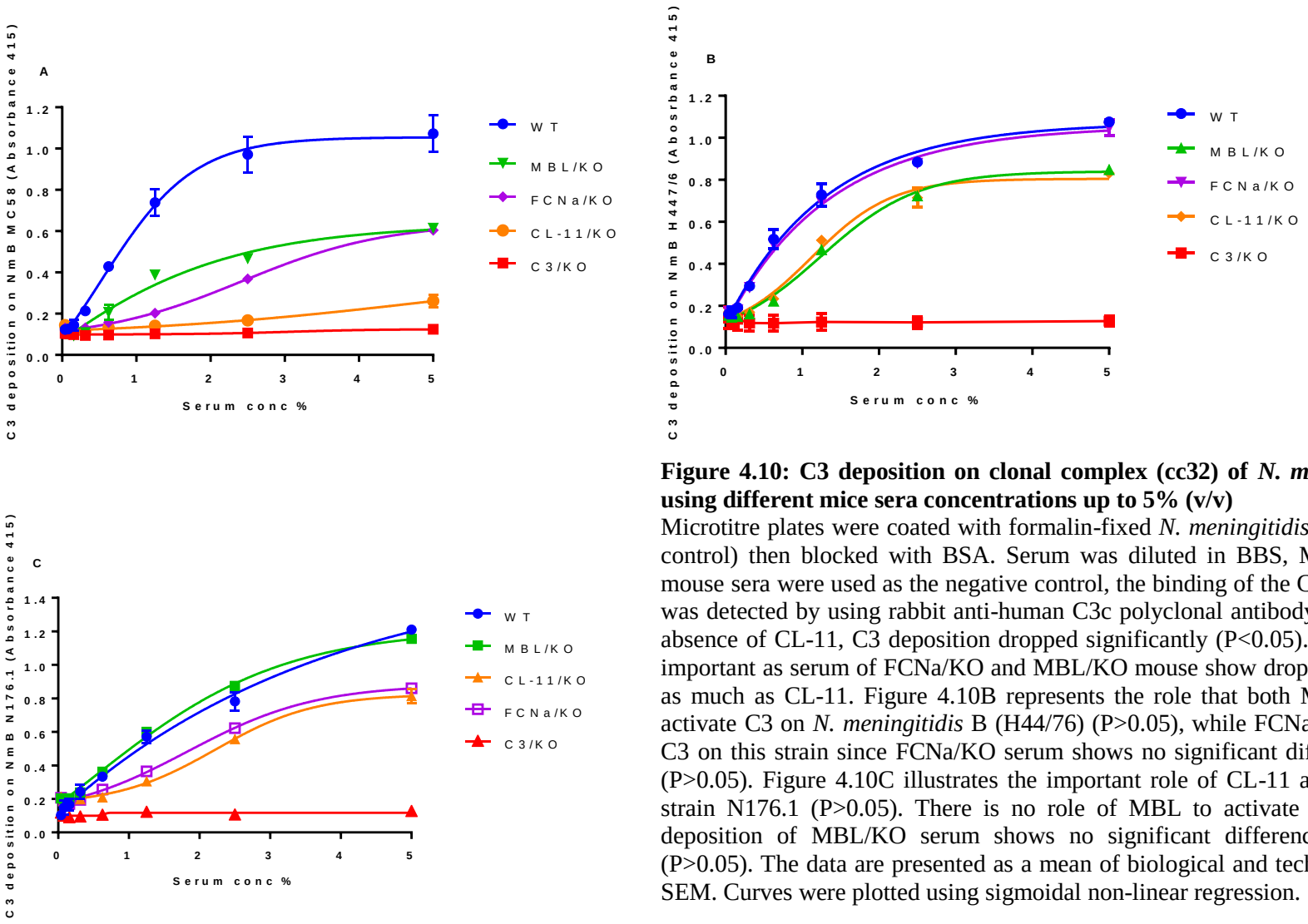


Figure 4.10: C3 deposition on clonal complex (cc32) of *N. meningitidis* genogroup B using different mice sera concentrations up to 5% (v/v)

Microtitre plates were coated with formalin-fixed *N. meningitidis* (or mannan as a positive control) then blocked with BSA. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. C3/KO mouse sera were used as the negative control, the binding of the C3 cleavage product (C3c) was detected by using rabbit anti-human C3c polyclonal antibody. In Figure 4.10A, in the absence of CL-11, C3 deposition dropped significantly ($P<0.05$). FCNa and MBL are less important as serum of FCNa/KO and MBL/KO mouse show drop in C3 deposition, but not as much as CL-11. Figure 4.10B represents the role that both MBL and CL-11 share to activate C3 on *N. meningitidis* B (H44/76) ($P>0.05$), while FCNa has no role in activating C3 on this strain since FCNa/KO serum shows no significant difference with WT control ($P>0.05$). Figure 4.10C illustrates the important role of CL-11 and FCNa to drive C3 on strain N176.1 ($P>0.05$). There is no role of MBL to activate C3 on this strain as C3 deposition of MBL/KO serum shows no significant difference with the WT control ($P>0.05$). The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

Table 4.11: Semi-quantitative scoring for the highest serum concentration and EC50 of the LP recognition molecules role in driving C3 deposition on clonal complex (cc32) of *N. meningitidis* genogroup B using genetically knockout different type of mice sera.
The score is based on the extent of the complement component deficiency impact on C3 deposition at highest serum concentration used and ranges +++ (<20% of the positive control as high impact), ++ (>20<50% of the positive control as medium impact), + (>50<80% of the positive control as low impact) and ± (>80<90% of the positive control as very low impact) and - (>90% of the positive control as no impact).

Isolate	Genogroup	Sequence type (Clonal complex)	MBL			CL-11			FCNa		
			% to the positive control	score	EC50 Control/strain ± SEM	% to the positive control	score	EC50 Control/strain ± SEM	% to the positive control	score	EC50 Control/strain ± SEM
MC58	B	ST-74 (cc32)	57	+	1.42/2.54 ± 0.69	20	+++	1.42/1.83 ± 2.59	56	+	1.42/2.06 ± 8.60
H44/76	B	ST-32 (cc32)	79	+	1.29/3.39 ± 1.06	77	+	1.29/1.38 ± 0.93	97	-	1.29/2.83 ± 0.17
N176.1	B	ST-5682 (cc32)	95	-	5.50/4.14 ± 0.68	67	+	5.50/63.5 ± 140	71	+	5.50/11.34 ± 7.29

In clonal complex (cc213), CL-11 and FCNa share the role to drive C3 on strains of this group as there is no significant difference ($P>0.05$) between both recognition molecules on all strains. There is no role of MBL in activation of C3 by strains of this clonal complex. MBL/KO serum shows no significant ($P>0.05$) difference from the WT control as shown in Figure 4.11 and Table 4.12.

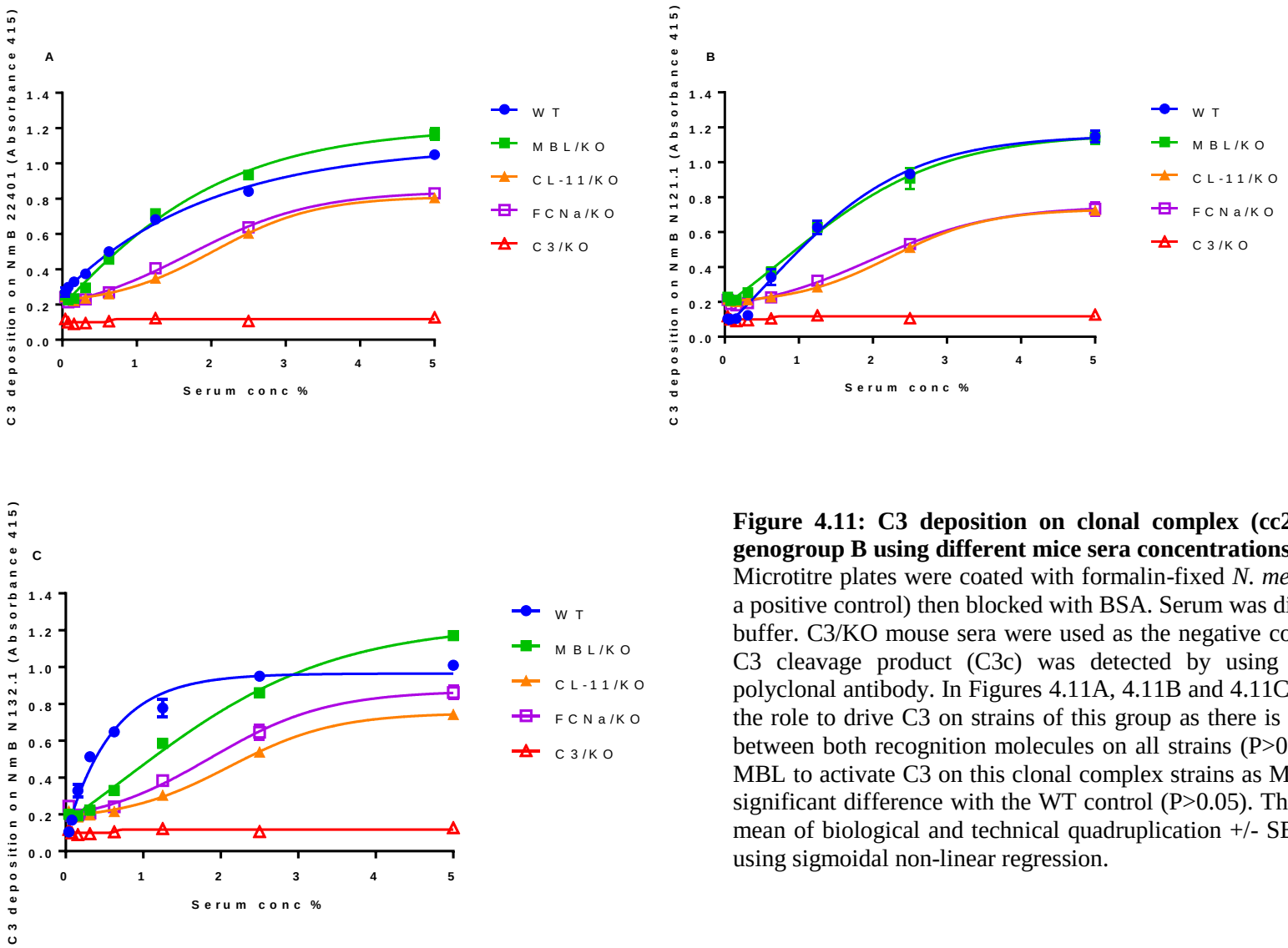


Figure 4.11: C3 deposition on clonal complex (cc213) of *N. meningitidis* genogroup B using different mice sera concentrations up to 5% (v/v)
Microtitre plates were coated with formalin-fixed *N. meningitidis* (or mannan as a positive control) then blocked with BSA. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. C3/KO mouse sera were used as the negative control, the binding of the C3 cleavage product (C3c) was detected by using rabbit anti-human C3c polyclonal antibody. In Figures 4.11A, 4.11B and 4.11C, CL-11 and FCNa share the role to drive C3 on strains of this group as there is no significant difference between both recognition molecules on all strains (P>0.05). There is no role of MBL to activate C3 on this clonal complex strains as MBL/KO serum shows no significant difference with the WT control (P>0.05). The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

Table 4.12: Semi-quantitative scoring for the highest serum concentration and EC50 of the LP recognition molecules role in driving C3 deposition on clonal complex (cc213) of *N. meningitidis* genogroup B using genetically knockout different type of mice sera.
The score is based on the extent of the complement component deficiency impact on C3 deposition at highest serum concentration used and ranges +++ (<20% of the positive control as high impact), ++ (>20<50% of the positive control as medium impact), + (>50<80% of the positive control as low impact) and ± (>80<90% of the positive control as very low impact) and - (>90% of the positive control as no impact).

Isolate	Genogroup	Sequence type (Clonal complex)	MBL			CL-11			FCNa		
			% to the positive control	score	EC50 Control/strain ± SEM	% to the positive control	score	EC50 Control/strain ± SEM	% to the positive control	score	EC50 Control/strain ± SEM
22401	B	ST-213 (cc213)	111	-	2.26/2.51 ± 0.40	77	+	2.26/16.99 ± 12.35	79	+	2.26/8.26 ± 3.12
N121.1	B	ST-213 (cc213)	100	-	2.53/3.53 ± 0.76	63	+	2.53/79.29 ± 22.76	64	+	2.53/16.12 ± 10.25
N132.1	B	ST-213 (cc213)	116	-	0.39/4.93 ± 0.90	74	+	0.39/24.8 ± 24.93	85	±	0.39/11.1 ± 6.47

In clonal complex (cc41/44), results show that FCNa is essential to drive the deposition of C3 on strains of this group. In the absence of FCNa, C3 deposition dropped significantly ($P<0.05$). Collectin-11 and MBL are less important as serum of CL-11/KO and MBL/KO show significant ($P<0.05$) drop in C3 deposition but not as like as FCNa as shown in Figure 4.12 and Table 4.13.

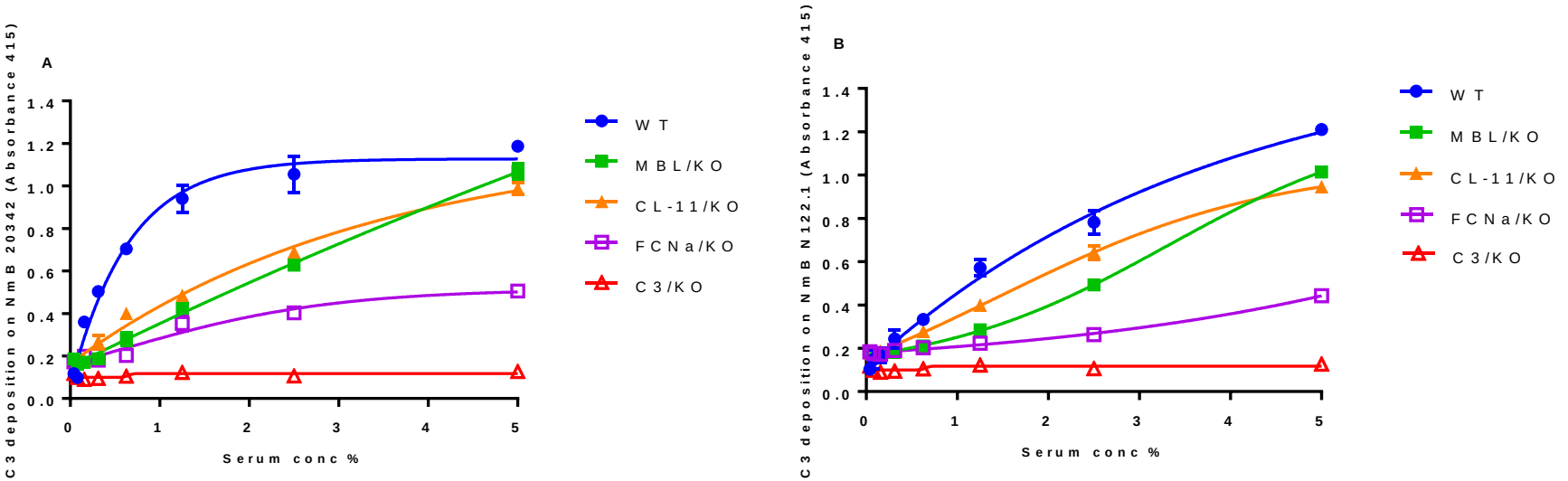
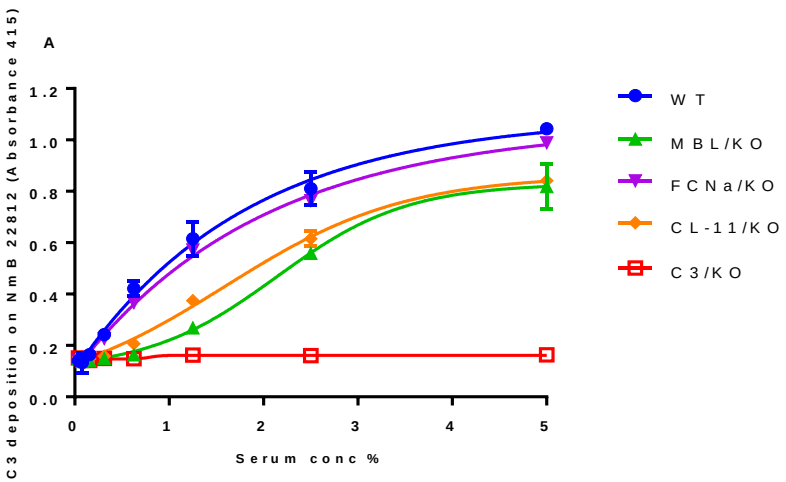


