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# Complement Factor H Related proteins and their biological role during bacterial infection

By:

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A thesis submitted in accordance with the requirements of The Open University for the  
degree of Doctor of Philosophy

The Open University

Faculty of Science

Department of Life, Health and Chemical Sciences

30-September-2016

## ABSTRACT

*Neisseria meningitidis* is a major cause of meningitis and sepsis worldwide despite available polysaccharide and protein vaccines. Individuals with rare defects in the terminal complement pathway are susceptible to meningococcal disease but further genetic factors that contribute to disease susceptibility are less well understood. A genome wide association study has linked polymorphisms in the Complement Factor H (CFH) locus with meningococcal disease in the general population. CFH is a negative regulator of the alternative complement pathway whereas CFH-related proteins (CFHR), encoded in the CFH locus, act as antagonists of CFH. Investigations of the biological role of the CFHRs have been hampered by lack of reagents, therefore a panel of specific monoclonal antibody reagents to CFHR2-5 were generated and characterised.

Previously, *N. meningitidis* has been shown to bind CFH *via* a surface exposed lipoprotein, factor H binding protein (fHbp), at high affinity. This study demonstrates that CFHR3 binds to the bacterial surface in a fHbp dependent-manner. Furthermore, CFHR3 competes with CFH for binding to fHbp. Bound CFHR3 increases susceptibility of *N. meningitidis* to complement-mediated lysis which was dependent on the sequence of the fHbp variant. The ability of *N. meningitidis* to evade the host immune system is likely to be determined by the relative levels of CFH and CFHR3 on the bacterial surface, providing a molecular mechanism for how variation in *cfhr3* may predispose individuals to meningococcal disease.

Furthermore this work demonstrates that *Neisseria cinerea*, which colonises the respiratory mucosa, expresses fHbp and binds CFH at similar affinities as meningococcal fHbp promoting bacterial survival in serum. The recently developed meningococcal vaccine, Bexsero<sup>®</sup>, includes fHbp as an antigen and antibodies elicited by Bexsero<sup>®</sup> are bactericidal against *N. cinerea* suggesting that the introduction of this vaccine could affect nasopharyngeal carriage of *N. cinerea*.

## Contributions and acknowledgements

The CFHR2 monoclonal antibody (mAb), used a positive control for screening of mAbs produced in Chapter 4, was provided by Professor Paul Morgan, University of Cardiff.

Recombinant CFH and CFHR CCP domains were produced in collaboration with Professor Susan Lea's laboratory, University of Oxford, by Dr. Joseph Caesar and Dr. Philip Ward. Antigens for the production of mAb produced in Chapter 4, were conjugated to carrier proteins (KLH) by Dr. Joseph Caesar.

Dr. Talat Malik and Professor Matthew Pickering from Imperial College, London, provided full length recombinant CFHR3 and CFHR5 and Dr. Elena Goicoechea De Jorge, also from Imperial College, London, provided  $\Delta CFHR3/CFHR1$  sera.

Work produced in Chapter 6 was conceived and designed by myself, but ELISA and SPR data analysing the binding affinity of *Neisseria cinerea* fHbp for CFH, and the surface localisation of *N. cinerea* fHbp (Figure 6.3 and 6.4) was performed by Katy Poncin a masters student from Belgium under my direct supervision. Data was analysed and interpreted by myself and Katy Poncin. pET-21b expression vector containing *N. cinerea* CCUG 346 T fHbp V1.110 and *N. cinerea* CCUG 346 T  $\Delta fHbp$  was generated by Katy Poncin under my direct supervision.

## Publications resulting from this study

Competition between antagonistic complement factors for a single protein on *N. meningitidis* rules disease susceptibility.

Caesar JJ\*, **Lavender H\***, Ward PN\*, Exley RM, Eaton J, Chittock E, Malik TH, Goiecochea De Jorge E, Pickering MC, Tang CM, Lea SM. (2014) *Elife*. Dec 23;3. DOI: 10.7554/eLife.04008

\* These authors contributed equally to this work

## Other publications not included in this study during the period of registration

(October 2013 - September 2016)

Structure and mechanism of a molecular rheostat, an RNA thermometer that modulates immune evasion by *Neisseria meningitidis*.

Barnwal RP, Loh E, Godin KS, Yip J, **Lavender H<sup>†</sup>**, Tang CM, Varani G. (2016) *Nucleic Acids Res*. Nov 2;44(19):9426-9437. DOI: 10.1093/nar/gkw584

<sup>†</sup> Contribution: Examination of the thermodynamic activity of the *Neisseria meningitidis* polysaccharide capsule, *CssA*, by mutagenesis of 5' untranslated region in a GFP reporter system in *Escherichia coli* (Figure 3).

Thermoregulation of Meningococcal fHbp, an Important Virulence Factor and Vaccine Antigen, Is Mediated by Anti-ribosomal Binding Site Sequences in the Open Reading Frame.

Loh E, **Lavender H<sup>†</sup>**, Tan F, Tracy A, Tang CM. (2016) *PLoS Pathog*. Aug 25;12(8):e1005794. DOI: 10.1371/journal.ppat.1005794.

<sup>†</sup> Contribution: Analysis of temperature-dependent fHbp expression in clinical isolates of *N. meningitidis* and evaluation of surface localised fHbp after growth at different temperatures (Unpublished data and Figure 1A).

## Abbreviations

$\beta$ 2-AR	$\beta$ 2-adrenergic receptor
aHUS	atypical haemolytic-uraemic syndrome
APS	Ammonium Persulfate
AMD	Age-related macular degeneration
AP	Alternative Pathway
BHI	Brain Heart Infusion
BSA	Bovine Serum Albumin
C3GN	C3 glomerulonephritis
C4bp	C4 binding protein
Carb	Carbenicillin
CCP	Complement Control Protein
CEACAMs	Carcinoembryonic antigen cell adhesion molecules
CFH	Complement Factor H
CFHL	Complement Factor H like
CFHR	Complement Factor H Related proteins
CNV	Copy Number Variation
CP	Classical Pathway
CR3	Complement Receptor 3
CRASPs	Complement regulator-acquiring surface proteins
CRP	C-reactive protein
DAMPs	Damage-associated molecular patterns
DEAP HUS	Deficiency of CFHR plasma proteins and Autoantibody Positive Haemolytic Uremic Syndrome
DDD	Dense Deposit Disease
Ecb	Staphylococcal extracellular complement binding protein

ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
Efb	Extracellular fibrinogen-binding protein
EGTA	ethylene glycol-bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
Erm	Erythromycin
Fab	Fragment antigen-binding
FACs	Fluorescence-activated cell sorting
FB	Factor B
FBA	Fructose-1,6-bisphosphate
FBS	Foetal Bovine Serum
FD	Factor D
FDA	Food and Drug Administration
FetA	Ferric enterobactin receptor A
FI	Factor I
fHbp	Factor H binding protein
GAG	Glycosaminoglycan
GHfp	Gonococcal Homologue of Factor H binding protein
GWAS	Genome Wide Association Study
HAT	hypoxanthine-aminopterin-thymidine medium
HI-NHS	Heat Inactivated-Normal Human Serum
Hib	<i>Haemophilus influenzae</i> type B glycoconjugate vaccine
HRP	Horseradish Peroxidase
HT	hypoxanthine -thymidine medium
IP	Intraperitoneal
IPTG	Isopropyl $\beta$ -D-1-thiogalactopyranoside

Kan	Kanamycin
KLH	Keyhole limpet hemocyanin
LB	Luria-Bertani
LP	Lectin Pathway
LPS	Lipopolysaccharide
mAb	Monoclonal Antibody
MAC	Membrane attack complex
MASPs	MBL-associated serine proteases
MBL	Mannan Binding lectin
MLEE	Multilocus Enzyme Electrophoresis
MLST	Multi Locus Sequence Typing
Msf	Meningococcal surface fibril
NadA	<i>Neisseria</i> adhesin A
NHBA	<i>Neisseria</i> heparin binding antigen
NHS	Normal Human Serum
NspA	Neisserial surface protein A
OMV	Outer Membrane Vesicles
Opa	Class five outer membrane opacity associated protein
Opc	Class five C outer membrane protein
ORF	Open Reading Frame
P	Properdin
pAb	Polyclonal Antibody
PAFr	Platelet Activating Factor receptor
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate Buffered Solution
PCR	Polymerase Chain Reaction



PFA	Paraformaldehyde
PRM	Pattern recognition molecule
PspC	Pneumococcal Surface Protein C
PTX3	Pentraxin related protein 3
PVDF	Polyvinylidene Fluoride
RCA	Regulators of Complement Activation
SAP	Serum amyloid P
SBA	Serum Bactericidal Activity
Sbi	<i>Staphylococcus aureus</i> immune evasion protein
SCR	Short Consensus Repeats
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SNP	Single Nucleotide Polymorphism
SPR	Surface Plasmon resonance
TEMED	Tetramethylethylenediamine
Tfp	Type four pili
TP	Terminal complement Pathway
WB	Western Blotting
WGS	Whole Genome Sequencing
YadA	<i>Yersinia</i> adhesin A

# Table of Contents

ABSTRACT.....	i
Contributions and acknowledgements .....	ii
Publications resulting from this study.....	iii
Abbreviations.....	iv
List of Figures .....	xiii
List of Supplementary Figures .....	xv
List of Tables .....	xvi
<b>1. INTRODUCTION .....</b>	<b>1</b>
1.1 The Complement System: Pathway overview.....	1
1.1.1 The Classical pathway.....	1
1.1.2 The Lectin pathway .....	2
1.1.3 The Alternative pathway .....	2
1.1.4 The Terminal complement pathway .....	4
1.2 Complement Factor H protein family.....	4
1.2.1 Complement Factor H .....	5
1.2.1.1 CFH Structure .....	6
1.2.1.2 Role in complement regulation.....	6
1.2.1.3 The role of CFH in discriminating self from non-self surfaces.....	7
1.2.1.4 Recruitment of CFH as a mechanism for complement evasion by bacterial pathogens .....	8
1.2.2 Complement Factor H Related proteins .....	11
1.2.2.1 CFHR1 .....	12
1.2.2.2 CFHR2 .....	12
1.2.2.3 CFHR3 .....	12
1.2.2.4 CFHR4 .....	13
1.2.2.5 CFHR5 .....	13
1.3 CFHRs and interactions with the Complement system .....	13
1.3.1 Interaction of CFHRs and the pentraxins .....	13
1.3.2 CFHR interactions with C3 and function .....	14

1.4 Association of CFHRs with human disease .....	15
1.5 CFHR interactions with bacterial pathogens .....	16
1.6 <i>Neisseria</i> .....	18
1.6.1 Commensal <i>Neisseria</i> .....	18
1.7 <i>Neisseria meningitidis</i> .....	19
1.7.1 Classification .....	19
1.7.2 Pathogenesis .....	20
1.7.3 Epidemiology .....	22
1.7.4 Natural immunity against <i>N. meningitidis</i> .....	23
1.7.5 Susceptibility to meningococcal disease .....	24
1.7.5.1 Complement deficiencies .....	24
1.7.5.2 Terminal complement inhibitors .....	25
1.7.5.3 Genome Wide Association Studies (GWAS) .....	25
1.7.6 Complement evasion by <i>N. meningitidis</i> .....	26
1.7.6.1 Surface structures that facilitate complement evasion .....	26
1.7.6.2 Acquisition of complement regulators .....	27
1.7.6.3 Protease activity against complement components .....	28
1.7.7 <i>N. meningitidis</i> vaccines .....	29
1.7.7.1 Correlates of protection .....	29
1.7.7.2 Polysaccharide vaccines .....	29
1.7.7.3 Challenges for meningococcal B vaccines .....	31
1.7.7.4 Recombinant protein vaccines against meningococcal B .....	31
1.7.7.5 Components of licensed meningococcal B vaccines .....	32
1.7.7.5.1 fHbp .....	32
1.7.7.5.2 NadA .....	33
1.7.7.5.3 NHBA .....	33
1.7.7.6 Improving immunogenicity of meningococcal vaccines .....	34
1.7.7.7 Conservation of meningococcal vaccine antigens .....	35
1.7.7.7.1 fHbp .....	36
1.7.7.7.2 NadA .....	36