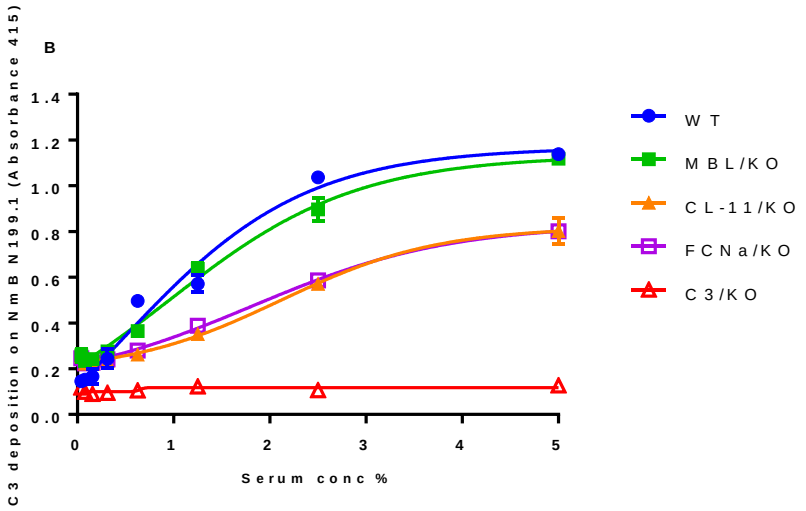
Figure 4.12: C3 deposition on clonal complex (cc41/44) of *N. meningitidis* genogroup B using different mice sera concentrations up to 5% (v/v)
Microtitre plates were coated with formalin-fixed *N. meningitidis* (or mannan as a positive control) then blocked with BSA. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. C3/KO mouse sera were used as the negative control, the binding of the C3 cleavage product (C3c) was detected by using rabbit anti-human C3c polyclonal antibody. In Figures 4.12A and 4.12B, FCNa is essential to drive the deposition of C3 on strains of this group. In the absence of FCNa, C3 deposition dropped significantly ($P<0.05$). Collectin-11 and MBL are less important as serum of CL-11/KO and MBL/KO, show drop in C3 deposition but not as like as FCNa. The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

Table 4.13: Semi-quantitative scoring for the highest serum concentration and EC50 of the LP recognition molecules role in driving C3 deposition on clonal complex (cc41/44) of *N. meningitidis* genogroup B using genetically knockout different type of mice sera.
The score is based on the extent of the complement component deficiency impact on C3 deposition at highest serum concentration used and ranges +++ (<20% of the positive control as high impact), ++ (>20<50% of the positive control as medium impact), + (>50<80% of the positive control as low impact) and ± (>80<90% of the positive control as very low impact) and - (>90% of the positive control as no impact).

Isolate	Genogroup	Sequence type (Clonal complex)	MBL			CL-11			FCNa		
			% to the positive control	score	EC50 Control/strain ± SEM	% to the positive control	score	EC50 Control/strain ± SEM	% to the positive control	score	EC50 Control/strain ± SEM
20342	B	ST-1433 (cc41/44)	90	-	0.48/35.8 ± 22.78	83	±	0.48/4.59 ± 0.99	43	++	0.48/3.68 ± 1.54
N122.1	B	ST-8511 (cc41/44)	84	±	5.50/1.27 ± 5.99	78	+	5.50/11.5 ± 2.26	37	++	5.50/2.02 ± 1.84

Last tested clonal complex for *N. meningitidis* B is (cc269). As shown in Table 4.14, variations between all strains were observed. For strain 22812, MBL plays the major role of C3 activation, while the absence of CL-11 showed very low impact on driving C3 as shown in Figure 4.13A. In strain (N199.1), CL-11 and FCNa share the role of activating C3 as shown in Figure 4.13B. Finally, in strain (N207.1), FCNa is the only recognition molecule that was capable to activate C3 deposition as shown in Figure 4.13C.





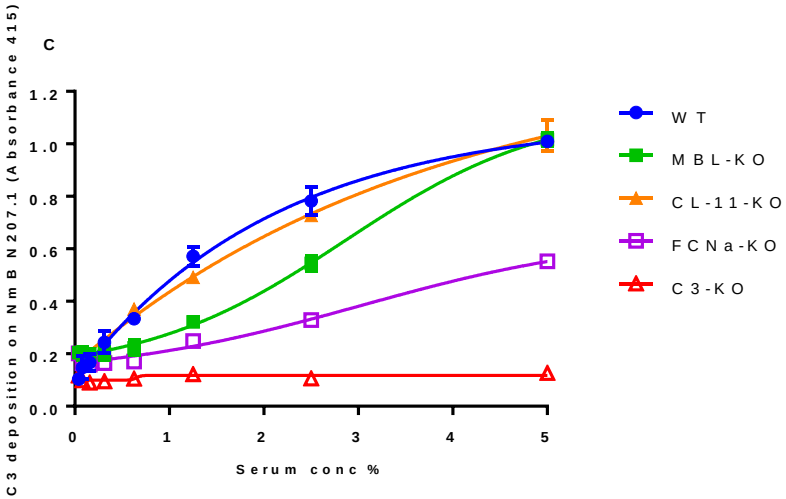


Figure 4.13: C3 deposition on clonal complex (cc269) of *N. meningitidis* genogroup B using different mice sera concentrations up to 5% (v/v)
Microtitre plates were coated with formalin-fixed *N. meningitidis* (or mannan as a positive control) then blocked with BSA. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. C3/KO mouse sera were used as the negative control, the binding of the C3 cleavage product (C3c) was detected by using rabbit anti-human C3c polyclonal antibody. In Figure 4.13A, CL-11 and MBL share the role to drive C3 on strains of this group as there is no significant difference between both recognition molecules strain 22812 (P>0.05). There is no role of FCNa to activate C3 on this strain as FCNa/KO serum shows no significant difference with the WT control (P>0.05). Figure 4.13B represents the role that both FCNa and CL-11 share to activate C3 on strain N199.1 (P>0.05), while MBL has no role in activating C3 on this strain since MBL/KO serum shows no significant difference with WT control (P>0.05). Figure 4.13C illustrates the important role of FCNa to drive C3 on strain N207.1 (P<0.05). There is no role of MBL or CL-11 to activate C3 on this strain as C3 deposition of both MBL/KO and CL-11, show no significant difference with the WT control (P>0.05). The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

Table 4.14: Semi-quantitative scoring for the highest serum concentration and EC50 of the LP recognition molecules role in driving C3 deposition on clonal complex (cc269) of *N. meningitidis* genogroup B using genetically knockout different type of mice sera

The score is based on the extent of the complement component deficiency impact on C3 deposition at highest serum concentration used and ranges +++ (<20% of the positive control as high impact), ++ (>20<50% of the positive control as medium impact), + (>50<80% of the positive control as low impact) and ± (>80<90% of the positive control as very low impact) and - (>90% of the positive control as no impact).

Isolate	Genogroup	Sequence type (Clonal complex)	MBL			CL-11			FCNa		
			% to the positive control	score	EC50 Control/strain ± SEM	% to the positive control	score	EC50 Control/strain ± SEM	% to the positive control	score	EC50 Control/strain ± SEM
22812	B	ST-269 (cc269)	78	+	2.02/28.5 ± 34.8	81	±	2.02/7.61 ± 2.37	95	-	2.02/2.37 ± 0.22
N199.1	B	ST-283 (cc269)	98	-	2.10/3.68 ± 3.68	70	+	2.10/27.9 ± 27.9	70	+	2.10/12.5 ± 12.46
N207.1	B	ST-269 (cc269)	101	-	2.52/4.44 ± 5.13	102	-	2.52/5.64 ± 1.08	55	+	2.52/4.55 ± 5.33

Using same types of sera, clonal complex (cc167) of genogroup Y showed similarities in the role of lectin pathway recognition molecules to activate C3. It is clear as shown from Figure 4.14 and Table 4.15 that MBL has the major role in activating C3 on strains of this group. However, in strain N417.1, CL-11 is important as well but not as much as MBL. There is no role of FCNa in driving C3 on this group strains as serum from FCNa/KO mouse shows no significant difference ($P>0.05$) compared to WT control.

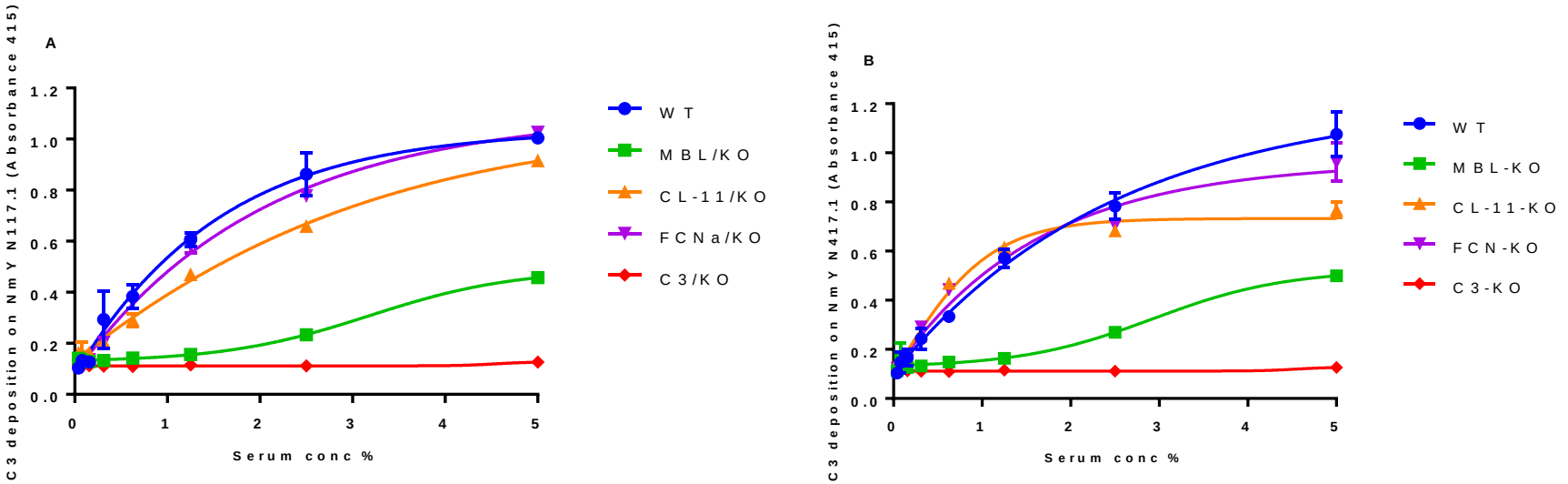


Figure 4.14: C3 deposition on clonal complex (cc167) of *N. meningitidis* genogroup Y using different mice sera concentrations up to 5% (v/v)
Microtitre plates were coated with formalin-fixed *N. meningitidis* (or mannan as a positive control) then blocked with BSA. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. C3/KO mouse sera were used as the negative control, the binding of the C3 cleavage product (C3c) was detected by using rabbit anti-human C3c polyclonal antibody. For both strains, MBL has the major role in activating C3 on strains of this group as the absence of MBL dropped C3 deposition significantly ($P<0.05$). However, CL-11 is important as well but not as much as MBL. There is no role of FCNa in driving C3 on this group strains as serum from FCNa/KO mouse shows no significant difference with WT control ($P>0.05$). The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

Table 4.15: Semi-quantitative scoring for the highest serum concentration and EC50 of the LP recognition molecules role in driving C3 deposition on clonal complex (cc167) of *N. meningitidis* genogroup Y using genetically knockout different type of mice sera.
The score is based on the extent of the complement component deficiency impact on C3 deposition at highest serum concentration used and ranges +++ (<20% of the positive control as high impact), ++ (>20<50% of the positive control as medium impact), + (>50<80% of the positive control as low impact) and ± (>80<90% of the positive control as very low impact) and - (>90% of the positive control as no impact).

Isolate	Genogroup	Sequence type (Clonal complex)	MBL			CL-11			FCNa		
			% to the positive control	score	EC50 Control/strain ± SEM	% to the positive control	score	EC50 Control/strain ± SEM	% to the positive control	score	EC50 Control/strain ± SEM
N117.1	Y	ST-767 (cc167)	45	++	1.66/1.81 ±1.74	91	-	1.66/4.79 ± 0.81	102	-	1.66/2.41 ± 0.28
N417.1	Y	ST-767 (cc167)	46	++	3.20/2.92 ± 3.65	71	+	3.20/0.78 ± 0.15	89	±	3.20/1.56 ± 0.31

In Figure 4.15A, *N. meningitidis* Y (N59.1) strain represent clonal complex (cc174). With this strain, MBL plays the major role to drive C3. There is very low role of CL-11 and no role of FCNa in driving C3 to this strain as there is no significant difference (P>0.05) between FCNa and WT control. Other *N. meningitidis* Y strain is (N222.1), which is a member of clonal complex (cc23). This strain showed the important role of FCNa and MBL to activate C3 on its surface, while CL-11 showed less important role as shown in Figure 4.15B and Table 4.16.

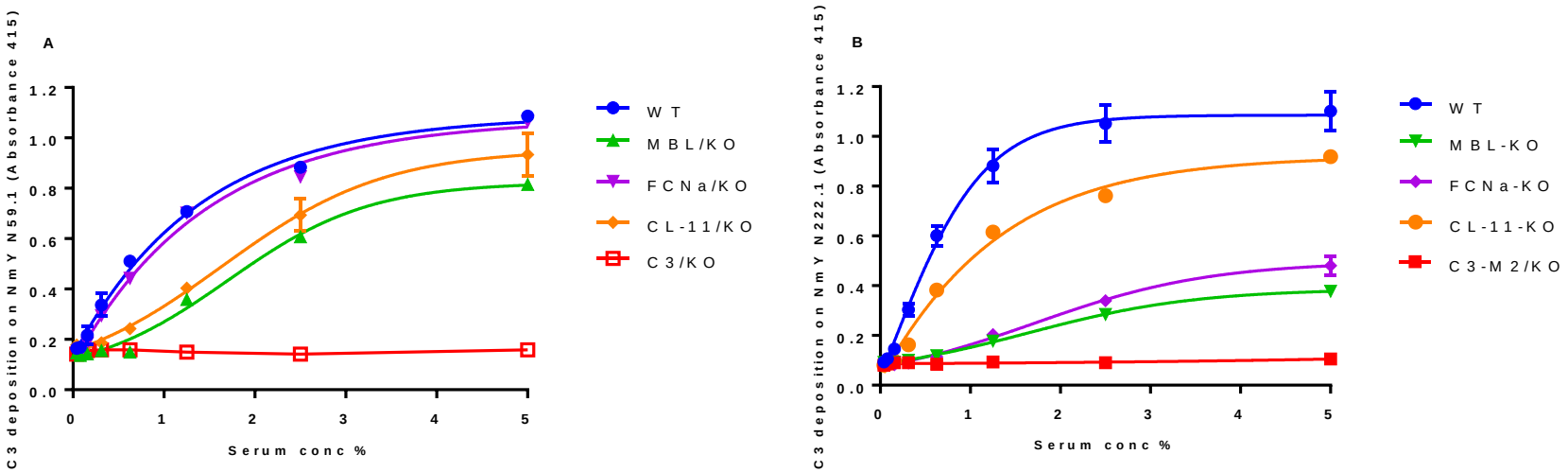


Figure 4.15: C3 deposition on clonal complexes (cc174) and (cc23) of *N. meningitidis* genogroup Y using different mice sera concentrations up to 5% (v/v)

Microtitre plates were coated with formalin-fixed *N. meningitidis* (or mannan as a positive control) then blocked with BSA. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. C3/KO mouse sera were used as the negative control, the binding of the C3 cleavage product (C3c) was detected by using rabbit anti-human C3c polyclonal antibody. In Figure 4.15A, strain N59.1 of clonal complex (cc174) showed that MBL and CL-11 are both sharing the role to drive C3 as there is no significant difference between them ($P>0.05$). There is no role of FCNa in driving C3 to this strain as there is no significant difference shown with WT control ($P>0.05$). The other strain in Figure 4.15B is N222.1 of clonal complex (cc23). In this strain, the absence of either FCNa or MBL dropped C3 significantly ($P<0.05$). Collectin-11 is less important to drive C3 on this strain. The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

Table 4.16: Semi-quantitative scoring for the highest serum concentration and EC50 of the LP recognition molecules role in driving C3 deposition on clonal complexes (cc174) and (cc23) of *N. meningitidis* genogroup Y using genetically knockout different type of mice sera.
The score is based on the extent of the complement component deficiency impact on C3 deposition at highest serum concentration used and ranges +++ (<20% of the positive control as high impact), ++ (>20<50% of the positive control as medium impact), + (>50<80% of the positive control as low impact) and ± (>80<90% of the positive control as very low impact) and - (>90% of the positive control as no impact).

Isolate	Genogroup	Sequence type (Clonal complex)	MBL			CL-11			FCNa		
			% to the positive control	score	EC50 Control/strain ± SEM	% to the positive control	score	EC50 Control/strain ± SEM	% to the positive control	score	EC50 Control/strain ± SEM
N59.1	Y	ST-1466 (cc174)	75	+	1.38/8.11 ± 3.69	86	±	1.38/7.59 ± 3.00	98	-	1.38/1.51 ± 0.16
N222.1	Y	ST-1655 (cc23)	34	++	0.76/8.00 ± 8.00	83	±	0.76/1.38 ± 1.38	44	++	0.76/9.74 ± 9.74

To summarise, using mice sera, previous results showed similarities in driving C3 on different strains and differences on others. In *N. meningitidis* genogroup B, the tested clonal complexes were CL-11 dependant much more than other recognition molecules except, N207.1. In *N. meningitidis* genogroup Y, MBL is consistently the major recognition molecule.

From all the previous findings, with few exceptions, I suggest that the LP recognition molecules reacting accumulatively to activate C3 on different clonal complexes. However, in genogroup B, CL-11 is the major recognition molecule and MBL plays a major role in recognising all strains of different clonal complexes of genogroup Y.

4.2. C5b-9 deposition using human serum

To assess the ability of complement system to drive MAC formation on *N. meningitidis* different strains, C5b-9 deposition assays were performed using normal human serum. In these assays, bacteria strains were grouped based on their clonal complexes and genogroups. C5b-9 ability to drive MAC formation on *N. meningitidis* reflects the ability of the complement system to kill bacteria. In these assays, an antibody against human C5b-9 was used to detect MAC formation on tested strains as described in section 2.1.1.4. Formalin-fixed *N. meningitidis* different strains were coated on ELISA microtitre plates. Strain H44/76 is very susceptible to MAC formation. Therefore, it was used as positive control and BSA was used as negative control. Used type of serum has no antibody against BEXSERO, which eliminate the CP activity via anti-group B antibody. Also, a concentration of 20% (v/v) of normal human serum was used, to permit AP activation beside the LP, which will lead to the highest amount of MAC that can be formed on each strain.

All data were used to calculate an EC50 of C3 deposition and a semi-quantitative deposition score relative to the positive control. The score is based on the extent of the deposition at highest serum concentration used and ranges from - (no deposition), ± (>0<10% of the positive control), + (>10<50% of the positive control), ++ (>50<80% of the positive control) and +++ (>80% of the positive control).

For clonal complex (cc32), although H44/76 is a part of the same clonal complex, H44/76 is much more susceptible to MAC formation than MC58 and N176.1 as shown in Figure 4.16 and Table 4.17. Formation of MAC on MC58 and N176.1 is significantly low ($P<0.05$). Therefore, I suggest that the complement system role with MC58 and N176.1 is opsonisation by C3 and both strains have the ability to escape downstream complement activation, which protect them from MAC formation.

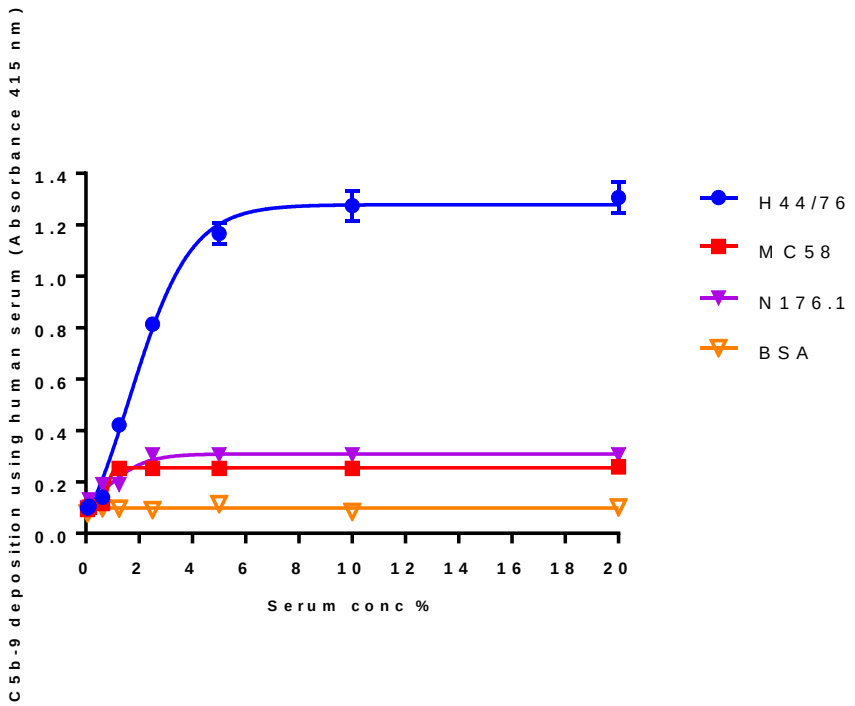


Figure 4.16: C5b-9 deposition on clonal complex (cc32) of *N. meningitidis* genogroup B using different normal human serum concentrations up to 20% (v/v)
Microtitre plates were coated with formalin-fixed *N. meningitidis* (or *N. meningitidis* strain H44/76 as a positive control) then blocked with BSA that was used as the negative control. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. The binding of the C5b-9 (MAC) was detected by using mouse anti-human C5b-9 antibody. H44/76 is much more susceptible to MAC formation than MC58 and N176.1. Strains other than H44/76 of this clonal complex are protected from MAC formation. The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

Table 4.17: Semi-quantitative scoring for the highest serum concentration and EC50 of C5b-9 deposition on clonal complex (cc32) of *N. meningitidis* genogroup B using normal human serum.
The score is based on the extent of the deposition at highest serum concentration used and ranges from - (no deposition), ± (>0<10% of the positive control), + (>10<50% of the positive control), ++ (>50<80% of the positive control) and +++ (>80% of the positive control).

Isolate	Genogroup	Sequence type (Clonal complex)	% to the positive control	score	EC50 Control/strain ± SEM
MC58	B	ST-74 (cc32)	20	+	2.65/0.63 ± 0.33
H44/76	B	ST-32 (cc32)	100	+++	2.65/2.65 ± 0.62
N176.1	B	ST-5682 (cc32)	23	+	2.65/0.90 ± 0.32

In clonal complex (cc213), results show that strain 22401 has slightly but insignificantly (P>0.05) lower MAC formation than other strains of the same clonal complex as shown in Figure 4.17 and Table 4.18.

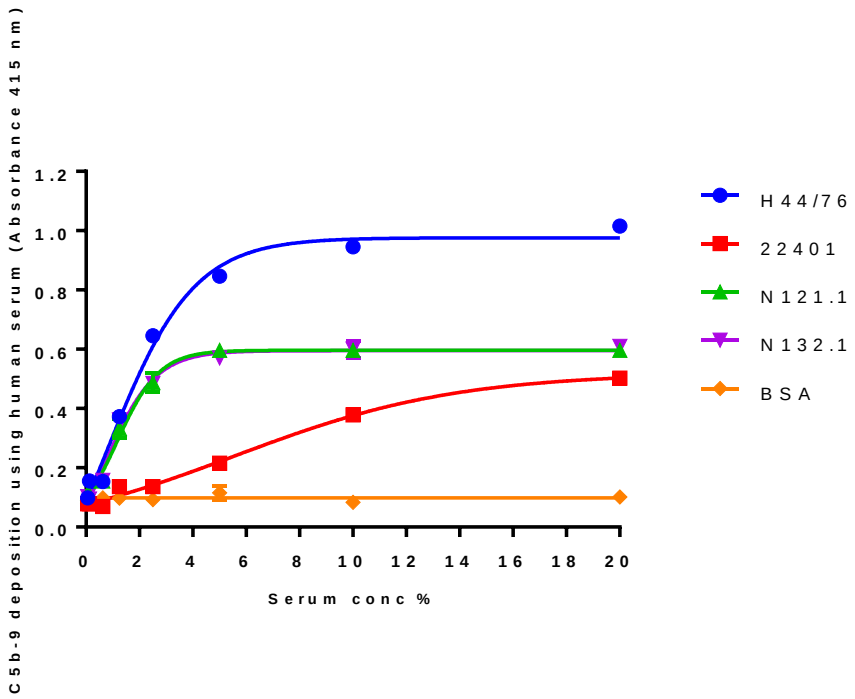


Figure 4.17: C5b-9 deposition on clonal complex (cc213) of *N. meningitidis* genogroup B using different normal human serum concentrations up to 20% (v/v)
Microtitre plates were coated with formalin-fixed *N. meningitidis* (or *N. meningitidis* strain H44/76 as a positive control) then blocked with BSA that was used as the negative control. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. The binding of the C5b-9 (MAC) was detected by using mouse anti-human C5b-9 antibody. All strains show no significant differences on MAC formation at the highest serum concentration (P>0.05). The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

Table 4.18: Semi-quantitative scoring for the highest serum concentration and EC50 of C5b-9 deposition on clonal complex (cc213) of *N. meningitidis* genogroup B using normal human serum.
The score is based on the extent of the deposition at highest serum concentration used and ranges from - (no deposition), ± (>0<10% of the positive control), + (>10<50% of the positive control), ++ (>50<80% of the positive control) and +++ (>80% of the positive control).

Isolate	Genogroup	Sequence type (Clonal complex)	% to the positive control	score	EC50 Control/strain ± SEM
22401	B	ST-213 (cc213)	49	+	2.72/24.9 ± 7.41
N121.1	B	ST-213 (cc213)	59	++	2.72/1.55 ± 0.41
N132.1	B	ST-213 (cc213)	60	++	2.72/1.44 ± 0.32

In clonal complex (cc41/44), strain 20342 shows significantly (P<0.05) low MAC formation compared to N122.1. Suggesting that strain 20342 has the ability to escape complement attack as shown in Figure 4.18 and Table 4.19.

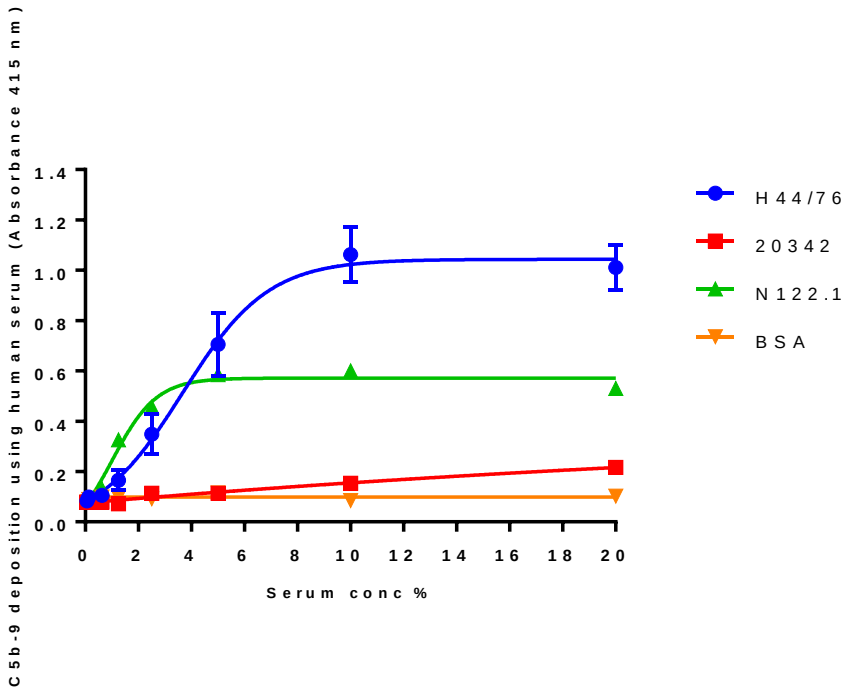


Figure 4.18: C5b-9 deposition on clonal complex (cc41/44) of *N. meningitidis* genogroup B using different normal human serum concentrations up to 20% (v/v)
Microtitre plates were coated with formalin-fixed *N. meningitidis* (or *N. meningitidis* strain H44/76 as a positive control) then blocked with BSA that was used as the negative control. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. The binding of the C5b-9 (MAC) was detected by using mouse anti-human C5b-9 antibody. MAC deposition on strain N121.1 is significantly higher than 20342 (P<0.05). Strain 20342 has the ability to escape complement attack. The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

Table 4.19: Semi-quantitative scoring for the highest serum concentration and EC50 of C5b-9 deposition on clonal complex (cc41/44) of *N. meningitidis* genogroup B using normal human serum.
The score is based on the extent of the deposition at highest serum concentration used and ranges from - (no deposition), ± (>0<10% of the positive control), + (>10<50% of the positive control), ++ (>50<80% of the positive control) and +++ (>80% of the positive control).

Isolate	Genogroup	Sequence type (Clonal complex)	% to the positive control	score	EC50 Control/strain ± SEM
20342	B	ST-1433 (cc41/44)	21	+	2.31/64.5 ± 52.3
N122.1	B	ST-8511 (cc41/44)	52	++	2.31/0.42 ± 1.38

As Figure 4.19 and Table 4.20 show, in clonal complex (cc269), MAC formation on strain N199.1 is significantly lower (P<0.05) than other strains of the same group. At higher serum concentration, both 22812 and N207.1 are significantly higher in MAC formation than N199.1 (P<0.05).

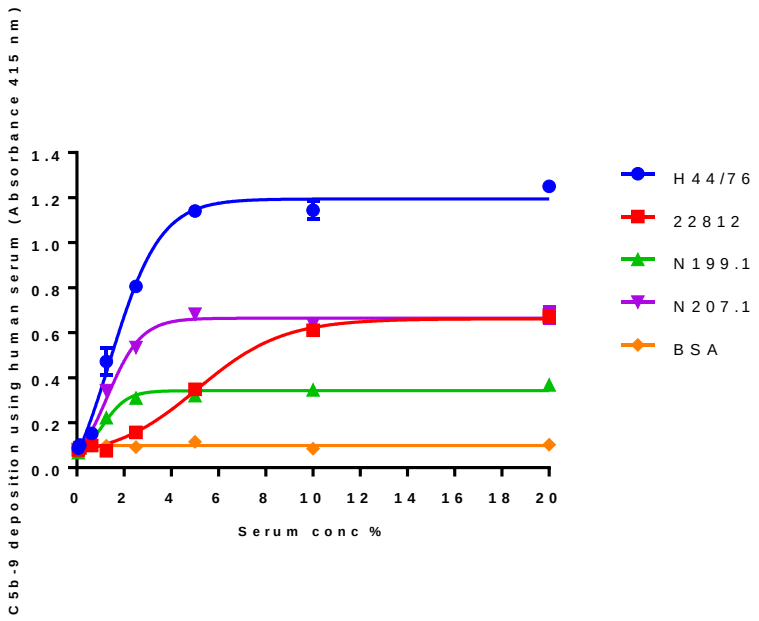


Figure 4.19: C5b-9 deposition on clonal complex (cc269) of *N. meningitidis* genogroup B using different normal human serum concentrations up to 20% (v/v)
Microtitre plates were coated with formalin-fixed *N. meningitidis* (or *N. meningitidis* strain H44/76 as a positive control) then blocked with BSA that was used as the negative control. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. The binding of the C5b-9 (MAC) was detected by using mouse anti-human C5b-9 antibody. MAC formation on strain N199.1 is significantly lower than other strains of the same group (P<0.05). At higher serum concentration, both 22812 and N207.1 are significantly higher in MAC formation than N199.1 (P<0.05). The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

Table 4.20: Semi-quantitative scoring for the highest serum concentration and EC50 of C5b-9 deposition on clonal complex (cc269) of *N. meningitidis* genogroup B using normal human serum.
The score is based on the extent of the deposition at highest serum concentration used and ranges from - (no deposition), ± (>0<10% of the positive control), + (>10<50% of the positive control), ++ (>50<80% of the positive control) and +++ (>80% of the positive control).