1.7.7.3 NHBA .....	36
1.8 <i>Neisseria cinerea</i> .....	36
1.8.1 <i>N. cinerea</i> carriage .....	36
1.8.2 Infections caused by <i>N. cinerea</i> .....	37
<b>2. AIMS .....</b>	<b>38</b>
<b>3. MATERIALS AND METHODS .....</b>	<b>39</b>
3.1. Antibodies .....	39
3.2. Mouse immunisations .....	40
3.2.1 Generation of mAbs .....	40
3.2.2 Generation of immune sera .....	41
3.3. Monoclonal antibody production .....	41
3.3.1. Media and supplements for mAb production .....	41
3.3.2. Spleen preparation prior to cell fusion .....	42
3.3.3. Cell fusion .....	42
3.3.4. Cloning hybridomas by limiting dilution .....	43
3.3.6. Monoclonal antibody isotyping .....	43
3.3.7. Monoclonal antibody production and purification .....	44
3.4. Enzyme-linked immunosorbent assay (ELISA) .....	44
3.4.1 mAb screening .....	44
3.4.2. Evaluation of CFH binding .....	45
3.4.3. Evaluation of immunogenicity sera .....	45
3.5. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) .....	46
3.6. Western Blotting .....	47
3.6.1. mAb specificity screening .....	47
3.6.2. Detection of fHbp .....	48
3.6.3. Evaluation of CFH binding .....	48
3.7. Bacterial strains and growth .....	48
3.8. Antibiotics .....	50
3.9. Molecular Cloning .....	51

3.9.1. Genomic DNA preparation .....	51
3.9.2. Polymerase Chain Reaction (PCR) .....	51
3.9.3. Site directed mutagenesis .....	53
3.9.4. Bacterial transformation .....	54
3.9.5. Construction of complemented <i>N. meningitidis</i> and <i>N. cinerea</i> strains.....	54
3.9.6. Generation of fHbp expression constructs .....	55
3.9.7. Generation of <i>N. cinerea</i> CCUG 346 T $\Delta$ fHbp .....	56
3.10. Sequence analysis and model predication of <i>N. cinerea</i> fHbp .....	56
3.11. fHbp purification.....	56
3.12. Surface Plasmon Resonance (SPR) .....	57
3.13. Flow cytometry .....	57
3.13.1. Evaluation of CFHR3 and CFH binding .....	58
3.13.2 Competition assay .....	58
3.14. Bacterial serum survival assay.....	59
3.14.1. Evaluating the effect of CFHR3 on bacterial survival.....	59
3.15. Serum Bactericidal Assay (SBA) .....	60
<b>4. GENERATION AND CHARACTERISATION OF CFHR MONOCLONAL ANTIBODIES .....</b>	<b>61</b>
4.1. Introduction .....	61
4.2. Results.....	64
4.2.1. Generation of anti-human CFHR antibodies .....	64
4.2.2. Phase II screening: Cell fusion and screening of mAbs against the immunising antigen. 68	
4.2.3. Phase III screening: Determining the specificity of mAbs against CFH and CFHRs .....	68
4.2.4. Phase IV: Screening of anti-human CFHR mAbs cloned by limiting dilution .....	73
4.2.5. Production and purification of HSL-1 (Phase V).....	75
4.3. Discussion .....	77
<b>5. COMPETITION BETWEEN HOST MOLECULES INFLUENCES SUSCEPTIBILITY TO MENINGOCOCCAL DISEASE .....</b>	<b>83</b>
5.1. Introduction .....	83
5.2. Results.....	85

5.2.1. CFHR3 binds <i>N. meningitidis</i> in an fHbp-dependent manner.....	85
5.2.2. Development of an assay to investigate the effect of CFHR3 on bacterial survival.....	85
5.2.3. <i>N. meningitidis</i> susceptibility to complement mediated lysis is altered by the acquisition of CFHR3 .....	89
5.2.4. Generation of <i>N. meningitidis</i> isogenic strains expressing different fHbp variants .....	90
5.2.5. Development of Alternative Pathway serum assays to assess the functional consequences of CFHR3 binding .....	90
5.2.6. fHbp sequence affects strain sensitivity to complement mediated lysis after incubation with CFHR3 .....	94
5.2.7. CFHR3 competes with CFH for binding of <i>N. meningitidis</i> fHbp.....	95
5.3. Discussion .....	98
<b>6. CHARACTERISATION OF FHBP FROM NEISSERIA CINEREA AND POTENTIAL IMPLICATIONS OF VACCINATION AGAINST MENB.....</b>	<b>102</b>
6.1. Introduction .....	102
6.2. Results.....	105
6.2.1. <i>N. cinerea</i> fHbp is predicted to be structurally similar to meningococcal fHbp. ....	105
6.2.2. Functional characterisation of <i>N. cinerea</i> fHbp. ....	106
6.2.3. <i>N. cinerea</i> isolates express fHbp on the bacterial surface.....	109
6.2.4. Binding of CFH promotes complement resistance of <i>N. cinerea</i> .....	111
6.2.5. Mice immunised with Bexsero® elicit a serum bactericidal activity response which is comparable to mice immunised with fHbp alone.....	114
6.3. Discussion .....	119
<b>7. REFERENCES.....</b>	<b>126</b>
<b>8. SUPPLEMENTARY INFORMATION.....</b>	<b>162</b>
<b>9. Publication: Competition between antagonistic complement factors for a single protein on <i>N. meningitidis</i> rules disease susceptibility. Caesar JJ. <i>et al.</i> (2014) <i>Elife</i> .....</b>	<b>165</b>

## List of Figures

### 1. INTRODUCTION

Figure 1.1: Activation of CP and LP.....	2
Figure 1.2: Initiation of the AP.....	3
Figure 1.3: Activation of the TP.....	4
Figure 1.4: Homology of CFH and CFHR protein family.....	5
Figure 1.5: Regulation of the AP by CFH.....	7

### 4. GENERATION AND CHARACTERISATION OF CFHR MONOCLONAL ANTIBODIES

Figure 4.1: Available antibodies cross-react with other CFH family members.....	62
Figure 4.2: Schematic representation of mAb generation and identification.....	64
Figure 4.3: Peptides used to generated mAbs against CFHR4 and CFHR5 are surface localised.....	65
Figure 4.4: Schematic showing the generation of mAbs.....	66
Figure 4.5: Specificity of mAbs during Phase III-a screening.....	69
Figure 4.6: Specificity of novel mAbs during Phase III-b screening.....	70
Figure 4.7: Recognition of CFH and CFHR2-CFHR5 in NHS.....	72
Figure 4.8: Recombinant Fab fragment of HSL-1 retains specificity for CFHR3.....	76

### 5. COMPETITION BETWEEN HOST MOLECULES INFLUENCES SUSCEPTIBILITY TO MENINGOCOCCAL DISEASE

Figure 5.1: <i>N. meningitidis</i> binds CFHR3 on its surface in an fHbp-dependent manner.....	86
Figure 5.2: Optimisation of serum assays.....	88
Figure 5.3: CFHR3 increases susceptibility of <i>N. meningitidis</i> to complement-mediated lysis.....	89
Figure 5.4: Characterisation of isogenic <i>N. meningitidis</i> expressing different fHbps.....	91
Figure 5.5: Optimisation of AP assays.....	93
Figure 5.6: Survival of <i>N. meningitidis</i> in AP assays is affected by the fHbp sequence.....	95
Figure 5.7: CFH and CFHR3 compete for binding to fHbp.....	97

## 6. CHARACTERISATION OF FHBP FROM NEISSERIA CINEREA AND POTENTIAL IMPLICATIONS OF VACCINATION AGAINST MENB

Figure 6.1: Sequence alignment of <i>N. meningitidis</i> fHbp V1.1 with <i>N. cinerea</i> fHbp from ( <a href="http://pubmlst.org/">http://pubmlst.org/</a> ).....	105
Figure 6.2: <i>N. cinerea</i> fHbp is predicted to be structurally similar to <i>N. meningitidis</i> fHbp .....	107
Figure 6.3: <i>N. cinerea</i> fHbp binds CFH at high affinity .....	108
Figure 6.4: Functional fHbp is expressed by all <i>N. cinerea</i> strains examined.....	109
Figure 6.5: <i>N. cinerea</i> fHbp is present on the bacterial surface .....	110
Figure 6.6: <i>N. cinerea</i> recruits CFH to its surface in a fHbp-dependent manner .....	112
Figure 6.7: Serum survival of <i>N. cinerea</i> .....	113
Figure 6.8: CFHR3 increases susceptibility of <i>N. cinerea</i> to complement mediated lysis .....	114
Figure 6.9: Immunisation with Bexsero® elicits immune responses against <i>N. cinerea</i> .....	115



## List of Supplementary Figures

SI 1: <i>N. cinerea</i> fHbp is predicted to be structurally similar to <i>N. meningitidis</i> fHbp. ....	162
SI 2: CFHR3 <sub>(4,5)</sub> does not influence susceptibility of <i>N. cinerea</i> to complement mediated lysis.....	163
SI 3: Immunogenicity of individual mouse sera .....	163
SI 4: Optimisation of rabbit complement concentration for <i>N. cinerea</i> SBA assays. ....	164
SI 5: Characterisation of isogenic <i>N. cinerea</i> expressing the homologous fHbp. ....	164

## List of Tables

### 1. INTRODUCTION

Table 1.1: Recruitment of CFH by bacterial pathogens ..... 10

Table 1.2: Recruitment of CFHRs by bacterial pathogens..... 17

### 3. MATERIALS AND METHODS

Table 3.1: Antibodies ..... 39

Table 3.2: Summary of antigens used in the generation of mouse anti-human CFHR mAbs ..... 40

Table 3.3: Media and supplements used in the generation of mAbs ..... 42

Table 3.4: Reagents for the preparation of polyacrylamide gels ..... 46

Table 3.5: *N. meningitidis* and *N. cinerea* strains ..... 50

Table 3.6: Concentration of antibiotics ..... 50

Table 3.7: Primers ..... 53

Table 3.8: *Neisseria* complementation plasmids ..... 55

Table 3.9: Plasmids used for protein expression in *E. coli* ..... 55

### 4. GENERATION AND CHARACTERISATION OF CFHR MONOCLONAL ANTIBODIES

Table 4.1: Summary of the selection of mouse anti-human CFHR mAbs in each phase of production  
..... 67

Table 4.2: Summary of characterised mouse anti-human CFHR mAbs cloned by limiting dilution... 74

### 6. CHARACTERISATION OF FHBP FROM NEISSERIA CINEREA AND POTENTIAL IMPLICATIONS OF VACCINATION AGAINST MENB

Table 6.1: Serum bactericidal titres of pooled sera from mice immunised with recombinant fHbp,  
Bexsero® or control mice against *N. cinerea* strains..... 118

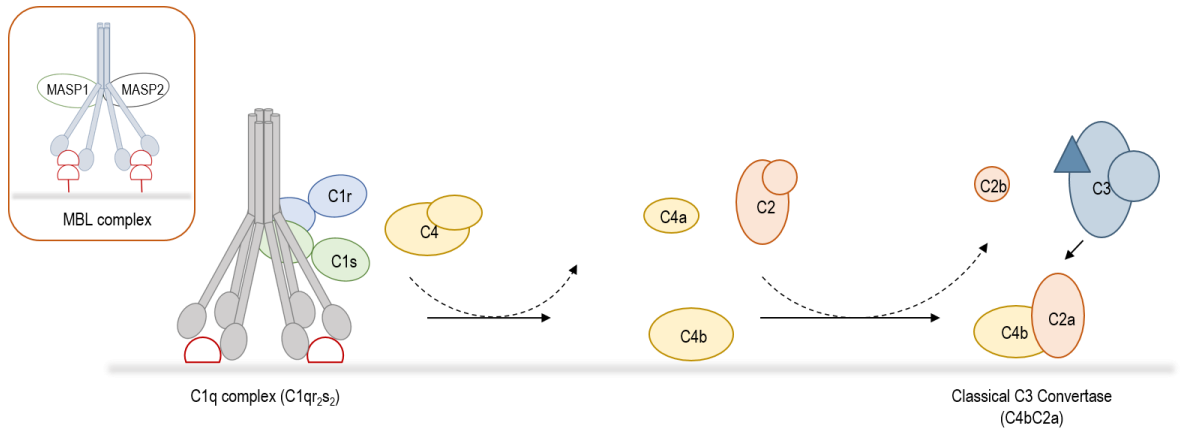
# 1. INTRODUCTION

## 1.1 The Complement System: Pathway overview

The Complement system is a crucial mechanism of host defence against pathogens and mediates both immunological and inflammatory responses [Dunkelberger, J. R. 2010]. Complement is able to discriminate self from non-self, eliminate cellular debris and apoptotic cells, and recognise foreign molecules including those on microbes [Medzhitov, R. 2002. Trouw, L. A. 2008. Ricklin, D. 2010]. The Complement system is comprised of over 50 fluid phase and membrane-associated proteins which are integrated in a tightly regulated proteolytic cascade, initiated through sequential cleavage of complement components and resulting in the activation of the innate immune system [Ricklin, D. 2010]. Complement activation can be initiated by three pathways: through the recognition of foreign surfaces (alternative pathway, AP), antibody:antigen complexes (classical pathway, CP) or pathogen glycans (lectin pathway, LP), which ultimately lead to lysis or phagocytosis of the complement target [Pangburn, M. K. 1981. Bindon, C. I. 1988. Nadesalingam, J. 2005. Ricklin, D. 2010].