Isolate	Genogroup	Sequence type (Clonal complex)	% to the positive control	score	EC50 Control/strain ± SEM
22812	B	ST-269 (cc269)	54	++	2.25/13.2 ± 5.02
N199.1	B	ST-283 (cc269)	29	+	2.25/1.38 ± 0.34
N207.1	B	ST-269 (cc269)	54	++	2.25/1.60 ± 0.47

Also, using normal human serum, clonal complexes of *N. meningitidis* genogroup Y were tested. Clonal complex (cc167) shows different amount of MAC formation between strains. However, strain N417.1 showed significantly (P<0.05) higher amount of MAC formed than strain N117.1 as shown in Figure 4.20 and Table 4.21.

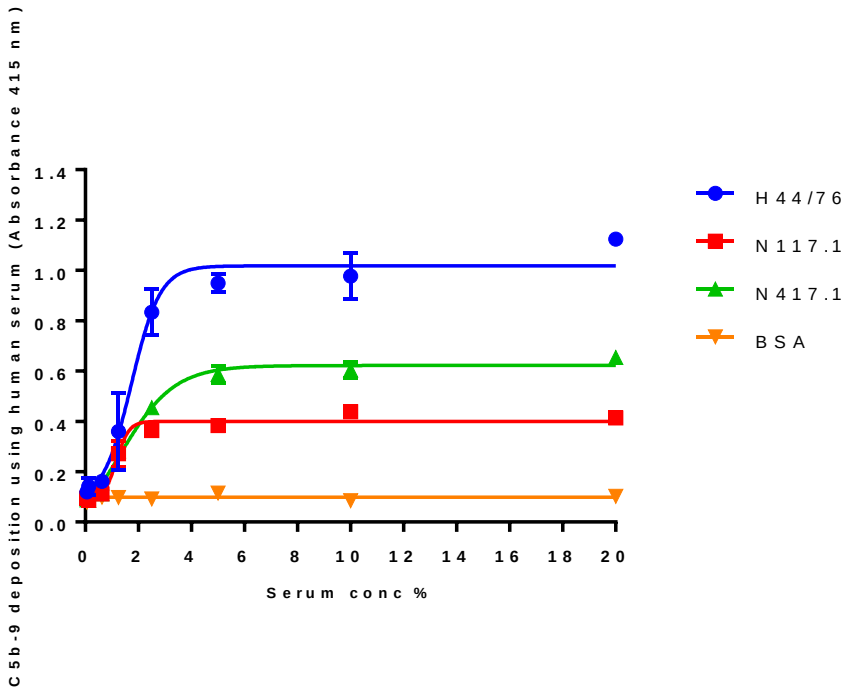


Figure 4.20: C5b-9 deposition on clonal complex (cc167) of *N. meningitidis* genogroup Y using different normal human serum concentrations up to 20% (v/v)
Microtitre plates were coated with formalin-fixed *N. meningitidis* (or *N. meningitidis* strain H44/76 as a positive control) then blocked with BSA that was used as the negative control. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. The binding of the C5b-9 (MAC) was detected by using mouse anti-human C5b-9 antibody. Significantly different amount of MAC formation was between strains. Nevertheless, MAC formation on strain N417.1 is significantly (P<0.05) higher than N117.1. The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

Table 4.21: Semi-quantitative scoring for the highest serum concentration and EC50 of C5b-9 deposition on clonal complex (cc167) of *N. meningitidis* genogroup Y using normal human serum.
The score is based on the extent of the deposition at highest serum concentration used and ranges from - (no deposition), ± (>0<10% of the positive control), + (>10<50% of the positive control), ++ (>50<80% of the positive control) and +++ (>80% of the positive control).

Isolate	Genogroup	Sequence type (Clonal complex)	% to the positive control	score	EC50 Control/strain ± SEM
N117.1	Y	ST-767 (cc167)	37	+	2.34/1.38 ± 1.38
N417.1	Y	ST-767 (cc167)	58	++	2.34/2.15 ± 2.15

Although the last two *N. meningitidis* genogroup Y strains were representing different clonal complexes, there was no significant difference (P>0.05) between the amounts of MAC formation on both strains as shown in Figure 4.21 and Table 4.22.

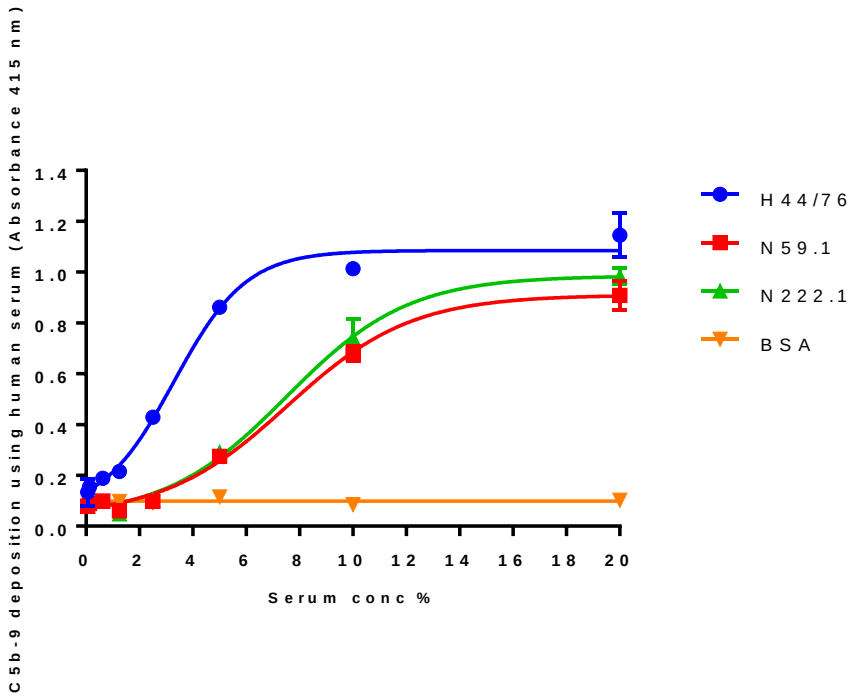


Figure 4.21: C5b-9 deposition on clonal complexes (cc174) and (cc23) of *N. meningitidis* genogroup Y using different normal human serum concentrations up to 20% (v/v)
Microtitre plates were coated with formalin-fixed *N. meningitidis* (or *N. meningitidis* strain H44/76 as a positive control) then blocked with BSA that was used as the negative control. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. The binding of the C5b-9 (MAC) was detected by using mouse anti-human C5b-9 antibody. Strain N59.1 is of clonal complex (cc174) and strain N222.1 is of clonal complex (cc23). There was no significant difference between the amounts of MAC formation on both strains (P>0.05). The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

Table 4.22: Semi-quantitative scoring for the highest serum concentration and EC50 of C5b-9 deposition on clonal complexes (cc174) and (cc23) of *N. meningitidis* genogroup Y using normal human serum.
The score is based on the extent of the deposition at highest serum concentration used and ranges from - (no deposition), ± (>0<10% of the positive control), + (>10<50% of the positive control), ++ (>50<80% of the positive control) and +++ (>80% of the positive control).

Isolate	Genogroup	Sequence type (Clonal complex)	% to the positive control	score	EC50 Control/strain ± SEM
N59.1	Y	ST-1466 (cc174)	80	++	6.13/44.7 ± 32.37
N222.1	Y	ST-1655 (cc23)	86	++	6.13/43.2 ± 31.29

Based on the previous findings, no specific pattern was seen within any clonal complex. Since terminal pathway activation and MAC formation followed C3 activation, it might be expected that C3 deposition correlates closely with MAC formation. However, as shown in Table 4.23, with few exceptions, there is a consistency between C3 deposition and MAC formation on tested strains. *N. meningitidis* uses several mechanisms to control complement activation as discussed earlier. The most important of which is the sequestration of factor H by fHbp with a variation from strain to strain. This might explain the ability of some strain to inactivate complement C3 which will lead to very low or no MAC formation.

Table 4.23: Comparison between C3 deposition and MAC formation semi-quantitative scoring on different clonal complexes of *N. meningitidis* genogroups B and Y using normal human serum.

The score is based on the extent of the deposition at highest serum concentration used and ranges from - (no deposition), ± (>0<10% of the positive control), + (>10<50% of the positive control), ++ (>50<80% of the positive control) and +++ (>80% of the positive control).

Isolate	Genogroup	Sequence type (Clonal complex)	C3 deposition	MAC deposition
MC58	B	ST-74 (cc32)	++	+
H44/76	B	ST-32 (cc32)	+++	+++
N176.1	B	ST-5682 (cc32)	++	+
22401	B	ST-213 (cc213)	+++	+
N121.1	B	ST-213 (cc213)	+++	++
N132.1	B	ST-213 (cc213)	+++	++
20342	B	ST-1433 (cc41/44)	+++	+
N122.1	B	ST-8511 (cc41/44)	+++	++
22812	B	ST-269 (cc269)	+++	++
N199.1	B	ST-283 (cc269)	+++	+
N207.1	B	ST-269 (cc269)	+++	++
N117.1	Y	ST-767 (cc167)	+++	+
N417.1	Y	ST-767 (cc167)	+++	++
N59.1	Y	ST-1466 (cc174)	++	++
N222.1	Y	ST-1655 (cc23)	++	++

4.3. The complement system pathway that contribute to complement activation on *N. meningitidis*

To assess the role of the LP and AP in activating the complement system on each strain, half maximal effective concentration (EC_{50}) of C3 deposition and C5b-9 deposition on all tested strains was calculated. For mouse serum, The AP EC_{50} value is equal to 5.2 (Figure 3.3A). Using human serum, the AP EC_{50} value is equal to 3.9 (Figure 3.3B).

Based on calculated EC_{50} values from C3 deposition assays using mouse serum, all the tested *N. meningitidis* strains are found to be LP pathway dependant, except N121.1, N132.1 and N199.1, which are AP dependant. Using data from C3 and C5b-9 deposition of normal human serum, all the tested *N. meningitidis* strains are found to be LP dependant in driving C3 or MAC formation on their surface. However, strains 22401, 20342, 22812, N59.1 and N222.1 rely on the AP to generate a significant amount of MAC on their surface. Based on the previous findings, I suggest that in the absence of specific antibodies against *N. meningitidis*, the LP is the major pathway to drive C3 on tested strains surface as shown in Table 4.24

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Table 4.24: calculated EC_{50} values for all strains tested and their corresponding complement pathway using C3 and c5b-9 deposition data of normal human serum and WT mouse serum
Any value (>5.2) using mouse serum or (>3.9) using human serum is mostly alternative pathway dependant. Less than that is lectin pathway dependant.

Clonal complex	Genogroup	Strain	C3 deposition EC_{50} (mouse serum)	C3 deposition EC_{50} (human serum)	C5b-9 deposition EC_{50} (human serum)
cc32	B	MC58	3.92	2.508	0.6324
		H44/76	1.162	0.516	2.647
		N176.1	4.218	1.263	0.902
cc213	B	22401	3.135	0.677	> 3.9
		N121.1	> 5.2	2.532	1.546
		N132.1	> 5.2	1.99	1.44
cc41/44	B	20342	1.583	0.910	> 3.9
		N122.1	4.55	2.532	1.378
cc269	B	22812	1.475	0.305	> 3.9
		N199.1	> 5.2	2.081	1.376
		N207.1	1.03	1.326	1.599
cc167	Y	N117.1	2.446	2.247	1.381
		N417.1	0.713	2.62	2.152
cc174	Y	N59.1	4.103	0.442	> 3.9
cc23	Y	N222.1	1.693	0.318	> 3.9

4.3.1. C3 deposition using mouse serum on phase variant strains of *N. meningitidis*

The expression of several genes involved in host adaptation by *N. meningitidis*, is a process known as phase variation (PV). To understand *N. meningitidis* cell wall proteins variation role in activating the complement system, phase variant isolates were tested. Four *N. meningitidis* genogroup B strains were isolated from the same volunteer in different time points. These isolates are; N199.1, N199.2, N378.1 and N378.2, which has its PorA varied in VR2 (Alamro *et al.*, 2014, Bidmos *et al.*, 2011). As shown in Figure 4.22, variant phases of in these strain of the same volunteer showed no significant difference (P>0.05) between C3 deposition on tested isolates using wild type mouse serum.

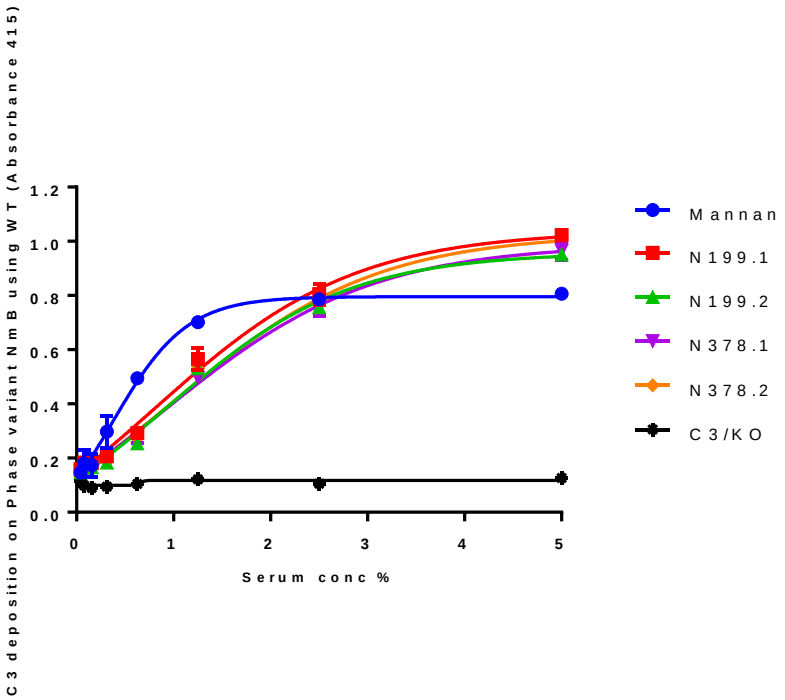

Figure 4.22: C3 deposition on phase variant *N. meningitidis* genogroup B using different wild type mouse serum concentrations up to 5% (v/v)
Microtitre plates were coated with formalin-fixed *N. meningitidis* (or mannan as a positive control) then blocked with BSA. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. C3/KO mouse sera were used as the negative control, the binding of the C3 cleavage product (C3c) was detected by using rabbit anti-human C3c polyclonal antibody. Variant phases of same clonal complex from same volunteer showed no significant (P>0.05) difference in C3 deposition onto these isolates using wild type mouse serum. The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

Another four phase variant isolates of *N. meningitidis* genogroup Y were tested using wildtype mouse serum, i.e. N222.1, N222.2, N459.5 and N459.6, which has its PorA varied in VR1 (Bidmos *et al.*, 2011). Similar to tested isolates of genogroup B, results showed no significant differences (P>0.05) between C3 depositions on tested isolates as shown in Figure 4.23.