### 1.1.1 The Classical pathway

The fluid phase pattern recognition molecule (PRM) C1q initiates the CP by direct binding onto the surface of a pathogen or apoptotic cell, or by recognising immune complexes formed by IgM or IgG binding to an antigen [Collins, C. 2002. Bindon, C. I. 1988.]. The CP can also be initiated by pentraxins, *e.g.* C-reactive protein (CRP), pentraxin related protein 3 (PTX3) or serum amyloid P (SAP), binding phosphorylcholine or phosphorylethanolamine on microbes or apoptotic cells [Szalai, A. J. 2002; Jiang, H. X. 1991. Schwalbe, R. A. 1992]. C1q is present in serum in a complex with C1r and C1s, C1qr<sub>2</sub>s<sub>2</sub> [Chen, C. H. 1998]. Binding of C1q results in cleavage of C1s by C1r, leading to the subsequent cleavage of C4, to C4a and C4b, by activated C1s [Lepow, I. H. 1963. Ziccardi, R. J. 1977. Bally, I. 2009]. The exposed thioester bond of C4b then covalently binds to the surface of the activating molecule, leading to opsonisation [Schreiber, R. D. 1974. Dodds, A. W. 1996]. Surface bound C4b recognises and binds C2 which is cleaved into C2a and C2b by C1s, [Müller-Eberhard, H. J. 1967. Nagasawa, S. 1977]. C2a remains bound by C4b to form the CP C3 convertase, C4bC2a, which cleaves C3 to activate the AP and the terminal complement pathway. Figure 1.1 summarises the CP.



**Figure 1.1: Activation of CP and LP**

The CP is initiated by the binding of the C1q complex; consisting of C1q (grey), two C1r molecules (blue) and two C1s molecules (green) to IgG or IgM on activating surfaces. Conformational changes in C1q result in the activation of the proteases C1r and C1s resulting in the cleavage of C4 by C1s. C4b is deposited on activator surfaces and binds C2 which is cleaved by C1s to C2a, that remains bound to C4b, and the C2b fragment is released. The C4bC2a complex is a C3 convertase that can cleave C3. The LP is initiated by the binding of the MBL complex (red box) to carbohydrates on microbes. The MBL complex is structurally similar to the C1q complex of the CP. MASP1 and MASP2 are functionally similar to C1r and C1s respectively, which results in cleavage of C4 and C2 to form the C3 convertase.

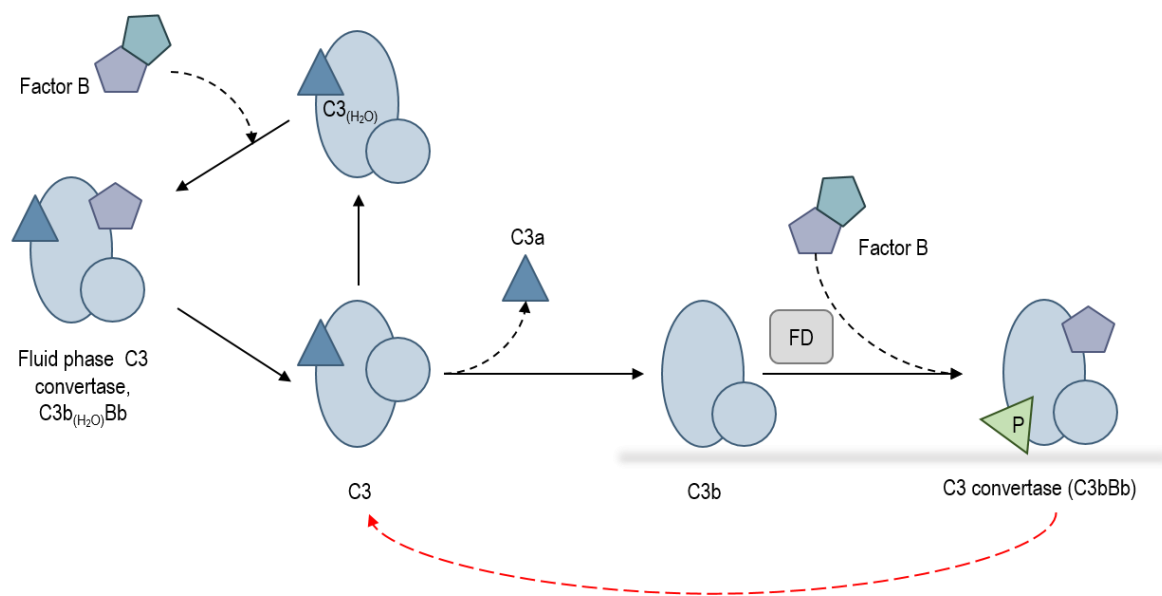
### 1.1.2 The Lectin pathway

The LP is functionally similar to the CP as it results in the formation of the same C3 convertase, C4bC2a [Degn, S. E. 2012]. The LP is initiated by the recognition of mannose or *N*-acetylglucosamine by Mannan Binding lectin (MBL) or acetylated carbohydrates on pathogens by ficolins. MBL or ficolins assemble with MBL-associated serine proteases (MASPs) into a complex similar to the C1 complex of the CP [Matsushita, M. 1992. Sato, T. 1994. Thiel, S. 1997. Nadesalingam, J. 2005]. MASP-1 and MASP-2 are functionally and structurally similar to C1r and C1s, respectively, with MASP-2 proteolytically cleaving both C4 and C2 leading to complement activation, whereas MASP-1 promotes the LP by specifically cleaving C2 [Héja, D. 2012. Degn, S. E. 2012].

### 1.1.3 The Alternative pathway

The formation of the CP convertase by the CP or LP results in the cleavage of C3 into C3a and C3b, which exposes a thioester moiety in C3b, that covalently binds to an available amine or carbohydrate group. This results in the attachment of C3b on the surface of the activating molecule [Fearon, D. T. 1975. Law, S. K. 1977. Pangburn, M. K. 1980. Sim, R. B. 1981. Fishelson, Z. 1984]. C3b then binds

Factor B (FB) which becomes susceptible to cleavage into Ba and Bb by the protease Factor D (FD) [Lesavre, P. H. 1978. Janssen, B. J. 2009. Hourcade, D. E. 2011]. Bb remains bound to C3b, forming the AP C3 convertase, C3bBb. The AP is also activated by C3 'tick-over' which results from spontaneous hydrolysis of C3 to C3<sub>H<sub>2</sub>O</sub>, leading to the generation of the soluble C3 convertase (C3<sub>H<sub>2</sub>O</sub>Bb) after binding and cleavage of FB [Pangburn, M. K. 1981]. The convertase is further processed to generate active C3b, leading to surface deposition of the complex [Hugli, T. E. 1975. Pangburn, M. K. 1981]. Further PRM-mediated activation of the AP can result from recognition of pathogen- or damage-associated molecular patterns (PAMPs or DAMPs, respectively) by properdin (P) which attracts C3b to targeted surfaces and also stabilises the AP C3 convertase, C3bBbP [Spitzer, D. 2007. Kimura, Y. 2008. Fearon, D. T. 1975.]. Once activated the AP acts as a positive amplification loop, accounting for 80-90% of total complement activation, regardless of the initiating pathway [Fung, M. 2001. Harboe, M. 2008]. The AP needs to be tightly regulated to avoid damage to host tissues and a number of proteins are involved in the negative regulation of the AP. Figure 1.2 summarises C3 tick-over and activation of the AP.

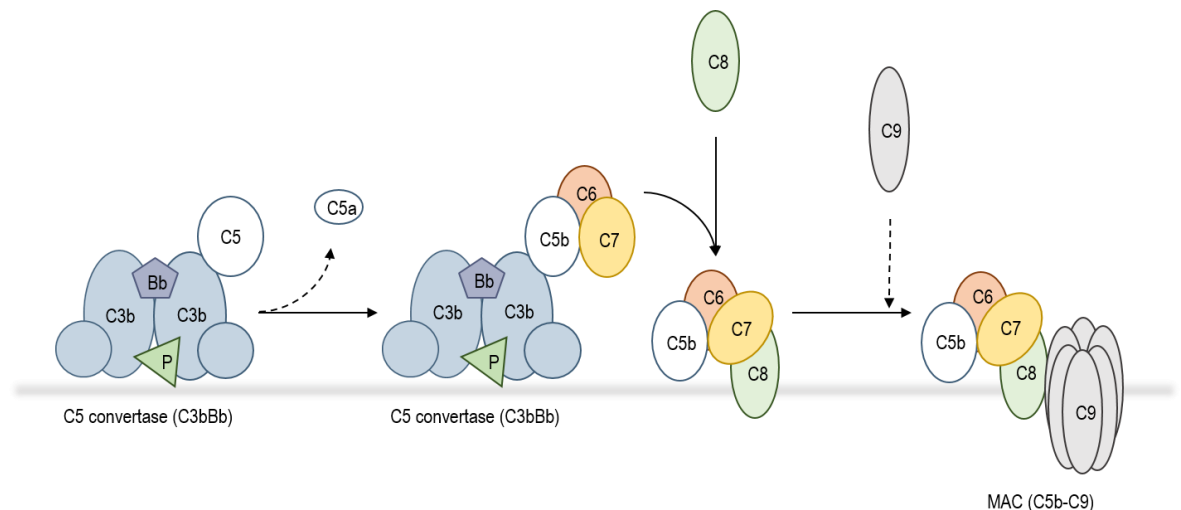


**Figure 1.2: Initiation of the AP**

C3 'tick over' occurs by the spontaneous hydrolysis of C3 (C3<sub>H<sub>2</sub>O</sub>) resulting in binding and subsequent cleavage of FB by FD to form the fluid phase C3 convertase, C3<sub>H<sub>2</sub>O</sub>Bb. The fluid phase C3 convertase cleaves C3 into C3b and C3a; C3b is deposited on activator surfaces. Deposited C3b binds FB making FB susceptible to cleavage by FD, resulting in the release of Ba and formation of the C3 convertase, C3bBb. Binding of Properdin (P) stabilises the C3 convertase. The C3 convertase cleaves further C3 molecules acting as a positive amplification loop (red dotted arrow) to increase C3 deposition on activator surfaces.

### 1.1.4 The Terminal complement pathway

Formation of the C5 convertase, whereby an additional C3b molecule is incorporated into the CP or AP C3 convertase resulting in C4bC2aC3b and C3bBbC3b, respectively, occurs following deposition of increased amounts of C3b on a target surface [Daha, M. R. 1976. Medicus, R. G. 1976. Sim, R. B. 1980. Pangburn, M. K. 2002]. The change of substrate from C3 to C5 initiates the Terminal Pathway (TP) through the cleavage of C5 to C5a and C5b, which results in binding of further complement molecules C6 and C7 (C5b-7) [Medicus, R. G. 1976]. The C5b-7 complex can then be inserted into a cell membrane where it associates with C8, causing the polymerisation of multiple C9 molecules to assemble into a pore, the membrane attack complex (MAC) [Müller-Eberhard HJ 1986]. Figure 1.3 summarises the activation of the TP and formation of the MAC.



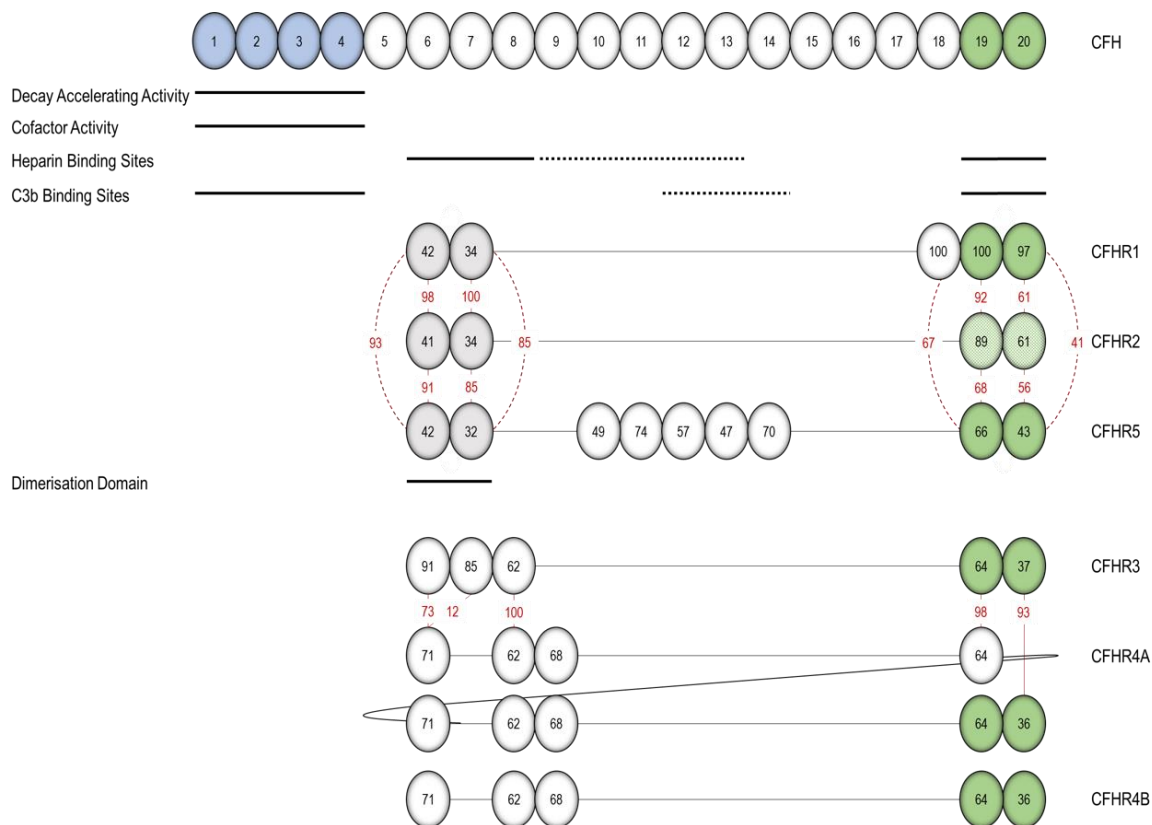
**Figure 1.3: Activation of the TP**

Binding of a further C3b molecule to the C3 convertase on an activating surface generates the C5 convertase, C3bBbC3b, and a switch in substrate from C3 to C5. The C5 convertase cleaves bound C5 to C5b and releases the C5a fragment. Bound C5b binds C6 and subsequently C7; the C5b-C7 complex binds to the membrane and recruits C8 and polymerisation of C9 to form the MAC, resulting in lysis of the cell.

## 1.2 Complement Factor H protein family

The human complement factor H (CFH) protein family are encoded by genes located within the Regulators of Complement Activation (RCA) gene cluster on chromosome 1q32. The protein family consists of six related proteins; Complement Factor H (CFH), the major negative regulator of the AP,

and five CFH Related proteins (CFHRs) encoded downstream of the *CFH* gene. The homology between CFH and the CFHRs is shown in Figure 1.4 together with their major known ligands.



**Figure 1.4: Homology of CFH and CFHR protein family**

CFH and CFHRs consist of between 4 and 20 CCP domains. Alignment of homologous CCP domains with CFH are shown; the percentage amino acid sequence identity with CFH is indicated in domains and sequence identity between homologous CFHR domains is indicated in red. The complement regulatory domains of CFH, CCP domains 1-4 (blue) are not conserved in the CFHRs. Shared binding motifs between CCP domains of CFH and CFHRs for C3b, heparin and the regulatory activity of CFH are also indicated. Dotted lines for heparin and C3b binding sites between CCP domains 9-14 are not confirmed. Confirmed C-terminal C3b/GAG binding domains (green), CCP domains 3-4 of CFHR2 retain C3b binding but have lost GAG binding (light green). Group 1 CFHRs (CFHR1, -2, and -5) share a conserved dimerisation motif in the first N-terminal CCP domain (grey) whereas group 2 CFHRs (CFHR-3 and -4) lack the motif.

### 1.2.1 Complement Factor H

CFH (originally called  $\beta$ 1H globulin) was identified in human serum by Nilsson and Muller-Eberhard in 1965, as an essential component of complement which functions in 'immune haemolysis' [Nilsson, U. R. 1965]. The CFH gene encodes for two proteins: CFH (155 kDa), and a truncated alternative splice variant, CFH Like 1 (CFHL1) (43 kDa), which is identical to Complement Control Protein (CCP) domains 1-7 of CFH but terminates in a unique amino acid sequence, Ser-Phe-Thr-Leu [Ripoche, J.

1988]. CFH is primarily produced in the liver by hepatocytes, and in addition is also produced by monocytes, thrombocytes, fibroblasts, endothelial cells, keratinocytes, and retinal pigment epithelial cells where it modulates local complement activation [Schulz, T. F. 1986. Halme, J. 2009. Whaley, K. 1980. Devine, D. V. 1987. Katz, Y. 1988. Brooimans, R. A. 1990. Timár, K. K. 2006. Chen, M. 2007]. Circulating plasma concentrations of CFH vary between 116–562 µg/mL, *i.e.* 0.7–3.6 µM, (mean concentration of 2.1 µM) with differences seen depending on age and ethnic groups [Esparza-Gordillo, J. 2004]. The major role of CFH is the regulation of the AP.

#### *1.2.1.1 CFH Structure*

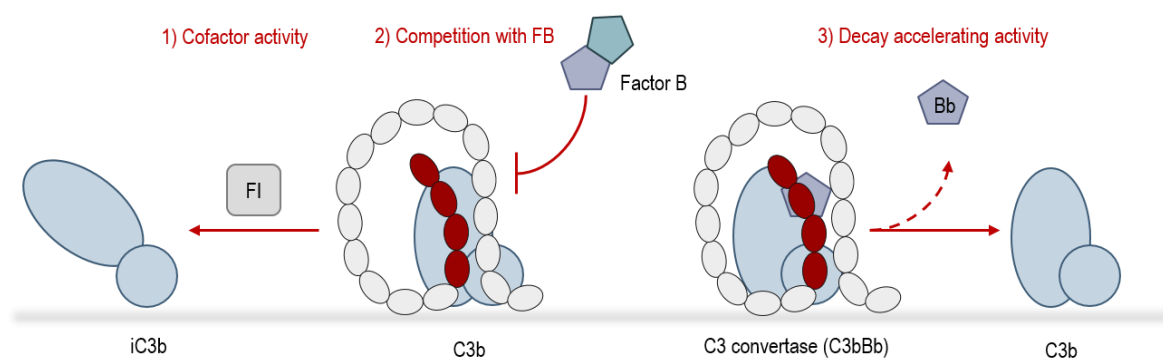
CFH is composed of 20 domains termed Complement Control Protein modules (CCP), also referred to as Short Consensus Repeats (SCRs) or sushi domains [Kristensen, T. 1986. Ripoche, J. 1988]. CCP domains are found in other complement regulatory proteins encoded by the RCA gene cluster [Lintin, S. J. 1988. Klickstein, L. B. 1987]. Each CCP domain of CFH is approximately 60 amino acids in length, stabilised by two disulphide bonds, and has three to eight amino acids between neighbouring CCP domains [Klickstein, L. B. 1987. Ripoche, J. 1988]. Low resolution X-ray structures indicate that CFH is flexible and is bent back on itself in solution, enabling it to engage ligands such as C3b and heparin, and bring them into close proximity with each other [Aslam, M. 2001]. Three C3b binding sites have been mapped to CCP domains 1-4, 12-14 and 19-20 of CFH, with the major C3b binding sites in CCP domains 1-4 and 19-20 [Schmidt, C. Q. 2008A. Schmidt, C. Q. 2008B]. Polyanion binding sites, which engage sulphated glycosaminoglycan or heparin on non-activating host surfaces, have been mapped to CCP domains 6-8 (with CCP domain 7 essential for this interaction), and domains 19-20 [Schmidt, C. Q. 2008A]. Another polyanion binding site was postulated to reside within CCP domains 9-15, although this has not been confirmed [Schmidt, C. Q. 2008B]. The complement regulatory functions of CFH can be attributed to CCP domains 1-4, while CCP domains 19-20 are essential for discriminating self from non-self surfaces.

#### *1.2.1.2 Role in complement regulation*

CFH has three mechanisms to down-regulate AP activation by acting as: (i) a co-factor for Factor I (FI) mediated proteolytic cleavage and inactivation of C3b to iC3b; (ii) competing with FB for binding to C3b, inhibiting the formation of both the AP C3 and C5 convertases; (iii) and accelerating the decay



of the AP C3 convertase by displacing bound Bb (Figure 1.5) [Weiler, J. M. 1976. Whaley, K. 1976. Pangburn, M. K. 1977]. The decay accelerating and FI cofactor activities of CFH reside in CCP domains 1-4 [Gordon, D. L. 1995. Kühn, S. 1995.]. The AP C3 convertase has a short half-life of approximately 90 seconds, unless stabilised by properdin which increases its half-life by 10-fold [Pangburn, M. K. 1986]. However, in the presence of bound CFH, the half-life of the AP C3 convertase is decreased to approximately 0.1 seconds [Pangburn, M. K. 1986]. Once Bb is displaced by CFH, no further FB can bind to form further C3 convertases. In the presence of FI, C3b in the CFH:C3b complex undergoes proteolysis in which the  $\alpha'$ -chain of C3b is cleaved to iC3b. CFHL1 retains co-factor and decay accelerating activities as the N-terminus is identical to CFH, although it lacks target recognition mediated by the C terminal domains of CFH [Kühn, S. 1995. Kühn, S. 1996]. Recent evidence demonstrates that CCP domain 4 of CFHL1 but not CFH, mediates attachment of CFHL1 to epithelial cells by a conserved sequence, Arg-Gly-Asp, and has been implicated as the major complement regulator protecting the Bruch's membrane in the human eye [Hellwage, J. 1997. Clark, S. J. 2015].



**Figure 1.5: Regulation of the AP by CFH**

CFH consists of 20 CCP domains with regulatory activity localised in CCP domains 1-4 (red domains). CFH regulates the activation of the AP by three ways: 1) Cofactor activity; binding of CFH to C3b promotes cleavage of C3b by FI to the inactive form of C3b, iC3b. 2) CFH competes with FB for binding to C3b preventing formation of the C3 convertase. 3) Decay accelerating activity; CFH binds to the C3 convertase promoting the dissociation of Bb from the complex.