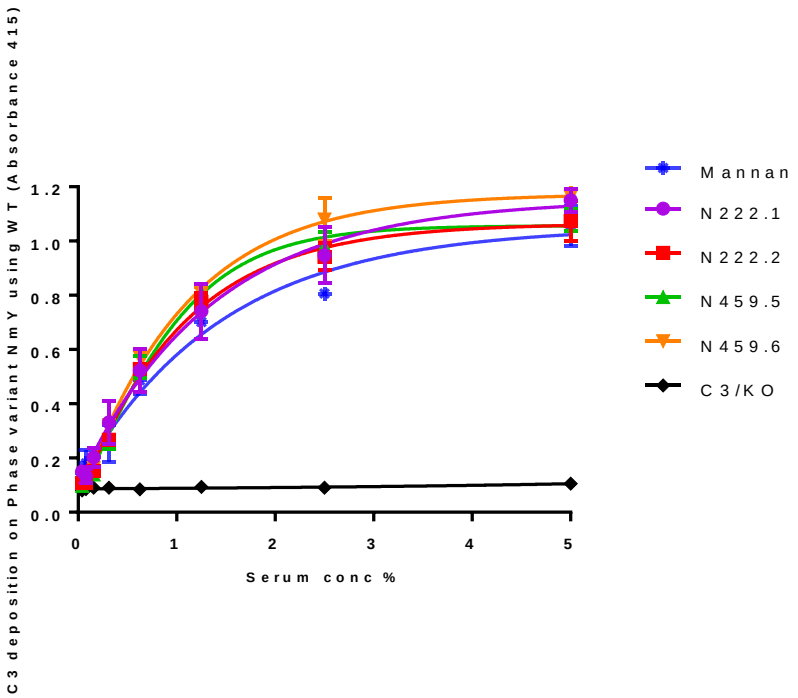

Figure 4.23: C3 deposition on phase variant *N. meningitidis* genogroup Y using different wild type mouse serum concentrations up to 5% (v/v)
Microtitre plates were coated with formalin-fixed *N. meningitidis* (or mannan as a positive control) then blocked with BSA. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. C3/KO mouse sera were used as the negative control, the binding of the C3 cleavage product (C3c) was detected by using rabbit anti-human C3c polyclonal antibody. No significant (P>0.05) differences between C3 depositions were shown on these isolates. The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

Based on the previous findings, I suggest that phase variation of OMP, specifically, PorA-VR does not change the complement activation behaviour and does not increase *N. meningitidis* resistance against complement attack.

4.3.2. Role of *N. meningitidis* capsule in the binding of different complement lectin pathway recognition molecules

Capsule has a well-known role in preventing bacteria phagocytosis. This prevention allows the bacteria to survive immune system innate cellular attack. The complement system is an important part of the innate immune system. Hence, to assess the role of the capsule in the binding of the LP recognition molecules, a genetically modified (capsule-KO) strain of *N. meningitidis* genogroup B MC58 was compared to wild type of the same strain. This comparison will narrow the differences between strains to be only with capsule. To achieve this, serum from normal human was tested on both MC58 and MC58-capsule/KO strains. As shown in Figure 4.24, C3 deposition was insignificantly (P>0.05) higher on encapsulated strain than other ligands.

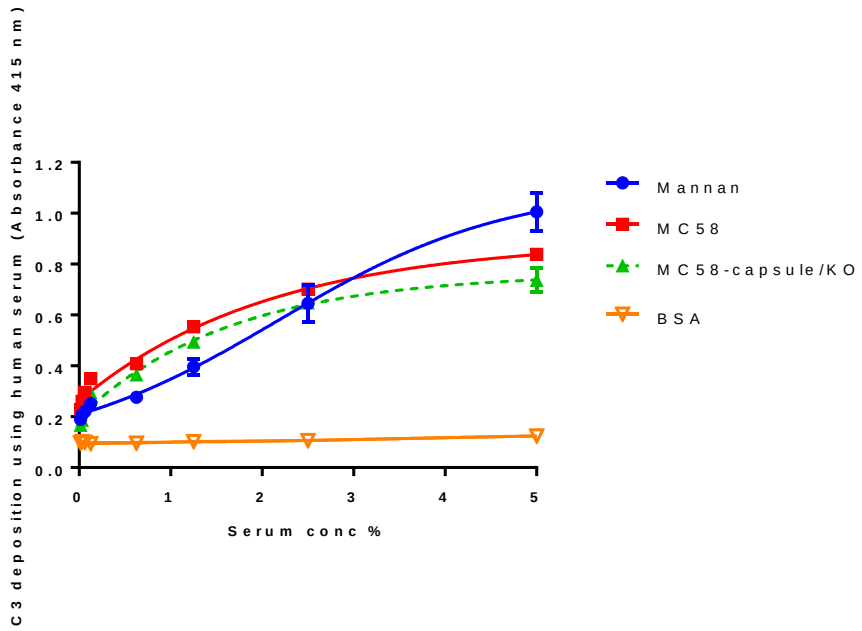

Figure 4.24: C3 deposition on *N. meningitidis* genogroup B (MC58 and MC58-capsule/KO) using different normal human serum concentrations up to 5% (v/v)
Microtitre plates were coated with formalin-fixed *N. meningitidis* (or mannan as a positive control) then blocked with BSA that was used as the negative control. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. The binding of the C3 cleavage product (C3c) was detected by using rabbit anti-human C3c polyclonal antibody. C3 deposition is slightly but insignificantly (P>0.05) higher on encapsulated strain. The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

To understand this difference, WT and genetically modified different type mouse sera were used, to figure out the role of each recognition molecule with the capsule. Using WT serum, capsule makes no significant difference ($P>0.05$) compared to non-capsulated strain as shown in Figure 4.25A. Using MBL/KO serum, either CL-11 or ficolin-A, binds to the capsule slightly but not significantly higher ($P>0.05$) than other ligands as shown in Figure 4.25B. Using FCNa/KO serum, either MBL or CL-11 binds significantly ($P<0.05$) higher on the capsule than other ligands as shown in Figure 4.25C. The absence of CL-11 in CL-11/KO serum has no significant ($P>0.05$) difference in binding the capsule as shown in Figure 4.25D. Previous results suggest that all the recognition molecules work accumulatively in the recognition of MC58. However, CL-11 is the most important molecule to recognise capsule and outer membrane ligands as CL-11/KO serum showed significant ($P<0.05$) drop in C3 deposition on both strains.

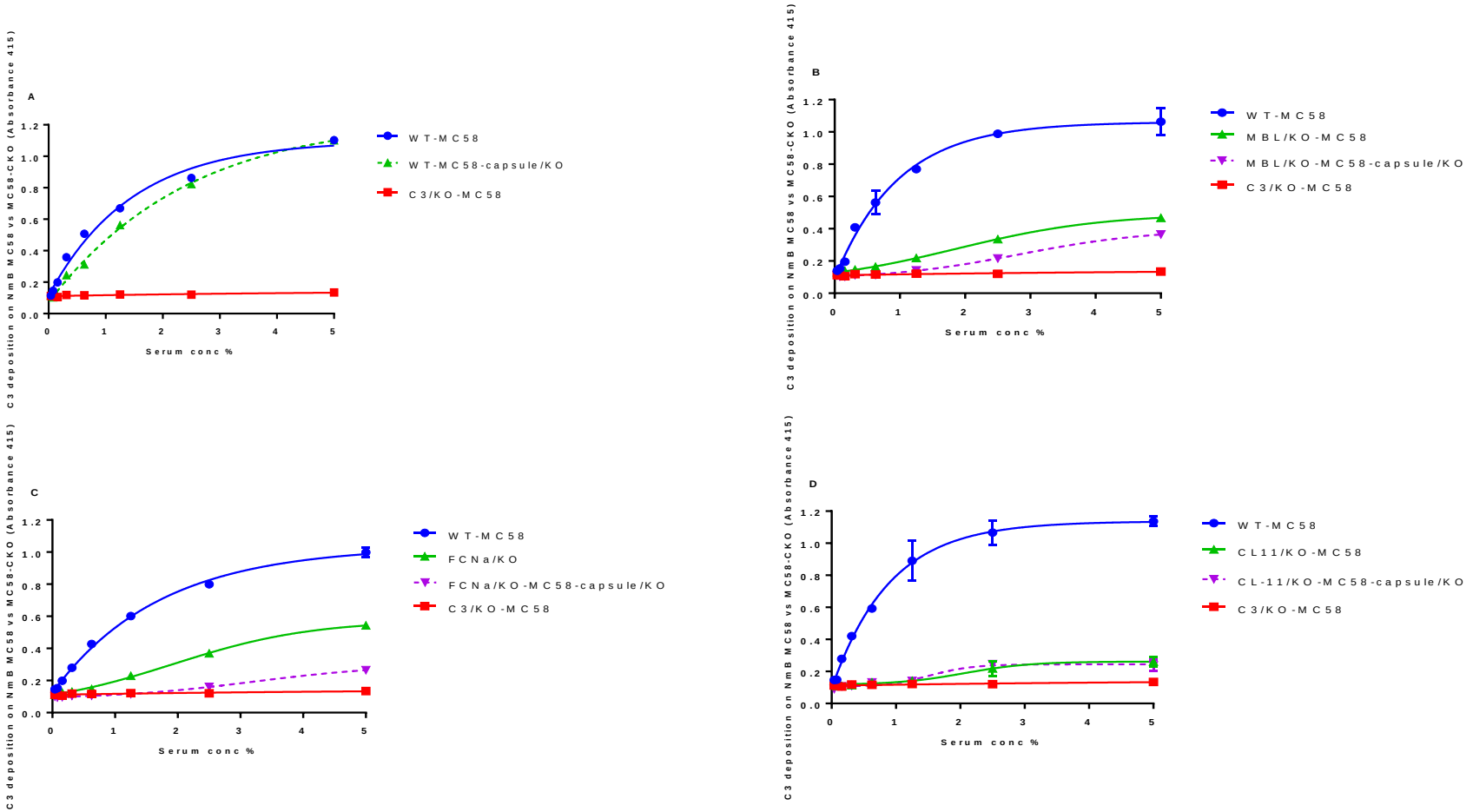

Figure 4.25: C3 deposition on *N. meningitidis* genogroup B (MC58 and MC58-capsule/KO) using different types of mouse sera of concentrations up to 5% (v/v)

Microtitre plates were coated with formalin-fixed *N. meningitidis* (or mannan as a positive control) then blocked with BSA. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. C3/KO mouse sera were used as the negative control, the binding of the C3 cleavage product (C3c) was detected by using rabbit anti-human C3c polyclonal antibody. Using WT serum, capsule makes no significant difference compared to non-capsulated strain ($P>0.05$). Furthermore, using MBL/KO and FCNa sera, there is a significant drop of C3 deposition ($P<0.05$). Using CL-11 has dropped the C3 deposition to nearly negative. In regard to capsule, CL-11 binds to capsule better than other cell wall ligands. Both ficolin-A and MBL have no role in binding to capsule. The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

To confirm the role of capsule in the recognition of *N. meningitidis* by the complement system, 2.5% (v/v) of standard pooled serum from 8 human volunteers and 1% (v/v) of 40 samples from neonate controls and therefore more likely to be immunologically naive. These samples ability to drive C3b on capsule were tested on MC58 and MC58-capsule/KO. Microtitre plates were coated with formalin-fixed *N. meningitidis* then blocked with BSA that was used as the negative control. 2.5% (v/v) of standard pooled human serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. 1% (v/v) of volunteer's samples was added in duplicates. The binding of the C3 cleavage product (C3c) was detected by using rabbit anti-human C3c polyclonal antibody. Standard curves of C3 deposition using standard pooled serum were created on both strains as shown in Figure 4.26. The result in Figure 4.27 confirms the previous findings by showing that MBL is not essential to drive C3 activation on *N. meningitidis* (MC58). Also, activation on the encapsulated MC58 strain is slightly, but not significantly (P>0.05) higher than on the capsule knockout, showing that the capsule of MC58 plays a minor role in the recognition of *N. meningitidis*.

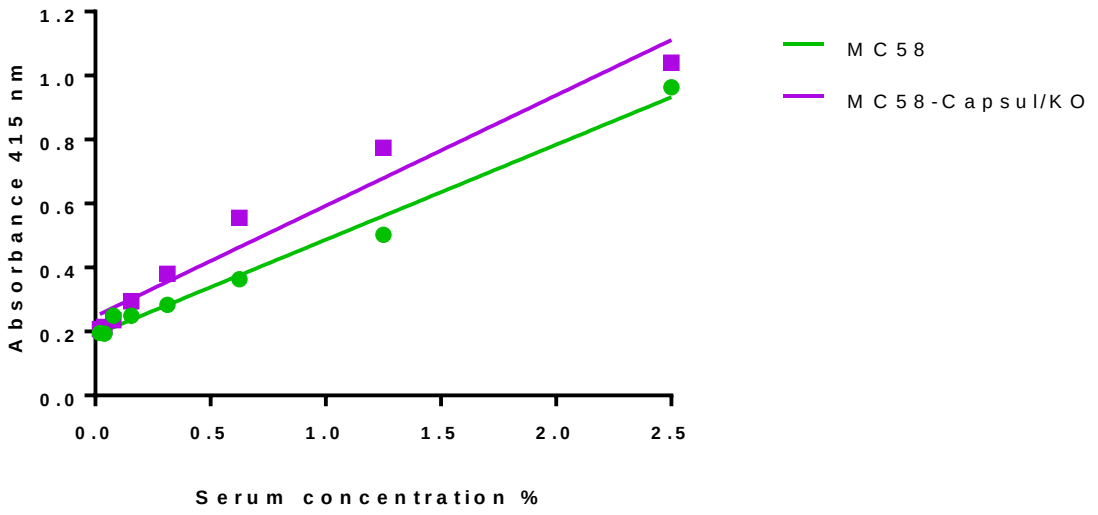

Figure 4.26: C3 deposition of Pooled NHS standard on MC58 and MC58-capsule/KO
Using 2.5% (v/v) standard pooled serum on both strains, activation on the encapsulated MC58 strain is slightly, but not significantly (P>0.05) higher than on the capsule knockout, showing that the capsule plays a minor role in the recognition of *N. meningitidis.*

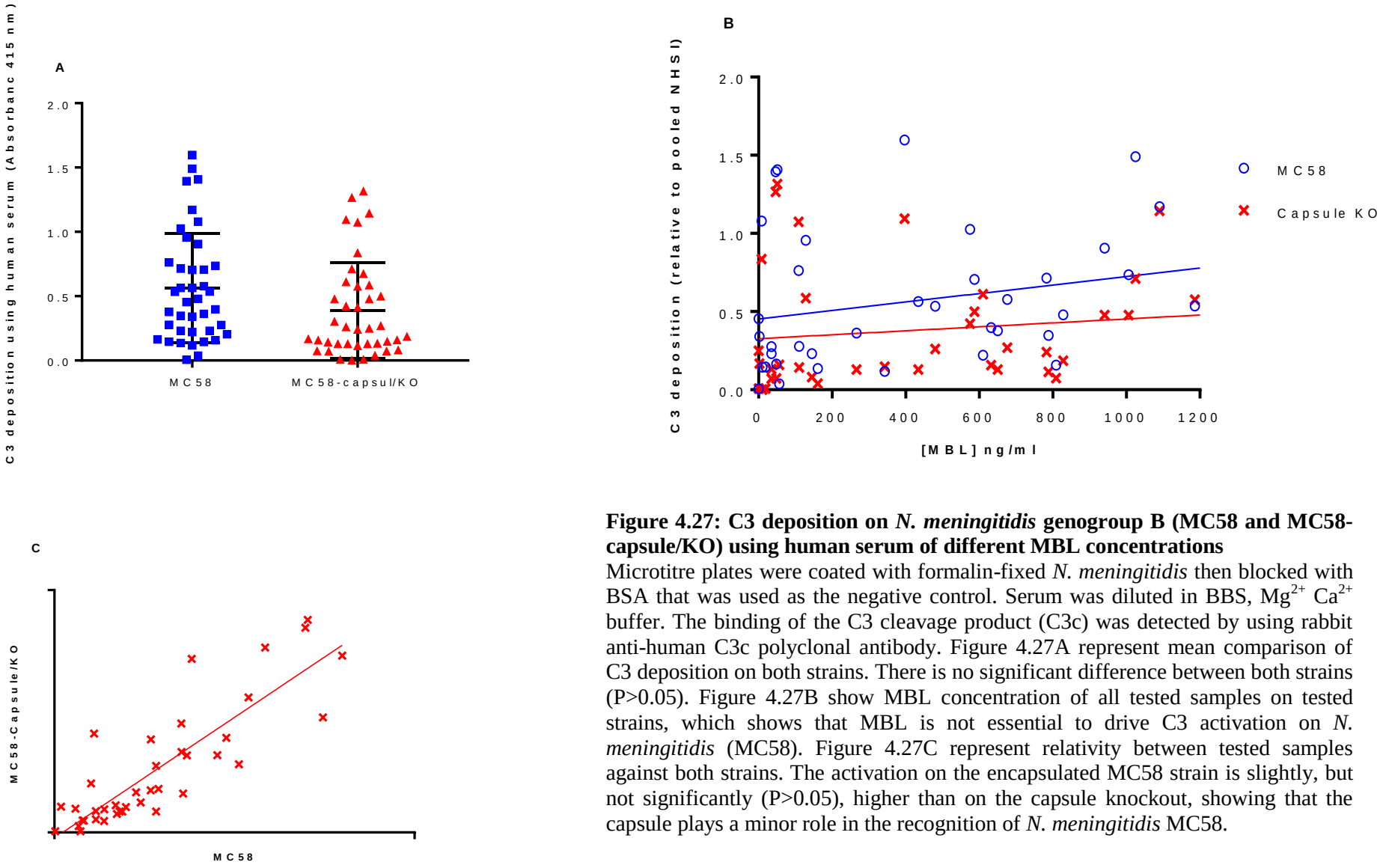

Figure 4.27: C3 deposition on *N. meningitidis* genogroup B (MC58 and MC58-capsule/KO) using human serum of different MBL concentrations

Microtitre plates were coated with formalin-fixed *N. meningitidis* then blocked with BSA that was used as the negative control. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. The binding of the C3 cleavage product (C3c) was detected by using rabbit anti-human C3c polyclonal antibody. Figure 4.27A represent mean comparison of C3 deposition on both strains. There is no significant difference between both strains ($P>0.05$). Figure 4.27B show MBL concentration of all tested samples on tested strains, which shows that MBL is not essential to drive C3 activation on *N. meningitidis* (MC58). Figure 4.27C represent relativity between tested samples against both strains. The activation on the encapsulated MC58 strain is slightly, but not significantly ($P>0.05$), higher than on the capsule knockout, showing that the capsule plays a minor role in the recognition of *N. meningitidis* MC58.

Previous results showed that capsule makes little difference in C3 deposition overall. To check the capsule role with MAC formation, C5b-9 deposition was tested on MC58 and MC58-capsule/KO strains. As can be seen from Figure 4.28, the capsule has no impact on MAC formation as there is no significant difference (P>0.05) between encapsulated and non-capsulated strains.

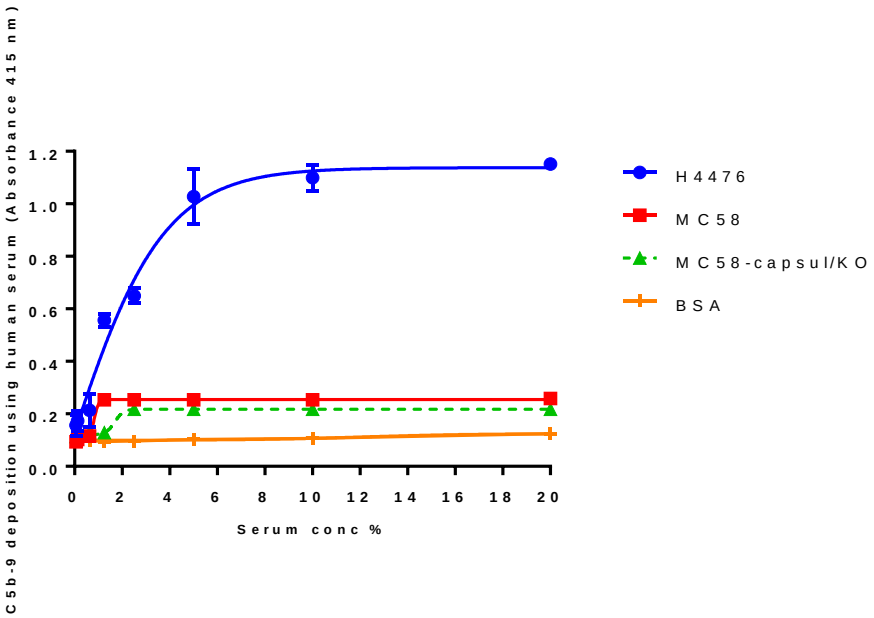

Figure 4.28: C5b-9 deposition on *N. meningitidis* genogroup B (MC58 and MC58-capsule/KO) using different normal human serum concentrations up to 5% (v/v)
Microtitre plates were coated with formalin-fixed *N. meningitidis* (or *N. meningitidis* strain H44/76 as a positive control) then blocked with BSA that was used as the negative control. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. The binding of the C5b-9 (MAC) was detected by using mouse anti-human C5b-9 antibody. Capsule does not affect the amount of MAC formation on MC58. The data are presented as a mean of biological and technical quadruplication +/- SEM. Curves were plotted using sigmoidal non-linear regression.

As the presence of capsule makes little difference overall, and the outer membrane proteins (OMP) variation show no differences in C3 deposition, I suggest that cell wall polysaccharides are the main ligands.

4.4. MBL and L-ficolin deficiency in a human volunteer

During the preliminary characterisation of human sera, volunteer 2 was found to have unique complement system component levels. As described in section 2.2.1.5, he had significantly low binding of mannan by MBL, no binding of N-acetylated BSA detected using anti-L-ficolin antibody and normal binding on zymosan detected with anti-CL-11 antibody (Figure 2.1). Interestingly, his serum was able to kill *N. meningitidis* (MC58) to the same extent as normal human serum in a serum bactericidal assay (SBA), as shown in Figure 4.29.

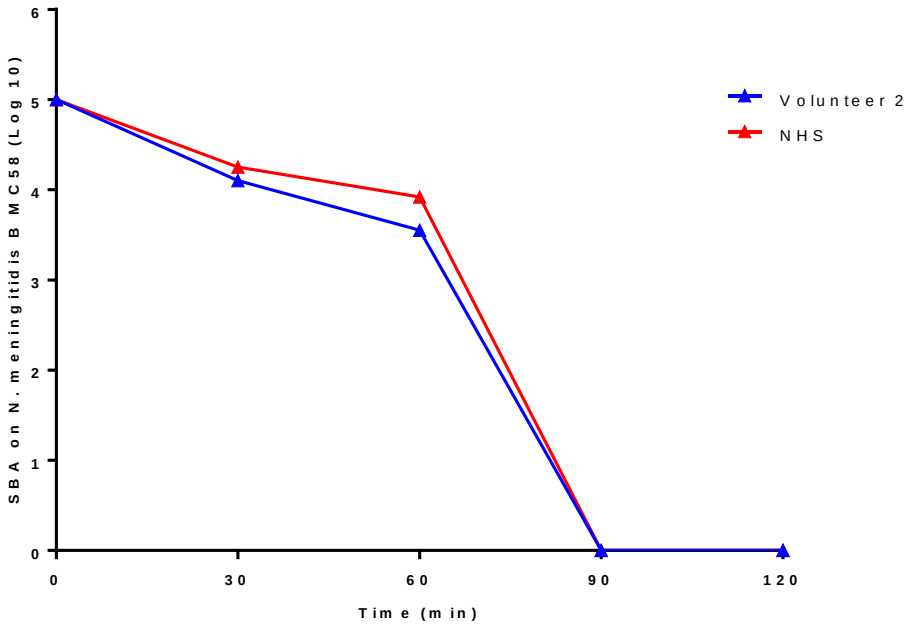

Figure 4.29: SBA on *N. meningitidis* genogroup B (MC58) using 20% (v/v) human serum. Serum Bactericidal Activity using human sera against *N. meningitidis* serogroup B (MC58). Bacteria and sera (20% serum concentration) were incubated at 37°C with shaking. Samples were taken at time points 0, 30, 60 And 90 minutes, plated out and the recoverable viable bacterial count was calculated. It was shown that volunteer 2 was able to kill the microbe in the same time that NHS did and no significant (P>0.05) difference between both volunteers.

Furthermore, Volunteer 2 had not been vaccinated against *N. meningitidis* genogroup B, as confirmed by ELISA (Figure 3.2). It was concluded that this volunteer's serum would be useful in determining the role of recognition molecules in the activation of LP and AP by *N. meningitidis*. To test the binding functionality of his L-ficolin, a sandwich ELISA was done by coating microtitre ELISA plates with rabbit Ficolin 2 polyclonal antibody (pAb) (biorbyte) in coating buffer and using volunteer 2 and control serum in conjunction with anti-human L-ficolin antibody (see section 2.2.1.5). No significant (P>0.05) binding to L-ficolin pAb was detected, as shown in Figure 4.30, whereas significantly (P<0.05) extensive binding was seen with normal serum.

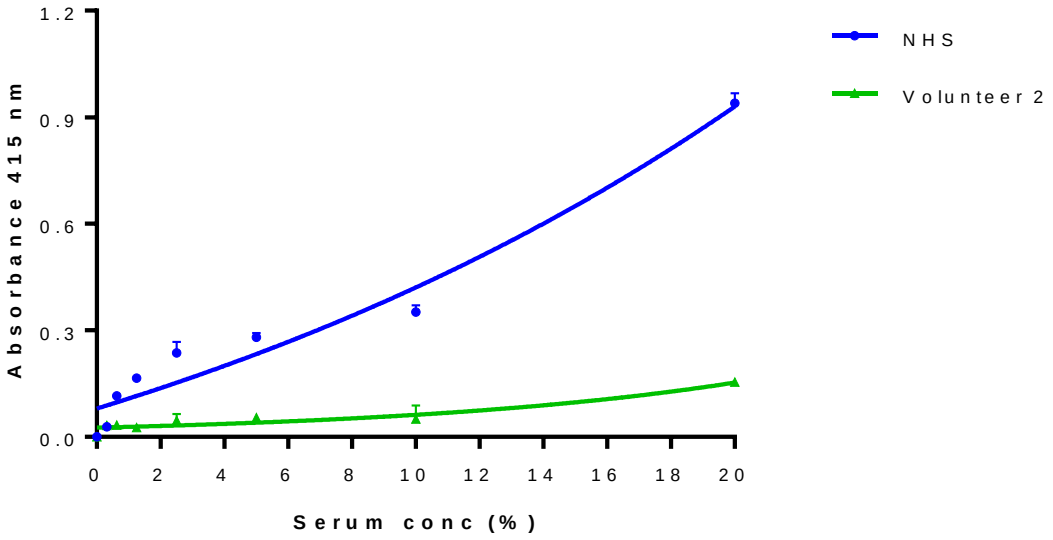

Figure 4.30: Sandwich ELISA using 20% (v/v) human serum to bind L-ficolin molecule using polyclonal and monoclonal antibody
Microtitre plates were coated with L-ficolin pAb then blocked with BSA. Serum was diluted in BBS, Mg^{2+} Ca^{2+} buffer. The binding of the L-ficolin was detected by using mouse anti-human L-ficolin mAb. Volunteer 2 showed significant low binding (P<0.05) compared to normal human serum. The data are presented as a mean of technical duplicates +/- SEM. Curves were plotted using sigmoidal non-linear regression.

To investigate the ficolin deficiency further, genotyping for his *FCN2* gene was done. Both volunteer and normal human were of similar *FCN2* bands size, as shown in Figure 4.31.

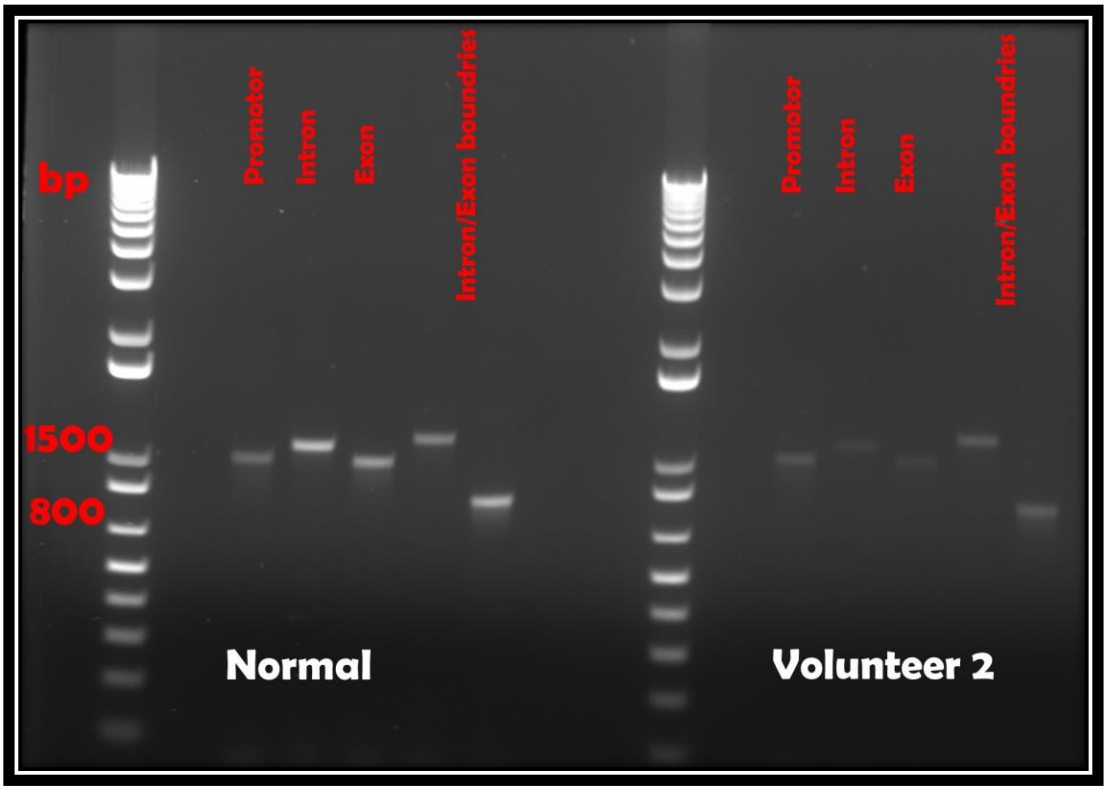

Figure 4.31: Genotyping of human *FCN2* gene
Genotyping results showed the amplified products, which was run with the PCR samples in a 1% agarose TAE-gel. There is no significant difference between NHS and volunteer 2. Bands represent the promotor, the exons, as well as the intron/exon boundaries and smaller introns

Studies showed that polymorphisms in *FCN2* gene promoter were associated with significant changes in the serum concentration of Ficolin-2 (Hummelshoj *et al.,* 2005). Hummelshoj *et al* also showed two polymorphisms in the exon encoding the fibrinogen-like domain (exon 8) were associated with the change in binding ability to GlcNAc (N-Acetylgalactosamine) (Hummelshoj *et al.,* 2005).

To study volunteer 2 findings, his *FCN2* gene sequencing was done by The Protein Nucleic Acid Chemistry Laboratory (PNACL) at The University of Leicester. The sequencing showed that the putative L-ficolin of volunteer 2 has a mutation located in exon 8 (*T236M*), the same mutation describes by Hummelshoj *et al*. Polymorphism of *T236M* was found to be associated with decreased binding ability to acetylated residues. However, this mutation was found to has no effect on serum concentration (Hummelshoj

et al., 2005). The sequence also had an insertion in the 3′ untranslated regions (UTR) of exon 8, which might affect mRNA stability, but it seems unlikely that it would lead to complete ablation of serum L-ficolin. Otherwise the DNA is identical to the normal one, with no detected changes in the exonic regions, the intron/exon boundaries or the promotor. These findings suggest that volunteer 2 L-ficolin polymorphism either caused an effect on his serum concentration level of L-ficolin or the technical limitation caused the inability to detect his L-ficolin. However, this polymorphism has decreased his L-ficolin binding ability. All the findings lead to the suggestion that volunteer 2 CL-11 is the only recognition molecule that could drive complement activation which led to *N.meningitidis* killing. However, this is yet to be confirmed.