### *1.2.1.3 The role of CFH in discriminating self from non-self surfaces*

C3b can be deposited on any surface, and therefore complement needs to be tightly regulated to avoid complement mediated attack of host cells. CFH is a key regulator of complement activation on host tissues due to its regulatory functions. Recognition of host cell surfaces by CFH is thought to be

mediated through sialic acids and glycosaminoglycans (GAG) *i.e.* heparin, heparan sulphate, and dextran sulphate [Jokiranta, T. S. 2005]. The polyanionic molecules on endothelial cells are generally host specific and have a 10-fold higher affinity for CFH than C3b, and therefore impair activation of complement [Fearon, D. T. 1978]. On host tissues, CFH can bind to human endothelial cells and basement membranes *via* CCP domain 20, while simultaneously binding C3b *via* CCP domain 19, and preventing complement-mediated attack *via* the regulatory CCP domains 1-4 [Jokiranta, T. S. 2005. Ferreira, V. P. 2006. Pickering, M. C. 2002. Kajander, T. 2011].

#### *1.2.1.4 Recruitment of CFH as a mechanism for complement evasion by bacterial pathogens*

The ability of pathogenic bacteria to evade the immune system is essential for survival within the host. Many pathogens have evolved mechanisms to circumvent the complement system. Both Gram positive and Gram negative bacteria can recruit CFH to avoid complement-mediated attack by decreasing opsonisation and lysis [Lambris, J. D. 2008]. Table 1.1 summarises bacteria which are known to bind CFH. Two main binding sites, which are distinct from the regulatory CCP domains, are utilised by microbes to sequester CFH [Meri, T. 2013]. One binding site is located in CCP domains 6-7, and is utilised by *Streptococcus pyogenes*, *Neisseria meningitidis* and *Pseudomonas aeruginosa* [Meri, T. 2013. Pérez-Caballero, D. 2004. Schneider, M. C. 2009. Kunert, A. 2007]. The other main interaction site is in the carboxy terminus of CFH in CCP domains 19-20; *Streptococcus pneumoniae*, *Neisseria gonorrhoeae* and *Staphylococcus aureus* utilise this second binding site [Meri, T. 2013. Hammerschmidt, S. 2007. Ngampasutadol, J. 2008. Haupt, K. 2008].

Host endothelial cells bind CFH *via* a charged groove between CCP domains 6-7 and also *via* 19-20 [Prosser, B. E. 2007]. Of note, *N. meningitidis*, binds CFH at the same site as host cells by CCP domains 6-7, and engages the GAG binding site in CCP domain 7 *via* meningococcal factor H binding protein (fHbp) [Schneider, M. C. 2009. Meri, T. 2013]. fHbp forms a high affinity complex with CFH, while the closely-related organism, *N. gonorrhoeae* binds CFH *via* an outer membrane porin, PorB.1B [Ram, S. 1998]. CFH binding to PorB.1B is enhanced by sialylation of the lipooligosaccharide of the gonococcus [Ram, S. 1998].

Bacterial spp.	Ligand	Ligand binding domain with CFH	Reference(s)
<i>Bordetella pertussis</i>	?	—————	Amdahl, H. 2011
<i>Bordetella parapertussis</i>	?	—————	Amdahl, H. 2011
<i>Borrelia burgdorferi</i>	CRASP-1 [1]	—————	Siegel, C. 2010
	CRASP-2 [1]	—————	Siegel, C. 2010
	OspE	—————	Hellwage, J. J. 2001. Bhattacharjee, A. 2013
<i>Borrelia hermsii</i>	CRASP-1	—————	Rossmann, E. 2007
<i>Escherichia coli</i>	Stx2 [1]	—————	Orth, D. 2009; Poolpol, K. 2014
<i>Fusobacterium necrophorum</i>	? [1]	—————	Friberg, N. 2008
<i>Haemophilus influenzae</i>	PH [1]	—————	Fleury, C. 2014
<i>Neisseria gonorrhoeae</i>	PorB.1A [1]	—————	Ram, S. 1998. Ngampasutadol, J. 2008
	PorB.1B [sia]	—————	Ram, S. 1998. Ngampasutadol, J. 2008
<i>Neisseria meningitidis</i>	fHbp	—————	Schneider, M. C. 2009
	NspA	—————	Lewis, L. A. 2010
	PorB2 [1]	—————	Lewis, L. A. 2013
<i>Leptospira interrogans</i>	Lig (A, B) [1]	—————	Castiblanco-Valencia, M. M. 2012
	LfHA	—————	Verma, A. 2006
	LenA	—————	Meri, T. 2005
	LenB [1]	—————	Stevenson, B. 2007
<i>Pseudomonas aeruginosa</i>	Tuf [1]	—————	Kunert, A. 2007
	LpD [1]	—————	Hallström, T. 2012
<i>Rickettsia conorii</i>	OmpB**	—————	Riley, S. P. 2012
<i>Salmonella enterica</i>	Rck	—————	Ho, D. K. 2010
<i>Staphylococcus aureus</i>	Sbi*	—————	Haupt, K. 2008
	SdrE**	—————	Sharp, J. A. 2012
<i>Streptococcus agalactiae</i>	$\beta$ protein	—————	Jarva, H. 2004

	SHT**	—————	Maruvada, R. 2009
<i>Streptococcus pneumoniae</i>	CbpA	—————      —————	Lu, L. 2006
	Hic	—————	Jarva, H. 2002
	PspC	—————      —————	Dave, S. 2004; Hammerschmidt, S. 2007; Duthy, T. G. 2002
<i>Streptococcus pyogenes</i>	M protein [1]	—————	Kotarsky, H. 1998. Pérez-Caballero, D. 2004
	Fba [1]	—————	Pandiripally, V. 2003
	Scl1.6; Scl1.55	—————	Reuter, M. 2010
<i>Streptococcus suis</i>	Fhb**	—————	Pian, Y. 2012
<i>Treponema denticola</i>	FhbB [1]	—————	McDowell, J. V. 2005; Miller, D. P. 2012
<i>Yersinia enterocolitica</i>	YadA*	—————	Schindler, M. K. 2012
	Ail	—————	Biedzka-Sarek, M. 2008

**Table 1.1: Recruitment of CFH by bacterial pathogens**

Reported domains of CFH (black line) which bind to the bacterial spp. indicated, with the known CFH receptor. Bacteria shown to bind CFH, but the domains of CFH that bind to the receptors have not been identified are indicated.

\* Complexes \*\* Unknown CFH binding region [1] Binds CFHL along with CFH [sia] Requires sialylation for CFH binding

Abbreviations: CRASP, complement regulator acquiring surface proteins; OspE, outer surface protein; Stx-2, Shiga toxins; PH, protein H; Por, Porin; fHbp, factor H binding protein; NspA, Neisserial Surface Protein A; Lig, Leptospiral Immunoglobulin-like; Lfh, Leptospiral factor H binding protein; Len, Leptospiral Endostatin-Like Protein; Tuf, Translation elongation factor Tu; LpD, Dihydrolipoyl dehydrogenase; Omp, outer membrane protein; Rck, resistance to complement killing; Sbi, second immunoglobulin-binding protein; SHT, streptococcal histidine trial; CbpA, choline-binding protein; Hic, factor H binding inhibitor of complement; Psp, pneumococcal surface protein; Fba, fibronectin binding protein; Scl, streptococcal collagen like protein; Fhb, factor H binding protein; YadA, Yersinia adhesin; Ail, attachment invasion locus protein.

More recently, CFH has been shown to enhance the activity of the *S. aureus* immune evasion protein, Sbi [Haupt, K. 2008. Amdahl, H. 2013. Meri, T. 2013]. This staphylococcal protein inhibits binding of IgG to Fc $\gamma$  receptors, and binds CFH and C3 in a tripartite complex [Burman, J. D. 2008. Haupt, K. 2008]. Staphylococcal extracellular complement binding protein (Ecb) and to a more limited extent, extracellular fibrinogen-binding protein (Efb) also bind CFH and C3b in a complex. In contrast to Sbi, Ecb and Efb are secreted proteins and do not recruit CFH to the bacterial surface [Amdahl, H. 2013].

Recent evidence has also shown that CFH binds to *Yersinia enterocolitica* by *Yersinia* adhesin A (YadA), in complex with C3b mediating enhanced resistance to serum [Schindler, M. K. 2012]. The identification of distinct molecules (Sbi, Ecb, Efb and YadA) in two bacterial species that bind CFH by alternative mechanisms that enhance down regulation of the AP emphasises the importance of recruitment of CFH to pathogenic bacteria [Meri, T. 2013].

Recruitment of CFH by pathogens can also promote bacterial invasion of host epithelial cells and attachment to neutrophils [Agarwal, V. 2010]. CFH is a ligand for human polymorphonuclear leukocytes *via* the integrin, Complement Receptor 3 (CR3) [DiScipio, R. G. 1998]. *S. pneumoniae* recruits CFH to its surface by Pneumococcal Surface Protein C (PspC). Binding of CFH to *S. pneumoniae* enhances adhesion and internalisation by interactions with CR3, with CFH acting as a molecular bridge. [Agarwal, V. 2010]. A similar mechanism by *N. gonorrhoeae* also enhances adherence to cells expressing CR3 [Agarwal, S. 2010].

### 1.2.2 Complement Factor H Related proteins

All five CFHRs are structurally similar to CFH but are composed of four to nine CCP domains, which have varying sequence identity with CCP domains of CFH (Figure 1.4) [Díaz-Guillén, M. A. 1999]. *cfhr1*, *cfhr2*, *cfhr3* and *cfhr4* were identified as mRNA transcripts of human liver that hybridise to CFH cDNA. CFHR5 was initially identified by using a monoclonal antibody (mAb) raised against human glomerular preparations and mRNA transcripts were subsequently detected in human liver preparations [Estaller, C. 1991. Skerka, C. 1992. Skerka, C. 1993. Skerka, C. 1997. McRae, J. L. 2001]. All CFHRs lack the regulatory domains of CFH (*i.e.* CCP domains 1-4), and therefore would be expected to lack FI cofactor and C3 convertase decay accelerating activity (Figure 1.4).

CFHRs can be divided into two groups based on their structure and sequence [Skerka, C. 2013]. Group I, classified by their conserved *N*-terminus and ability to form dimers, consists of CFHR1, CFHR2 and CFHR5 [Goicoechea de Jorge, E. 2013]. CFHR3 and CFHR4 lack the dimerisation motif yet have a high sequence identity with each other and form group II. Structural studies demonstrate that CFHR1, -2 and -5 assemble as head-to-tail dimers with three key residues (Tyr<sup>34</sup>, Ser<sup>36</sup> and Tyr<sup>39</sup>)

mediating this interaction [Goicoechea de Jorge, E. 2013]. Dimeric complexes can be isolated from serum, with the composition of dimers dependent on the relative abundance of each CFHR [Goicoechea de Jorge, E. 2013]. Dimerisation of the CFHRs increases their avidity for substrates such as C3b, and dimeric CFHR5 has also been demonstrated to have an increased ability to bind to activated murine C3 on the glomerular basement membrane compared to monomeric CFHR5 [Goicoechea de Jorge, E. 2013]. It is not known whether CFHR3 and CFHR4 form dimers although both lack the key residues required for dimer formation found in Group I CFHRs.

#### 1.2.2.1 CFHR1

CFHR1 is a five CCP domain protein with a high sequence identity to CCP domains 6-7 and 18-20 of CFH, and with CFHR2 and CFHR5 (Figure 1.4). CFHR1 occurs as two variants; CFHR1A has the amino acid sequence His-Leu-Glu in CCP domain 3, whereas this sequence is Tyr-Val-Gln in CFHR1B [Skerka, C. 1991. Susukida, R. 2007. Abarategui-Garrido, C. 2009]. CFHR1 also circulates in the blood stream as two isoforms, CFHR1 $\alpha$  with a single glycosylation site, while CFHR1 $\beta$  is glycosylated at residues Asn 126 and Asn 194 [Skerka, C. 1991. Liu, T. 2005]. Predicted to be the most abundant of the CFHRs, the serum concentration of CFHR1 is approximately 70–100  $\mu\text{g/ml}$  (1.6–2.4  $\mu\text{M}$ ) [Heinen, S. 2009].

#### 1.2.2.2 CFHR2

CFHR2 is composed of four CCP domains homologous to CCP domains 6-7 and 19-20 of CFH. Serum CFHR2 concentrations are estimated to be approximately 50  $\mu\text{g/ml}$  (1.2  $\mu\text{M}$ ) [Goicoechea de Jorge, E. 2013]. CFHR2 exists as two isoforms; CFHR2 is unglycosylated, whereas CFHR2 $\alpha$  is glycosylated at position Asn 126 [Skerka, C. 1992. Chen, R. 2009].

#### 1.2.2.3 CFHR3

CFHR3 consists of five CCP domains homologous to CCP domains 6-8 and 19-20 of CFH. Initial estimates of the serum concentration of CFHR3 indicate it is present at similar levels as CFHR1 *i.e.* 50–80  $\mu\text{g/ml}$  (1–1.6  $\mu\text{M}$ ) [Fritsche, L. G. 2010]. However many available antibodies against CFHR3 cross react with CFHR4 due to the high amino acid identity between CFHR3 and CFHR4 [Fritsche, L. G. 2010. Skerka, C. 2013]. Therefore estimates of CFHR3 levels may be inaccurate and the actual concentration may be significantly lower. Indeed, recent estimates of serum CFHR3 levels suggest

that the circulating concentration of CFHR3 is approximately 100-fold lower, and range from 0–2.7 µg/ml with a mean of 0.69 µg/ml (18.7 nM) [Pouw, R. B. 2016]. Like CFHR4, CFHR3 lacks an N terminal dimerisation motif, while multiple isoforms of CFHR3 have been detected in sera indicating four potential glycosylation variants [Skerka, C. 1993. Chen, R. 2009].

#### 1.2.2.4 CFHR4

*cfhr4* encodes for two alternative splice variants, CFHR4A and CFHR4B. CFHR4A is a nine CCP domain protein resulting from an internal duplication, with identical CCP domains 1-3 and 5-7 [Józsi, M. 2005]. CFHR4B is an alternative splice variant of CFHR4 composed of five CCP domains. The CCP domains of CFHR4B and domains 5-9 of CFHR4A are also homologous to CCP domains 6, 8, 9 and 19-20 of CFH. Serum concentrations of both isoforms of CFHR4 have been estimated to be approximately 25 µg/mL [Hebecker, M. 2012].

#### 1.2.2.5 CFHR5

CFHR5 forms heterodimers with CFHR1 and CFHR2 [Goicoechea de Jorge, E. 2013]. CFHR5 is composed of nine CCP domains which are homologous to CCP domains 6-7, 10-14 and 19-20 of CFH. Serum concentrations have been estimated at 3–6 µg/ml (0.05–0.09 µM) [McRae, J. L. 2005].

### 1.3 CFHRs and interactions with the Complement system

The complexity of studying CFHRs is evident from the literature in which CFHRs have been proposed to act as negative complement regulators, given their sequence identity with CFH, while recent evidence indicates that CFHRs enhance complement activation [Heinen, S. 2009. Hebecker, M. 2012. Goicoechea de Jorge, E. 2013. Tortajada, A. 2013].

#### 1.3.1 Interaction of CFHRs and the pentraxins

The monomeric pentraxin CRP binds phospholipids from damaged or apoptotic cells, inducing an inflammatory response by recruiting C1q and activating the CP [Volanakis, J. E. 1979; Volanakis, J. E. 1981]. CRP also binds CFH independently of Ca<sup>2+</sup> via CCP domains 7 and 8-11, modulating localised complement activation [Jarva, H. 1999]. Both CFHR4A and CFHR4B bind pentameric CRP in the fluid phase in the presence of Ca<sup>2+</sup> via CCP domain 1; the difference in binding region between CFH and

CFHR4 may explain why CFH binds monomeric CRP whereas CFHR4 binds pentameric CRP [Mihlan, M. 2009. Hebecker, M. 2010.]. Binding of CFHR4A to CRP also initiates the CP by increasing deposition of C4 and C3 [Hebecker, M. 2010.]. CRP also activates the AP by binding C3b, and mediating the opsonisation of biological surfaces and modulates complement activation [Hebecker, M. 2010.]. CFHR5 binds CRP *via* CCP domains 5-7 and is predicted to be recruited to sites of host tissue damage and potentially enhance complement activation [McRae, J. L. 2005. Kopp, A. 2012].

PTX3 has been shown to bind CFH and CFHL in a  $\text{Ca}^{2+}$  dependent manner by CCP domain 7 and 19-20 of CFH, leading to clearance of apoptotic cells [Deban, L. 2008. Kopp, A. 2012]. PTX3 bound to extracellular matrix components prevents complement activation by binding CFH, whereas damaged tissues are unprotected by regulators [Deban, L. 2008. Kopp, A. 2012]. PTX3 also binds CFHR1 by CCP domains 4-5 which are related to CFH CCP domains 19-20 [Kopp, A. 2012]. More recent evidence suggests that CFHR5 competes with CFH for binding to PTX3 and CRP enhancing C1q binding and results in increased deposition of C3b on surfaces [Csincsi, ÁI. 2015]. Therefore the balance between pentraxins, CFH, CFHR1 and CFHR5 will determine the level of complement activation on surfaces. Currently there is no evidence that CFHRs -2, -3, or -4 bind PTX3 [Csincsi, ÁI. 2015].

### 1.3.2 CFHR interactions with C3 and function

CFHR1, CFHR3, CFHR4A, CFHR4B and CFHR5 have been shown to bind C3b, iC3b and C3d but lack both cofactor activity for FI and decay accelerating activity [Heinen, S. 2009. Hellwage, J. 1999. Hebecker, M. 2012. Eberhardt, H. U. 2013. Goicoechea de Jorge, E. 2013]. At non-physiological concentrations, (*e.g.* 10  $\mu\text{M}$ ) or extended incubation times (*e.g.* 5 hours), CFHR3 and CFHR4B have weak FI cofactor activity, but this is unlikely to be biologically relevant [Hellwage, J. 1999. Hebecker, M. 2012.]. Other studies have used high concentrations of CFHR3 (1.8  $\mu\text{M}$ ) and CFHR5 (0.15  $\mu\text{M}$ ) to demonstrate weak FI cofactor activity [Fritsche, L. G. 2010. McRae, J. L. 2005]. CFHR1 has been proposed to act as a complement regulator by directly binding C5 or C5b-6 and preventing MAC formation [Heinen, S. 2009]. Subsequent studies have shown that CFHR3 also prevents the generation of C5a [Fritsche, L. G. 2010.]. Conversely Goicoechea de Jorge, E. *et al* could not demonstrate any significant interaction between CFHR1 with C5 at physiological protein



concentrations and instead demonstrated a novel role for CFHR1, CFHR2 and CFHR5. These CFHRs were shown to activate complement and increase erythrocyte lysis which was further enhanced by dimerisation of CFHRs [Goicoechea de Jorge, E. 2013]. Furthermore, CFHR1, CFHR2 and CFHR5 act as competitive antagonists of CFH by competing for binding C3b [Goicoechea de Jorge, E. 2013]. The ability of CFHR1, CFHR2 and CFHR5 to act as antagonists of CFH has been confirmed by others [Tortajada, A. 2013].

CFHR5 is also a PRM which specifically binds to necrotic endothelial cells and recruits properdin, by CCP domains 1-2 [Chen, Q. 2016]. Multimerisation of CFHR5 increases binding to cell surfaces and enhances properdin binding due to increased avidity [Chen, Q. 2016]. Therefore while CFH is essential for discriminating self from non-self, the CFHRs can help fine-tune complement regulation to maintain tissue homeostasis.

#### 1.4 Association of CFHRs with human disease

The *CFH/CFHR* gene locus is susceptible to gene rearrangements including replication, deletion, and duplication as well as mutations and polymorphisms. Rearrangements of this region are associated with human diseases, providing some insights into the function of CFHRs.

Lack of CFHR1, through  $\Delta CFHR3/CFHR1$  or  $\Delta CFHR1/CFHR4$ , and gene fusions of *CFH/CFHR1* or *CFH/CFHR3* predispose individuals to atypical haemolytic-uraemic syndrome (aHUS) associated with the appearance of auto-antibodies against CFH (DEAP HUS) [Holmes, L. V. 2013. Zipfel, P. F. 2007. Moore, I. 2010. Dragon-Durey, M. A. 2009. Francis, N. J. 2012; Francis, N. J. 2012]. The  $\Delta CFHR3/CFHR1$  deletion also confers protection against age-related macular degeneration (AMD) and IgA nephropathy [Hughes, A. E. 2006. Gharavi, A. G. 2011]. Both a duplication of CCP domains 1-4 of CFHR1 and *CFHR3/CFHR1* gene fusions are associated with the development of C3 glomerulonephritis (C3GN) [Tortajada, A. 2013. Malik, T. H. 2012]. A recent genome wide association study (GWAS) of susceptibility to meningococcal disease identified a single nucleotide polymorphism (SNP) in *cfhr3* which is associated with a decreased risk of developing meningococcal disease [Davila, S. 2010]. This GWAS study is discussed in further detail in section 1.7.5.3. Furthermore, the same

*cfhr3* SNP (rs426736) was identified in meningococcal GWAS study was also associated with leprosy caused by *Mycobacterium leprae* [Zhang, D. F. 2014].

Polymorphisms in *cfhr5* are associated with dense deposit disease (DDD) characterised by dense deposits of C3 in the glomerular basement membrane of the kidneys, whereas an internal duplication of *cfhr5* is associated with nephropathy in a family of Cypriot origin [Abrera-Abeleda, M. A. 2006. Gale, D. P. 2010]. Loss of function mutations in *cfhr5* have also been reported in aHUS patients [Maga, T. K. 2010. Westra, D. 2012]. In one study, individuals with sequence variants in *cfhr5* were reported to have a significant increased risk of AMD, whereas another study reported that the risk of AMD was unchanged [Zhang, H. 2008. Narendra, U. 2009]. Recently a single nucleotide insertion in *cfhr5* resulting in a premature stop codon presented with C3 deposits in the kidneys which were consistent with C3 glomerulopathy suggesting this sequence variant was a risk factor for developing chronic kidney disease. [Vernon, K.A. 2012].

### 1.5 CFHR interactions with bacterial pathogens

There are several examples of CFHRs recruited to the surface of pathogens, although in many instances the consequences of CFHR binding remain unknown. PorB.1A and sialylated PorB.1B of *N. gonorrhoeae* bind CFHR1 preferentially over CFH and CFHL1; however, no role for CFHR1 binding was determined [Ngampasutadol, J. 2008]. Of note, CFHR binding to pathogens is often mediated by the same proteins that recruit CFH. A notable exception is *Borrelia burgdorferi* which binds CFHR1, CFHR2, CFHR5 and CFH by distinct proteins. Complement regulator-acquiring surface proteins (CRASPs) of *B. burgdorferi* are complement evasion molecules which act by binding CFH [Haupt, K. 2007]. CRASPs have also been shown to bind preferentially to CFH or CFHRs, with CRASP-3 and -5 binding CFHR1, CFHR2 and CFHR5 rather than CFH, while CRASP-4 preferentially binds CFHR1 and CFHR2 [Haupt, K. 2007. Siegel, C. 2010. Hammerschmidt, C. 2012]. Recruitment of CFHR1, CFHR2 and CFHR5 does not enhance serum resistance of *B. burgdorferi* in the absence of CFH [Siegel, C. 2010]. Competition of CFHR1 with CFH for binding to Shiga toxin 2 of Enterohemorrhagic *Escherichia coli* via CCP domains 3-5 has been shown to decrease C3b cleavage [Poolpol, K. 2014]. Table 1.2 summarises the bacteria which bind CFHRs.