4.5. Discussion

Many published works have illustrated that complement has an important role in fighting against *N. meningitidis*. However, the recognition of different *N. meningitidis* strains by the lectin pathway recognition molecules of complement still needs to be assessed. By using different types of sera, my study could identify which clonal complexes of meningococcal strains are susceptible to complement attack via MBL recognition. MBL/KO serum show significant ($P<0.05$) loss of C3 deposition on all *N. meningitidis* genogroup Y of all strains tested. However, this doesn't apply on strains of genogroup B, as C3 deposition is still present even in the absence of MBL as showed in Table 4.11 to Table 4.16 and Figure 4.10 to Figure 4.15. Yet, no study for the relation of *N. meningitidis* and ficolins has been published. In my study, I focused on the relation of ficolins in the recognition of different strains of different clonal complexes. Overall, ficolin showed a role in driving the complement LP by recognising N222.1 strain of genogroup Y of clonal complex (cc23). There is no published study yet that address the role of CL-11 in fighting *N. meningitidis*. In my study, I found that CL-11 is crucial for driving C3 deposition on different clonal complexes of genogroup B, especially, MC58, as deficiency of CL-11 in CL-11/KO serum reduced the C3 deposition significantly ($P<0.05$) as shown in Figure 4.10. Also, CL-11 deficiency in CL-11/KO mouse serum results in a significant ($P<0.05$) drop in C3 deposition on capsule of MC58 strain. Neither MBL nor ficolin-A could overcome that deficiency.

To assess the role of the capsule in the binding of the LP recognition molecules, capsule deficient MC58 strain of *N. meningitidis* genogroup B was compared to wild type MC58. The result confirms that MBL is not essential to drive C3 activation on *N. meningitidis* (MC58) as showed in Figure 4.25B and Figure 4.27. That is also applied on ficolin-A as in Figure 4.25C. CL-11 is the major recognition molecule that recognised MC58 capsule (Figure 4.25D). Using WT serum, there was no difference in C3 deposition on MC58 either with or without capsule (Figure 4.25A). In my phase variation study on 4 strains of *N. meningitidis* genogroup B, which has variation in PorA VR2, and 4 strains of genogroup Y, which has a variation in PorA VR1, and found no significant ($P>0.05$) differences in C3 deposition among strains of both genogroups (Figure 4.22 and Figure 4.23).

Furthormore, with few exeptions, I found consistency between C3 deposition and MAC formation on *N. meningitidis* strains tested as showed in Table 4.23. However, two strains of genogroup B, i.e. MC58 and 20342, have shown significant ($P<0.05$) low MAC formation but high C3 deposition (Figure 4.1, Figure 4.3, Figure 4.16 and Figure 4.18), suggesting an evasion mechanism that acts, downstream of the C3 convertase. Further discussion will be in chapter 6.

5. Chapter 5: *In-vivo* studies

The *in-vitro* studies showed that the LP and AP are crucial in fighting *N. meningitidis*. MASP-2 is the key to activate the LP, as it cleaves C4 and C2 to form C4bC2a (the LP C3-convertase). Blocking MASP-2 will therefore inhibit the activity of the LP. Moreover, MASP-3 was found to be the key to activate the AP, as it converts pro-factor D to its active form, factor D. Factor D is the protein responsible of cleaving factor B in C3bB complex to form C3bBb (the AP C3-convertase). Hence, blocking MASP-3 will result in the inhibition of the AP. Anti-MASP-2 and anti-MASP-3 are used in some clinical trials to control the inflammatory consequences of autoimmune diseases, such as aHUS and PNH. Blocking these pathways might render the patients susceptible to meningococcal infection. That led to *in-vivo* infection experiments in pre-treated mice with inhibitory antibodies, either anti-MASP-2 or anti-MASP-3. Also, by vaccinating animals, these studies will show if and to what extent the CP can overcome the defects in any of the other pathways. As MC58 is the major strain that causes invasive disease in UK, it was chosen to be the experimental model.

5.1. Dosing study

Dosing study is important in order to determine the proper dose to be used in the study. The infectious dose must have an impact of 50% mortality, so protection and susceptibility effects can be observed. To assess the virulence of *N. meningitidis*, three different dosage, i.e. ($1.6X10^7$, $1.6X10^6$ and $4X10^5$ CFU/mouse), were given to three groups of C57BL/6 wildtype mice, each has five mice (n=5). As shown in Figure 5.1, the group of high dose ($1.6X10^7$ CFU/mouse) couldn't survive more than 9 hours. Eighty percent (80%) of the second group of moderate dose ($1.6X10^6$ CFU/mouse), died at the same time of the first group. The other 20% died 12 hours post-infection. The third group of lowest dose ($4X10^5$ CFU/mouse) showed variation in mortality time post-infection, in which, 40% died in 9 hours, 20% in 12 hours, 20% in 19 hours and 20% in 24 hours. Based on these data, doses were chosen for the subsequent experiments.

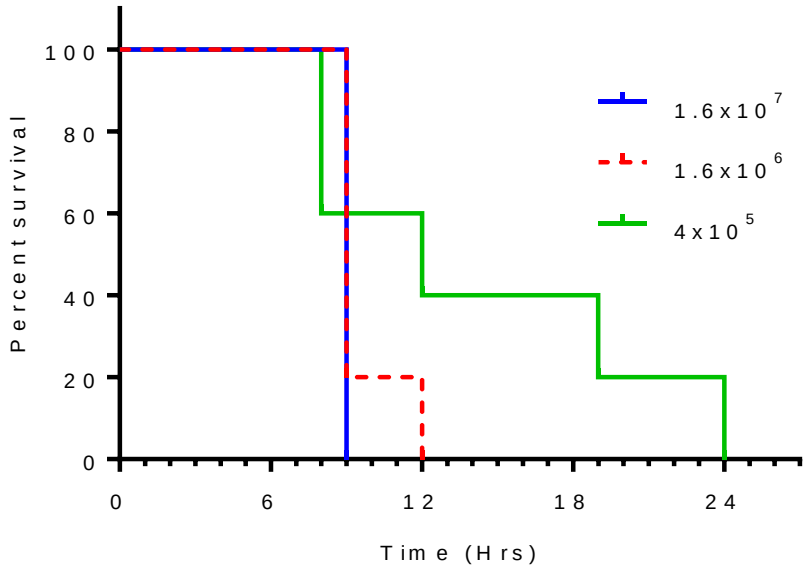

Figure 5.1: Virulence of *N. meningitidis* three different dosages
Survival time was corresponding to the administrated dose in three groups of C57BL/6 wild type mouse (n=5).

5.2. Infecting mice treated with complement components inhibitory antibodies with *N. meningitidis*

Based on the dosing study, a dose of $1X10^6$ CFU/mouse was chosen for the vaccinated group and a dose of $2X10^5$ CFU/mouse was chosen for the non-vaccinated group to achieve 50% mortality in each group. As shown in Figure 5.2, vaccination could increase survival of the same dose up to 40% comparing dosing study of $1X10^6$ CFU/mouse to vaccinated and treated mice with isotype control. Treating mice with anti-MASP-2 (OMS 721-HG4) antibody increased the protection of mice. I suggest that is because the CP with the AP could overcome the blocking of lectin pathway. Moreover, treating mice with anti-MASP-3 (13b1) was even more protective. I suggest that is due to the limitation of the inflammation, beside the functional LP and CP. Nevertheless, statistically, the differences between survival rates are insignificant (P>0.05) as shown in Table 5.1.

Table 5.1: P value of vaccinated mice groups
P value comparison shows no significant difference between tested groups.

Groups	P value	Interpretation
Anti MASP-3 vs. Control	>0.05	Insignificant
Anti MASP-3 vs. Anti MASP-2		
Control vs. Anti MASP-2		

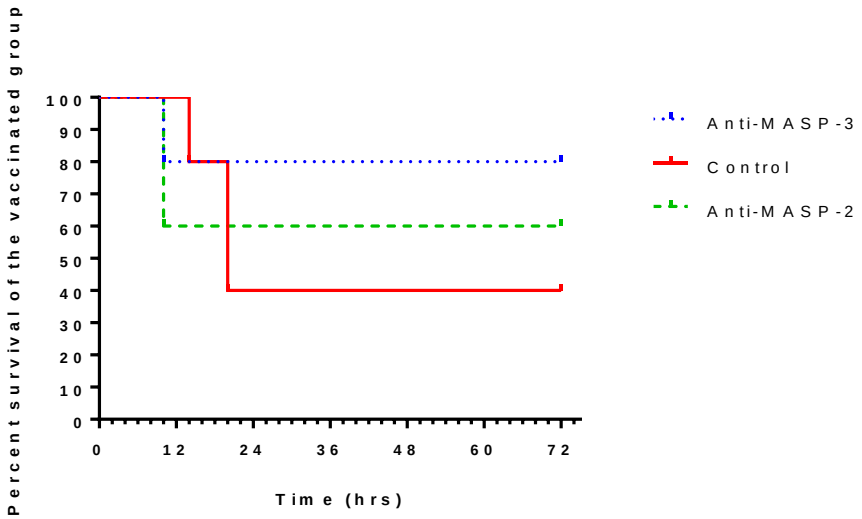

Figure 5.2: Survival of vaccinated and pre-treated mice with complement component inhibitory antibodies, which infected with $1X10^6$ CFU/mouse of *N. meningitidis*
The CP could overcome the blockage of either the LP or AP (n=10).

The CP is not functioning in the non-vaccinated groups, as there is no antigen-antibody complex, which is essential to the activation of the CP. Blocking the LP has dropped the survival percentage. There, there is evidence that in the absence of the CP, the LP is important. The blocking of the AP adds nothing, since blocking MASP-3 has no effect. I suggest that the LP could overcome the impaired function of the AP as shown in Figure 5.3. Nevertheless, statistically, the differences between survival rates are insignificant as shown in Table 5.2.

Table 5.2: P value of non-vaccinated mice groups
P value comparison shows no significant difference between tested groups.

Groups	P value	Interpretation
Anti MASP-3 vs. Control	>0.05	Insignificant
Anti MASP-3 vs. Anti MASP-2		
Control vs. Anti MASP-2		

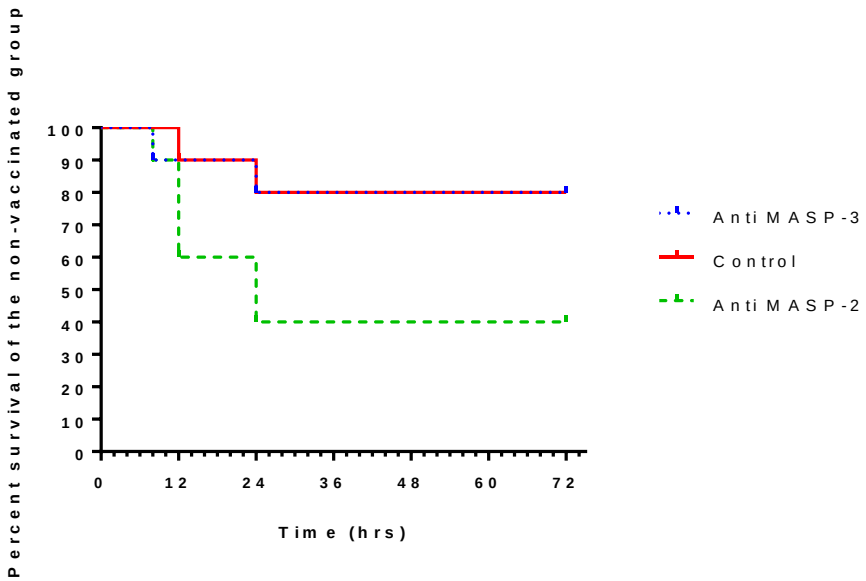

Figure 5.3: Survival of non-vaccinated and pre-treated mice with complement component inhibitory antibodies, which infected with 2X10^5 CFU/mouse of *N. meningitidis*
The inhibition of the LP decreased the survival percentage (n=10).

5.3. Discussion

The complement system is an important part of the immune system in fighting against meningococcal disease. As discussed earlier, the LP plays a crucial role in recognising and providing immediate killing against different strains of *N. meningitidis*. In my study, I treated different groups of mice with inhibitory antibodies, i.e. anti-MASP-2 and anti-MASP-3. Part of these groups was vaccinated with BEXSERO MenB vaccine. In the vaccinated group, pre-treated mice with anti-MASP-2 antibody (OMS721-HG4) showed increasing in protection against a lethal dose of *N. meningitidis* MC58 ($1X10^6$ CFU/mouse) (Figure 5.2). Perhaps that is because of the CP with the AP could overcome the blocking of lectin pathway by producing sufficient classical pathway C5 convertase (C4b2aC3b). In the non-vaccinated group, the CP is not functioning as there is no antigen-antibody complex, which is mainly essential to the activation of the CP. Although the infection dose was less than of vaccinated group ($2X10^5$ CFU/mouse), blocking the LP decreased the survival percentage (Figure 5.3). This is in line with my *in-vitro* studies, which showed that the deficiency of the LP recognition molecules led to decrease the C3 deposition on MC58.

In my study also, the vaccinated and pre-treated mice with anti-MASP-3 antibody (13b1) showed increase in protection against a lethal dose of *N. meningitidis* MC58 ($1X10^6$ CFU/mouse), perhaps the blocking of MASP-3 only, unlike MASP-1/3 KO, has bestowed in favour of the LP to provide higher response than depending on MASP-2 only (Figure 5.2). In the non-vaccinated group, infecting pre-treated mice with anti-MASP-3 antibody (13b1) with a lower dose of MC58 ($2X10^5$ CFU/mouse), showed no significant ($P>0.05$) difference compared to the control group (Figure 5.3). Perhaps this is due to the limitation of the inflammation. Further discussion will be in chapter 6.

6. Discussion and Conclusion

6.1. General Discussion

Many published works have illustrated that complement has an important role in fighting against *N. meningitidis.* However, the recognition of different *N. meningitidis* strains by the lectin pathway recognition molecules of complement still needs to be assessed. The LP activation takes place when one or more of the LP components, i.e. MBL, ficolin and CL-11, bind to pathogen-associated molecular patterns (PAMPs) on the pathogens (Schwaeble *et al.,* 2011). MBL is composed of three identical polypeptide chains. Each contains an N-terminal collagen-like domain linked by a neck region to a globular head part. That globular part contains the carbohydrate recognition domain (CRD) (McMullen *et al.,* 2006). Ficolins are composed of three of identical polypeptide chains. Each consists of a short N-terminal cysteine-rich domain with many cysteine residues, a collagen-like domain, a neck region and a fibrinogen-like carbohydrate recognition domain (FBG) (AmericanAcademyofPediatrics, 1996, Endo *et al.,* 2005, Garred *et al.,* 2010, Lynch *et al.,* 2004, Matsushita *et al.,* 2001). Collectin-11 is composed of an N-terminal domain, collagen-like domain, neck domain and a carbohydrate recognition domain (CRD). As a recognition molecule of the LP, CL-11 binds to D-mannose and L-fucose terminal saccharides on the microbe's surface and can interact with plasma MASP-1 and MASP-3 (Hansen *et al.,* 2010).

Serum MBL level in humans is different among individuals. This difference is age, gender and physical activity, independent (Ytting *et al.,* 2007). It was found that from 5 to 30% of the general population are deficient in MBL protein, according to ethnicity (Heitzeneder *et al.,* 2012). However, MBL deficient individuals that have inherited MBL deficiency seem relatively unaffected by infectious disease (Dahl *et al.,* 2004, Heitzeneder *et al.,* 2012). In regards to meningococcal infection, a study by Dahl *et al* (2004) on Caucasian population found no evidence for significant differences in infectious disease or mortality in MBL-deficient individuals versus controls. Another study found that MBL is a dependent opsonin, which accelerates microbial phagocytosis and killing (Jack *et al.,* 2005). A third study by Dahl *et al* (2004) indicates that a

combined deficiency of both properdin and MBL increases the risk of infection with *N. meningitidis*, in which, two out of six males with low MBL serum levels and also with undetectable properdin activity, had meningitis. This underlined the importance of epistatic genetic interactions in disease susceptibility (Bathum *et al.,* 2006). My results are consistent with these studies about the role of MBL in either opsonising or driving the killing to *N. meningitidis* certain strains (Jack *et al.,* 2001, Jack *et al.,* 2005). However, MBL needs CR1 to enhance phagocytosis and cannot activate it individually (Ghiran *et al.,* 2000, Jack *et al.,* 2005). By using different types of sera, my study could identify which clonal complexes of meningococcal strains are susceptible to complement attack via MBL recognition. MBL/KO serum show significant ($P<0.05$) loss of C3 deposition on all *N. meningitidis* genogroup Y of all strains tested. However, this doesn't apply on strains of genogroup B, as C3 deposition is still present even in the absence of MBL as showed in Table 4.11 to Table 4.16 and Figure 4.10 to Figure 4.15. This is again in line with what was found by Estabrook *et al* (2004) and Jack *et al* (1998) as they reported that MBL binding to *N. meningitidis* genogroups C and Y specifically to two outer membrane proteins, i.e. Opa and PorB.