Pathogen	CFHR	Ligand	CFHR Binding domain	CFH	Reference(s)
<i>Borrelia burgdorferi</i>	CFHR1 CFHR2 CFHR5	CRASP-3	CFHR2- SCR 4	Yes (Weak)	Haupt, K. 2007; Siegel, C. 2010
	CFHR1 CFHR2 CFHR5	CRASP-4		Yes (Weak)	Haupt, K. 2007; Hammerschmidt, C. 2012
	CFHR1 CFHR2 CFHR5	CRASP-5		Yes	Haupt, K. 2007; Siegel, C. 2010
<i>Borrelia hermsii</i>	CFHR1	CRASP-1	SCR 5	Yes	Rossmann, E. 2007
<i>Escherichia coli</i> (EHEC)	CFHR1	Stx2	SCRs 3–5	Yes	Poolpol, K. 2014;
<i>Fusobacterium necrophorum</i>	CFHR1 CFHR4	?	?	Yes	Friberg, N. 2008
<i>Leptospira interrogans</i>	CFHR1	LigA, LigB	SCRs 1–2; SCR 3–5	Yes	Castiblanco-Valencia, M. M. 2012
	CFHR1	LfHA	SCRs 3–5	Yes	Verma, A. 2006
	CFHR1	LenA	SCRs 3-5	Yes	Meri, T. 2005
	CFHR1	LenB	SCRs 1-2	Yes	Stevenson, B. 2007
<i>Neisseria gonorrhoeae</i>	CFHR1	PorB.1A	SCRs 4-5	Yes	Ngampasutadol, J. 2008
	CFHR1	PorB.1B [sia]	SCRs 4-5	Yes	Ngampasutadol, J. 2008
<i>Pseudomonas aeruginosa</i>	CFHR1	Tuf	SCRs 3–5	Yes	Kunert, A. J. 2007
		LpD	SCRs 3–5	Yes	Hallström, T. 2012
<i>Staphylococcus aureus</i>	CFHR1	Sbi	SCRs 4-5	Yes	Haupt, K. 2008
<i>Streptococcus pyogenes</i>	CFHR1 CFHR3	M protein	SCRs 1-2; SCR 1-2	Yes	Kotarsky, H. 1998
	CFHR1	Scl1.6; Scl1.55	SCRs 3-5	Yes	Reuter, M. 2010

**Table 1.2: Recruitment of CFHRs by bacterial pathogens**

Bacterial species and the receptors which bind CFHRs are indicated along with the known CCP domains which bind to the receptor. If the receptor also binds CFH this is also indicated.

[sia] Requires sialylation for CFHR1 binding

Abbreviations: CRASP, complement regulator acquiring surface proteins; Stx-2, Shiga toxins; Lig, Leptospiral Immunoglobulin-like; Lfh, Leptospiral factor H binding protein; Len, Leptospiral Endostatin-Like Protein; Tuf, Translation elongation factor Tu; LpD, Dihydrolipoyl dehydrogenase; Por, Porin; Sbi, second immunoglobulin-binding protein; Scl, streptococcal collagen like protein.

## 1.6 *Neisseria*

The genus *Neisseria* are Gram negative  $\beta$  proteobacteria and includes two human specific pathogens, *N. meningitidis* and *N. gonorrhoeae*. *N. meningitidis* is the major causative agent of meningitis and septicaemia while the gonococcus causes the sexually transmitted infection, gonorrhoea and is a major health concern due to the emergence of multi-drug resistance [Unemo, M. 2012]. The genus *Neisseria* also includes a number of other closely-related commensal species [Knapp, J. S. 1988].

### 1.6.1 Commensal *Neisseria*

Commensal *Neisseria* spp. are common inhabitants of the mucosal and dental surfaces of the human upper respiratory tract [Gold, R. 1978. Knapp, J. S. 1988. Sáez Nieto, J. A. 1998. Zaura, E. 2009. Kristiansen, P. A. 2012. Kraal, L. 2014. Sheikhi, R. 2015]. Some species of *Neisseria* have been isolated from other mammalian and non-mammalian hosts [Liu, G. 2015]. Frequent co-colonisation of multiple *Neisseria* spp. has been observed in carriage studies [Knapp, J. S. 1988. Sáez Nieto, J. A. 1998]. Horizontal gene transfer between these related organisms has made classifying *Neisseria* into distinct species problematic [Bowler, L. D. 1994; Zhou, J. 1997. Smith, N. H. 1999]. Several methods, including DNA-DNA hybridisation, 16s rRNA sequencing and Multi Locus Sequence Typing (MLST), have been utilised to designate the different species [Tønnum, T. 1989; Harmsen, D. 2001; Bennett, J. S. 2007; Maiden, M. C. 1998]. More recently, whole genome sequencing (WGS) approaches have been employed to distinguish and classify commensal *Neisseria* [Bennett, J. S. 2012].

The commensal *Neisseria*, *Neisseria lactamica* and *Neisseria polysaccharea* have been shown by DNA-DNA hybridisation and by WGS to be closely related to *N. meningitidis* and *N. gonorrhoeae* [Guibourdenche, M. 1986; Bennett, J. S. 2012]. Although more distinct than *N. lactamica* and *N. polysaccharea*, *N. cinerea* is also related to the proteotypic *Neisseria* pathogens as shown by DNA-DNA hybridisation [Guibourdenche, M. 1986]. While commensal *Neisseria* are generally considered to be harmless inhabitants of the human nasopharynx, there are case reports of commensal *Neisseria* causing disease in immuno-compromised individuals [Knapp, J. S. 1988. Liu, G. 2015]. However, *N. polysaccharea*, which is the most closely related to *N. meningitidis* and *N. gonorrhoeae*, has fewer case reports than *N. lactamica* and *N. cinerea* [Bennett, J. S. 2012]. Disease caused by *N.*

*lactamica* and *N. cinerea* has a range of presentations including meningitis, septicaemia and peritonitis [Kirchgesner, V. 1995. Everts, R. J. 2010. Taegtmeier, M. 2006].

In comparison with *N. meningitidis* and *N. gonorrhoeae*, the commensal *Neisseria* are less well studied. However, *N. lactamica* has been assessed for its ability to generate natural immunity and *N. lactamica* derived antigens from outer membrane vesicles (OMV) have been evaluated for the prevention of meningococcal disease [Gold, R. 1978. Evans, C. M. 2011. Vaughan, T. E. 2006. Gorringer, A. R. 2009].

## 1.7 *Neisseria meningitidis*

*N. meningitidis* is an obligate human pathogen and present in the nasopharynx of approximately 10% of the population [Stephens, D. S. 2007]. The bacterium is the major causative agent of meningitis and septicaemia with a case fatality rate of between 5-20% [Stephens, D. S. 2007]. Meningococcal disease has a rapid onset with non-specific early symptoms, and is often mistaken for viral infection [van Deuren, M. 2000]. Non-specific symptoms progress within a few hours and later patients can present with a purpuric skin rash, photophobia and neck stiffness which are characteristic of meningococcal disease. *N. meningitidis* can also cause uncommon clinical presentations including pericarditis, conjunctivitis, sinusitis, arthritis and urethritis [Tzeng, Y. L. 2000].

### 1.7.1 Classification

*N. meningitidis* is classified into serogroups based on its polysaccharide capsule; 13 serogroups of *N. meningitidis* have been identified (A, B, C, E, H, I, K, L, M, W, X, Y and Z). Serogroups A, B, C, W, X and Y account for the majority of disease [Frasch, C. E. 1985. Pollard, A. J. 2000. Jódar, L. 2002. Stephens, D. S. 2007]. The capsular polysaccharide of serogroups B and C strains consist of  $\alpha$ -2-8 and  $\alpha$ -1-9-linked polysialic acid, respectively, while serogroups W and Y express alternating sequences of D-galactose or D-glucose with sialic acid. The serogroup A capsule does not contain sialic acid but is composed of  $\alpha$  1-6 linked mannosamine phosphate [Liu, T. Y. 1971a. Liu, T. Y. 1971b. Bhattacharjee, A. K. 1976]. Meningococcal isolates are further classified based on the outer membrane proteins, PorA and PorB, into serosubtypes and subtypes, respectively [Frasch, C. E. 1985. van Deuren, M.

2000]. The properties of meningococcal lipopolysaccharide determine the immunotype of strains; so far 12 immunotypes have been identified [Tsai, C. M. 1987. Scholten, R. J. 1994].

Meningococcal surface proteins can undergo antigenic or phase variation, which complicates the classification of *N. meningitidis* in epidemiological studies [Caugant, D. A. 1998]. In addition, many carriage isolates do not express a capsule and therefore cannot be classified by serogrouping. Alternative methods for classifying *N. meningitidis* have been developed to overcome the issue of antigenic and phase variation. Multi Locus Sequence Typing (MLST, <http://pubmlst.org>) analyses the nucleotide sequence of seven genes with housekeeping functions (*abcZ*, *adk*, *aroE*, *fumC*, *gdh*, *pdhC*, and *pgm*) [Maiden, M. C. 1998]. A second classification system, Multilocus Enzyme Electrophoresis (MLEE), is based on the migration metabolic enzymes [Selander, R. K. 1986]. MLST has the advantage over MLEE that it can be standardised between laboratories [Maiden, M. C. 1998]. Recently WGS has been employed to characterise meningococcal isolates that are closely related [Lucidarme, J. 2015].

### 1.7.2 Pathogenesis

*N. meningitidis* is transmitted between individuals by aerosol spread or direct contact with secretions from the oropharynx and nasopharynx [Tzeng, Y. L. 2000]. Colonisation is an essential step in *N. meningitidis* pathogenesis, and initial adhesion to epithelial cells is mediated by bacterial type four pili (Tfp) [Kellogg, D. S. 1963. Nassif, X. 1993]. Tfp are hair-like filaments which project from the bacterial surface, and are important for colony formation, aggregation, and uptake of exogenous DNA [Lappann, M. 2006. Seifert, H. S. 1990]. There are several proposed epithelial host receptors for Tfp. For example, CD46, a membrane bound CP and AP regulator, has been proposed to mediate Tfp:epithelial cell attachment [Källström, H. 1997. Johansson, L. 2003]. However, CD46 is expressed mostly on the basolateral side of epithelial cells and knock down of CD46 did not affect gonococcal adhesion to epithelial cells [Maisner, A. 1997. Kirchner, M. 2005]. Tfp have also been shown to mediate the adherence of meningococci *via* the platelet activating factor receptor (PAFr) on human bronchial epithelial cells, although this has not been confirmed by others [Jen, F. E. 2013].

Meningococcal binding to epithelial cells is a two-step process. Tfp are required for the initial engagement of epithelial cells, while other adhesins mediate subsequent intimate adherence. Downregulation of the meningococcal polysaccharide capsule is essential for intimate adherence and temperature has been shown to be a key cue for the bacterium as capsule expression is lower at cooler temperatures, such as encountered by the bacterium in the nasopharynx [Hammerschmidt, S. 1996. Loh, E. 2013. Barnwal R, P. 2016]. The class five outer membrane opacity proteins, Opa and Opc mediate intimate adherence by binding members of the CD66 family, *i.e.* carcinoembryonic antigen cell adhesion molecules (CEACAMs) [Virji, M. 1996a. Virji, M. 1996b]. Of note, interactions between Opc and CEACAMs require high level expression of Opc [Sarkari, J. 1994]. *Neisseria* adhesin A (NadA) has also been implicated in the initial attachment of meningococci to epithelial cells by binding to  $\beta$ 1-integrins [Capecchi, B. 2005. Nägele, V. 2011]. Several other adhesins have been identified including the Adhesion and penetrating protein and Meningococcal surface fibril (Msf) [Serruto, D. 2003. Turner, D. P. 2006]. Of note, Msf has been suggested to bind the TP regulator, vitronectin [Griffiths, N. J. 2011].

Bacterial microcolonies form on the apical surface of epithelial cells, whereby the meningococcus multiplies and spreads over the cell. From this site, the bacterium can invade epithelial cells and then pass to the sub-epithelial layer by transversing the epithelial layer [Kirchner, M. 2005. Stephens, D. S. 1983. Sadlon, T. A. 1994. Sutherland, T. C. 2010].