Most normal healthy adults have serum L-ficolin within the range of 1000 to 6000 ng/mL. No value much below 1000 ng/mL has been detected in a healthy adult; therefore, absolute L-ficolin deficiency has not been shown to exist (Herpers *et al.,* 2006). Recent studies suggested that the insufficiency of L-ficolin might increase susceptibility to respiratory infections. In particular, protection from *Pseudomonas aeruginosa* (Kilpatrick and Chalmers, 2012). Yet, no study for the relation of *N. meningitidis* and ficolins has been published. In my study, I focused on the relation of ficolins in the recognition of different strains of different clonal complexes. Overall, ficolin showed a role in driving the complement LP by recognising N222.1 strain of genogroup Y of clonal complex (cc23).

Collectin-11 is expressed in plasma of normal humans in a very low concentration, of around 0.3µg/mL (Selman *et al.,* 2012, Yoshizaki *et al.,* 2012). Individuals with mutations in either *MASP1* gene or *COLEC11* gene encoding, display a wide spectrum of developmental disorders known as the 3MC syndrome (Carnevale, Mingarelli, Malpuech and Michels syndromes), which associated with inflammation/chemotaxis cascade in the aetiology of human developmental disorders (Rooryck *et al.,* 2011). Studies have defined

CL-11 as a novel initiator molecule of the lectin complement pathway activation upon recognition of microorganisms (Ma *et al.,* 2013). Another study found that CL-11 binds to *E. coli* and *P. aeruginosa*, whereas no significant (P>0.05) binding to *L. monocytogenes* has shown (Hansen *et al.,* 2010). There is no published study yet that address the role of CL-11 in fighting *N. meningitidis*. In my study, I found that CL-11 is crucial for driving C3 deposition on different clonal complexes of genogroup B, especially, MC58, as deficiency of CL-11in CL-11/KO serum reduced the C3 deposition significantly (P<0.05) as shown in Figure 4.10. This is consistent with other studies and findings, which all suggest the crucial role of CL-11 in the recognition of certain microorganisms (Hansen *et al.,* 2010). Also, CL-11 deficiency in CL-11/KO mouse serum results in a significant (P<0.05) drop in C3 deposition on capsule of MC58 strain. Neither MBL nor ficolin-A could overcome that deficiency.

The capsule of *N. meningitidis* has a well-known role in preventing bacteria from phagocytosis and providing resistance to CP attack (Uria *et al.,* 2008). A recent study demonstrated that B, C, W, and Y capsular polysaccharide inhibits the CP by inducing less C4b deposition, limiting the ability of antibodies to mediate bacterial killing, as meningococcal capsular polysaccharides of all of these groups, interfere with C4b deposition mediated by an anti-fHbp mAb (Agarwal *et al.,* 2014). Another study suggested that MBL can bind to encapsulated *N. meningitidis* serogroup C, particularly if the LOS is non-sialylated. It showed also that capsular polysaccharide and lipooligosaccharide glycan structure from *N. meningitidis* strains 7954, 7973, and 8026 of serogroup C, play critical roles in complement evasion meningococci to evade killing by human (Jack *et al.,* 2001). To assess the role of the capsule in the binding of the LP recognition molecules, capsule deficient MC58 strain of *N. meningitidis* genogroup B was compared to wild type MC58. The result confirms that MBL is not essential to drive C3 activation on *N. meningitidis* (MC58) as showed in Figure 4.25B and Figure 4.27. That is also applied on ficolin-A as in Figure 4.25C. CL-11 is the major recognition molecule that recognised MC58 capsule (Figure 4.25D). Using WT serum, there was no difference in C3 deposition on MC58 either with or without capsule (Figure 4.25A). This confirms the suggestions by Drogari *et al* (2002), who compared H44/76 strain to H44/76-capsule/KO, which are of genogroup B, and found no significant (P>0.05) differences in C3 deposition between both strains. He tested also C3 deposition on strains of PorA (+) and PorA (-), and found no significant (P>0.05) differences in C3 deposition (Drogari-

Apiranthitou *et al.,* 2002). These findings are also in line with my study on phase variation of 4 strains of *N. meningitidis* genogroup B, which has variation in PorA VR2, and 4 strains of genogroup Y, which has a variation in PorA VR1, and found no significant (P>0.05) differences in C3 deposition among strains of both genogroups (Figure 4.22 and Figure 4.23).

Besides its role in the opsonisation and immune-attraction, C3b is the main key to form C5 convertases which will lead to MAC formation (Podack *et al.,* 1982). MAC forms pores on target cell surface that allows free diffusion of salts and metabolites in and out of target cell. When adequate pores form, the cell is unable to survive any longer (Podack *et al.,* 1982). In humans, C5 is expressed in plasma in concentration, of 75µg/mL (Ram *et al.,* 2010). Many studies have illustrated the impact of C5 deficiency on immunity. About 50% of all persons deficient in a terminal complement component had invasive meningococcal disease (Figueroa and Densen, 1991), illustrating the importance of MAC formation in meningococcal killing. A Study by Drogari-Apiranthitou *et al* (2002), showed the differences of MAC formation between encapsulated and capsule/KO of H44/76 strain of genogroup B. He found high deposition on both strains with no significant (P>0.05) differences between them. In my study, H44/76 was used as a positive control in C5b-9 assays, as it showed the best curve fit during materials characterisation. Furthormore, with few exeptions, I found consistency between C3 deposition and MAC formation on *N. meningitidis* strains tested as showed in Table 4.23. However, two strains of genogroup B, i.e. MC58 and 20342, have shown significant (P<0.05) low MAC formation but high C3 deposition (Figure 4.1, Figure 4.3, Figure 4.16 and Figure 4.18), suggesting an evasion mechanism that acts, downstream of the C3 convertase.

To summarise, the *in-vitro* studies demonstrate the important role of the LP recognition molecules in driving C3 onto different strains of different clonal complexes of *N. meningitidis*. Although, all the recognition molecules work accumulatively to activate complement system, the results concluded that C3 deposition on genogroup Y was found to be more dependent on MBL than any other recognition molecule. Likewise, C3 deposition on genogroup B, either with or without capsule, was found to be of more of CL-11 dependant. The serogroup is not relevant for attack by the complement system, as the presence of capsule makes little difference overall and the capsule is permeable to the

LP recognition molecules and that cell wall polysaccharides are the main ligands. Also, the PorA of OMP has no impact on complement recognition, and finally, the evasion mechanism that acts on *N. meningitidis* is based on the C3 convertase.

As discussed earlier, the LP plays a crucial role in recognising and providing immediate killing against different strains of *N. meningitidis*. This recognition will activate mannan-binding lectin-associated serine proteases 1, 2, 3 (MASPs) (Dahl *et al.,* 2001, Matsushita and Fujita, 1992, Thiel *et al.,* 1997). MASP-1 cleaves C2, while MASP-2 cleaves C2 and C4 to form lectin pathway C3 convertase C4bC2a (Oroszlan *et al.,* 2016). Functional deficiency of MASP-2 is a primary immunodeficiency caused by homozygosity mutation in *MASP2* gene, which occurs in 1 out of 1700 individuals (Garcia-Laorden *et al.,* 2006, Garcia-Laorden *et al.,* 2008, Stengaard-Pedersen *et al.,* 2003). A study done by Stengaard-Pedersen *et al* (2003), found that in Caucasians, the functional MASP-2 deficiency is only found in individuals who have low levels of MASP-2, which cause an incapability of MASP-2 to be associated with MBL and ficolins. Other studies stated that only nine cases of MASP-2 deficiency have been reported so far (Cedzynski *et al.,* 2004, Garcia-Laorden *et al.,* 2006, Garcia-Laorden *et al.,* 2008, Olesen *et al.,* 2006, Sorensen *et al.,* 2005, Stengaard-Pedersen *et al.,* 2003, Stover *et al.,* 2005). Five of these cases did not show any symptoms, suggesting incomplete clinical penetrance of MASP-2 deficiency (Achtman, 1995, Garcia-Laorden *et al.,* 2006, Garcia-Laorden *et al.,* 2008, Stover *et al.,* 2005). However, these studies did not mention the vaccination status of those individuals and, if happened, the meningococcal strain that they dealt with. Also, a study by Schwaeble *et al* (2005) concluded that the inhibition of MASP-2 using relatively low doses of inhibitory antibody *in-vivo* may be therapeutically viable because of the strict absence of any extrahepatic MASP-2 biosynthesis and the relatively low abundance of MASP-2 in plasma. A recent study has successfully used anti-MASP-2 antibody *in-vivo* and showed that therapeutic application of MASP-2 inhibition reduced the morbidity and mortality associated with myocardial infarction (MI) with a functional CP and AP (Clark *et al.,* 2018). In my study, I treated different groups of mice with inhibitory antibodies, i.e. anti-MASP-2 and anti-MASP-3. Part of these groups was vaccinated with BEXSERO MenB vaccine. In the vaccinated group, pre-treated mice with anti-MASP-2 antibody (OMS721-HG4) showed increasing in protection against a lethal dose of *N. meningitidis* MC58 ($1X10^6$ CFU/mouse) (Figure 5.2). Perhaps that is because of the CP with the AP could overcome the blocking of lectin pathway by producing sufficient classical pathway

C5 convertase (C4b2aC3b). In the non-vaccinated group, the CP is not functioning as there is no antigen-antibody complex, which is mainly essential to the activation of the CP. Although the infection dose was less than of vaccinated group ($2X10^5$ CFU/mouse), blocking the LP decreased the survival percentage (Figure 5.3). This is in line with my *in-vitro* studies, which showed that the deficiency of the LP recognition molecules led to decrease the C3 deposition on MC58. Similar to that was suggested by other studies, which illustrate that MASP-2 deficient individual under certain conditions, show increase susceptibility to bacterial infection, especially when it is associated with immunocompromised conditions like HIV infection (Dahl *et al.,* 2004, Peterslund *et al.,* 2001, Sorensen *et al.,* 2005, Super *et al.,* 1989).

Another serine protease is MASP-3, which found to be the key to activate the AP, as it convert pro-factor D to factor D that is responsible of cleaving factor B in C3bB complex to form C3bBb (the AP C3-convertase). Hence, blocking MASP-3 will result in the inhibition of the AP. Individuals with mutations in *MASP1* gene display a wide spectrum of developmental disorders known as the 3MC syndrome (Carnevale, Mingarelli, Malpuech and Michels syndromes), which associated with inflammation/chemotaxis cascade in the aetiology of human developmental disorders (Rooryck *et al.,* 2011). The 3MC patients show no expression of MASP-3, the known key to activate the AP. Another study showed that MASP-1/3 deficient mouse has shown no functional AP (Sekine *et al.,* 2013). In my study, the vaccinated and pre-treated mice with anti-MASP-3 antibody (13b1) showed increase in protection against a lethal dose of *N. meningitidis* MC58 ($1X10^6$ CFU/mouse), perhaps the blocking of MASP-3 only, unlike MASP-1/3 KO, has bestowed in favour of the LP to provide higher response than depending on MASP-2 only (Figure 5.2). This is in line with what was demonstrated in recent studies, which suggested that MASP-1 is crucial in trans-activating MASP-2 under physiological circumstances, although MASP-2 was also reported to be sufficient itself, as a result of its ability to auto-activate C2 and C4 (Degn *et al.,* 2012, Takahashi *et al.,* 2008b). In the non-vaccinated group, infecting pre-treated mice with anti-MASP-3 antibody (13b1) with a lower dose of MC58 ($2X10^5$ CFU/mouse), showed no significant ($P>0.05$) difference compared to the control group (Figure 5.3). Perhaps this is due to the limitation of the inflammation.

To summarise, the *in-vivo* studies have shown the important role of vaccination in fighting MC58 via the CP. Therefore, I conclude that the CP can overcome the deficiency of either the LP or the AP. Nevertheless, inhibitory antibody being used in the treatment of complement-mediated autoimmune disease needs more investigation. That is to ensure its efficacy and safety profile against different strains of different microbes that known to be killed by the complement system.

6.2. Conclusion

The lectin pathway recognition molecules are crucial in driving C3 onto different strains of different clonal complexes of *N. meningitidis*. In general, these recognition molecules work accumulatively to activate complement system via the lectin pathway on *N. meningitidis.* On tested strains, MBL was found to be the major recognition molecule that drives C3b on *N. meningitidis* genogroup Y more than any other recognition molecule. Likewise, CL-11 was found to be the major recognition molecule that drives C3b on *N. meningitidis* genogroup B.

Neisseria meningitidis capsule is crucial for the survival of the bacteria in the blood (Uria *et al.,* 2008, Agarwal *et al.,* 2014). Tests were done on genogroup B capsule of (MC58) strain. The capsule of genogroup B is of α2-8-N-acetylneuraminic acid. Comparing encapsulated B strain with capsule knockout of the same strain has revealed that the presence of B strain capsule makes little but insignificant difference overall in C3b and C5b-9 deposition. Therefore it was concluded that the capsule is permeable to the lectin pathway recognition molecules and that cell wall polysaccharides are the main ligands.

In *Neisseria meningitidis,* the expression of several genes involved in host adaptation is a process known as phase variation (PV). Tested phase variant isolates in PorA from genogroup B and Y showed no significant difference ($P>0.05$) between C3 deposition using wild type mouse serum. This suggests that PV has no impact on complement recognition on tested strains.

Finally, studies on mice have revealed the important role of vaccination in fighting the infection of *N. meningitidis* (MC58) via the classical pathway even if either the lectin pathway or the alternative pathway is not functioning. Therefore, I conclude that the classical pathway can overcome the deficiency in any of the other complement pathways.

6.3. Future direction

With regard to the *in-vitro* work, the cause of strain to strain variation needs further study. This could be done using genetically modified strains of *N. meningitidis*, in which, each lacks an individual component of the outer membrane. Knowing which the ligand for each complement recognition molecule can answer the question of the cause of variation in interaction between Neisseria strains and complement. Also, testing other serogroups capsule role in the activation of the complement system is important, as it will give a clear idea about the specificity of the interaction between the complement system and different *N. meningitidis* encapsulated strains. That will help updating the treatment protocol if a genetic mutation, which might cause OMP evolution occur. Beside, many individuals are incapable to have the vaccine benefits, either for a medical situation, i.e. primary immune deficiency, or allergy to one of the vaccine components. Therefore, understanding the complement system exact behavior and its components specific ligands in different pathogenic *N.meningitidis* strains will help finding treatments for affected individuals. That will also help to understand the impact on infection and disease of inhibiting particular complement components; a therapeutic option for some inflammatory diseases, for example in treatment of complement mediated autoimmune diseases.

The *in-vivo* study needs confirmation. It also would be desirable to expand the inhibitory antibodies used to include those blocking the LP recognition molecules. This expansion will give a better understanding of the total immune response to the infection overall.

Also, further investigation of how volunteer 2's serum can kill *N. meningitidis* is warranted, for example, quantification of serum concentrations of (inactive) L-ficolin and CL-11. Purification of this L-ficolin would provide a reagent for the investigation of the basis of the lack of activity.

A recent unpublished study by Prof. Schwaeble showed that MASP-1/3 KO mice are psychologically instable. Therefore, another way to carry on from this point is to establish a psychological and immunological comparison between MASP-1/3 knockout mice and treated mice with MASP-1 and MASP-3 inhibitory antibodies.

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