Dissemination of *N. meningitidis*, in the circulation and its interactions with both epithelial and endothelial cells at the blood brain barrier are critical steps in meningococcal pathogenesis. Tfp interactions with CD147 initiate bacterial adherence to the endothelium and are important for meningococcal colonisation of the microvasculature [Bernard, S. C. 2014]. Engagement of CD147 initiates a signalling cascade involving the  $\beta$ 2-adrenergic receptor ( $\beta$ 2-AR), and leads to tight junction remodeling, allowing *N. meningitidis* to transverse the brain endothelial layer by a paracellular route [Coureuil, M. 2010. Lécuyer, H. 2012]. Both Opc and Opa also mediate intimate adhesion of bacteria to endothelial cells [Meyer, T. F. 1999]. Furthermore, Opc can bind vitronectin which acts as a molecular bridge by binding to  $\alpha_v\beta_3$  integrin on the apical surface of endothelial cells [Virji, M. 1994].

The clinical presentation of *N. meningitidis* infection occurs after the bacterium enters the bloodstream. Within the circulation, bacteria reach high densities of up to  $10^9$ /ml [Hackett, S. J. 2002]. The release of pro-inflammatory chemokines and cytokines, including interleukins -1, -6 and -8, and TNF- $\alpha$ , along with high levels lipopolysaccharide, lead to tissue damage and meningococcal sepsis [Brandtzaeg, P. 1989]. When bacteria cross the blood brain barrier, release of pro-inflammatory cytokines into the subarachnoid space result in inflammation of the meninges and meningitis [Brandtzaeg, P. 1992]. The pathophysiological factors which govern meningococcal disease progression are not limited to the interaction between bacteria with epithelial and endothelial cells, but also include the ability of the meningococcus to survive and replicate in the bloodstream.

### 1.7.3 Epidemiology

Meningococcal disease occurs worldwide but its prevalence varies depending on geographic location, age and season. There are estimated to be 1.2 million cases of meningococcal disease worldwide each year with 135,000 deaths [Stephens, D. S. 2007].

Epidemics occur every 5-10 years within the 'meningitis belt' in sub-Saharan Africa with approximately 500 cases per 100,000 population. Serogroup A meningococci are largely responsible for these outbreaks, and to a lesser extent cause epidemics in China and Russia [Maiden, M. C. 1998. Wang, J. F. 1992. Greenwood, B. 1999. Greenwood, B. M. 1987]. The high incidence of meningococcal disease in Africa coincides with the beginning of the dry season with humidity and dust associated with an increase in meningococcal disease [Molesworth, A. M. 2003].

In contrast, serogroup B disease is prevalent in Europe, while serogroups B, C and Y predominate in the USA [Jackson, L. A. 1995. Brooks, R. 2006]. The incidence of serogroup C disease fell in the UK following the introduction of a polysaccharide conjugate vaccine in 1999-2000 [Balmer, P. 2002. Maiden, M. C. 2008. Campbell, H. 2009]. Generally, outbreaks of meningococcal disease in developed countries are small and sporadic, affecting approximately 1 in 100,000 of the population [Achtman, M. 1995]. Epidemics of meningococcal disease have also been associated with the



movement of populations. For example, the 1987 Hajj pilgrimage to Mecca resulted in the spread of a virulent form of serogroup W *N. meningitidis* by returning pilgrims [Schwartz, D. 1987]. Therefore all individuals attending the Hajj pilgrimage are vaccinated against *N. meningitidis* serogroup A, C, W, Y with a conjugate vaccine.

#### 1.7.4 Natural immunity against *N. meningitidis*

Both *N. meningitidis* and commensal *Neisseria* spp. can colonise the human nasopharyngeal mucosa. Carriage of *N. lactamica* is high in infants and declines with age. Conversely, carriage of *N. meningitidis* is low in infancy and steadily rises in childhood and adolescence [Gold, R. 1978. Cartwright, K. A. 1987. Olsen, S. F. 1991. Bennett, J. S. 2005]. Incidence rates of meningococcal disease peak between 6 months and 2 years of age, coinciding with the waning of maternal antibodies and before the induction of bactericidal antibodies [Goldschneider, I. 1969a. Goldschneider, I. 1969b]. The level of serum bactericidal and cross-reactive IgG antibodies against *N. meningitidis* also increases with age in children even though meningococcal carriage remains low, suggesting that *N. lactamica* may protect against meningococcal disease [Gold, R. 1978. Cann, K. J. 1989]. Furthermore, individuals colonised by *N. lactamica* showed reduced carriage of *N. meningitidis* which also indicates that *N. lactamica* can inhibit colonisation of *N. meningitidis* [Gold, R. 1978. Bennett, J. S. 2005]. Human challenge with *N. lactamica* sought to determine the mechanism for the induction of natural immunity against *N. meningitidis* [Evans, C. M. 2011; Deasy, A. M. 2015]. Individuals colonised by *N. lactamica* generated cross-reactive salivary IgA and serum IgG antibodies against serogroup B meningococci supporting the hypothesis that colonisation by *N. lactamica* contributes to natural immunity against the meningococcus [Evans, C. M. 2011. Gold, R. 1978]. However, serum antibodies of *N. lactamica* carriers lacked bactericidal activity and furthermore, did not prevent colonisation of *N. meningitidis* [Evans, C. M. 2011]. A subsequent human challenge study observed a reduction in meningococcal carriage by both displacement of meningococci from the nasopharynx and further prevents meningococcal acquisition; potentially this can be achieved by altering conditions in the nasopharynx to favour *N. lactamica* colonisation [Deasy, A. M. 2015. Andrade, J. R. 1986. Vaughan, A. T. 2009. Wong, H. E. 2011].

### 1.7.5 Susceptibility to meningococcal disease

The molecular mechanisms which predispose some individuals to meningococcal disease are not fully understood. Age is an important determinant with the highest incidence of meningococcal disease seen in children between 6 and 24 months of age [Goldschneider, I. 1969. Moore, P. S. 1989]. Complement plays an important role in protection from meningococcal disease as individuals lacking complement components are highly susceptible to invasive disease and often have recurrent infections [Figueroa, J. E. 1991].

#### 1.7.5.1 Complement deficiencies

Individuals with rare, inherited deficiencies of complement components, particularly of the AP or TP, are predisposed to meningococcal disease [Figueroa, J. E. 1991]. Some deficiencies influence the severity of disease, while individuals with other defects suffer from recurrent infections [Pickering, M. C. 2008. Kuijpers, T. W. 2010].

Individuals with deficiencies in MAC components, *i.e.* C5-C9, tend to suffer from recurrent meningococcal infections of mild to moderate severity [Pickering, M. C. 2008. Barroso, S. 2010. Kuijpers, T. W. 2010; Orren, A. 2012]. Deficiency of any MAC component prevents formation of the MAC lytic pore on the bacterial surface. Of note, lack of C9 results in a reduction of complement mediated lysis, even though the C5-C8 complex can still lyse bacteria albeit to a lesser extent [Miyagawa, S. 1996]. These defects are rare in the general population but a higher prevalence is seen in certain ethnic groups. For example, C5, C6 and C8 deficiency is reported to be higher in the US, with a high prevalence of C6 deficiency in African Americans originally from South Africa (1 in 1600) [López-Lera, A. 2009. Orren, A. 2012. Zhu, Z. 2000. Pickering, M. C. 2008. Kojima, T. 1998. Tedesco, F. 1990.]. Cases of C7 deficiency have been reported worldwide, whereas the prevalence of C9 deficiency is higher in individuals of Japanese ancestry (0.095%) [Rameix-Welti, M. A. 2007. Barroso, S. 2006. Nishizaka, H. 1996. Fernie, B. A. 1996. Thomas, A. D. 2012. Barroso, S. 2010. Kuijpers, T. W. 2010. Khajooee, V. 2003. Fukumori, Y. 1989].

Deficiencies of components or regulators of the AP, such as Properdin and FD, also increase susceptibility to meningococcal disease. Properdin, stabilises the C3 convertase, while FD cleaves FB during the formation of the AP C3 convertase [Sjöholm, A. G. 1982. Braconier, J.H. 1983. Söderström, C. 1989. Sprong, T. 2006]. Deficiency of the complement regulators, CFH and FI, leads to a reduction in C3 levels with a clinical pathology similar to C3 deficiency [Zipfel, P. F. 1999. Vyse, T. J. 1994]. This is due to uncontrolled complement activation, resulting in excessive C3 cleavage in the fluid phase. Conversely, a SNP (C246T) in CFH which results in increased circulating CFH concentrations is also associated with predisposition to meningococcal disease [Haralambous, E. 2006]. Increased levels of CFH could render the bacterium less susceptible to complement mediated lysis [Haralambous, E. 2006].

Some studies have linked polymorphisms in the promoter of the *mbi* gene or in the gene itself to increased susceptibility to meningococcal disease [Bax, W. A. 1999. Hibberd, M. L. 1999. Kuipers, S. 2003]. However, this was not confirmed in a subsequent study [Kuijper, E. J. 1999].

#### 1.7.5.2 Terminal complement inhibitors

Eculizumab (Soliris®; Alexion Pharmaceuticals, Inc.) is a humanised mAb used to treat paroxysmal nocturnal hemoglobinuria and aHUS [Dmytrijuk, A. 2008. Kelly, R. J. 2011. Mache, C. J. 2009. Zuber, J. 2012]. Eculizumab binds C5 at high affinity, preventing both C5 cleavage by the C3 convertase and MAC formation [Dmytrijuk, A. 2008]. As deficiencies in TP components are linked with increased risk of meningococcal infection, therapies which inhibit the TP also predispose individuals to meningococcal disease [Figueroa, J. E. 1991. Struijk, G. H. 2013]. Due to the higher risk of meningococcal disease, patients are immunised against *N. meningitidis* prior to starting Eculizumab treatment [Dmytrijuk, A. 2008. Zlamy, M. 2012].

#### 1.7.5.3 Genome Wide Association Studies (GWAS)

A GWAS study published in 2010 by Davilla *et al.* identified SNPs in *CFH* (rs1065489) and *CFHR3* (rs426736) associated with meningococcal disease from 475 cases and 4,703 controls [Davila, S. 2010]. *CFH* SNP (rs1065489) coding for a nonsynonymous substitution resulted in a lower risk of meningococcal disease. Of note, sub-Saharan African populations have the highest incidence of

meningococcal disease, also had the lowest frequency of the *CFH* (rs1065489) polymorphism [Frazer, K. A. 2007]. Furthermore, *CFHR3* (rs426736) polymorphism also conferred a meningococcal disease risk reduction for minor allele carriers [Davila, S. 2010]. These findings were replicated in a further Western and South European cohort of 968 cases and 1,376 controls [Davila, S. 2010]. Of note, a SNP in *CFHR1* (rs16840658) was also strongly associated with meningococcal disease in the original and western European cohorts but failed genotyping in the southern European cohort [Davila, S. 2010]. Previous genetic studies had identified a SNP in the promotor region of NFκB (rs3753394) correlated with CFH levels but in the GWAS study by Davilla *et al.* this SNP showed a non-significant trend for association with disease in the southern and western Europe cohorts, potentially due to meningococcal strain differences [Haralambous, E. 2006]. A subsequent study confirmed the association of SNPs in *CFH* (rs1065489) and *CFHR3* (rs426736) with meningococcal disease from a central European cohort of 248 patients and 835 controls [Biebl, A. 2015]. However, the molecular mechanisms underlying these findings have not been examined.

#### 1.7.6 Complement evasion by *N. meningitidis*

When *N. meningitidis* enters the blood stream it is subject to complement-mediated attack which leads to bacterial lysis and clearance. The bacterium has evolved several mechanisms to avoid the human complement system.

##### 1.7.6.1 Surface structures that facilitate complement evasion

Expression of a polysaccharide capsule is important for serum resistance of *N. meningitidis* with almost all clinical isolates being encapsulated, whereas 50% of colonising *N. meningitidis* are unencapsulated [Jarvis, G. A. 1987. Claus, H. 2002]. The mechanisms by which the capsule protects *N. meningitidis* from complement are not fully understood, although it is thought to prevent insertion of the MAC [Drogari-Apiranthitou, M. 2002]. Furthermore, the chemical composition of the capsule may affect complement susceptibility; serogroup A strains bind more C3 and C4 compared with strains expressing sialic acid containing capsules (such as serogroups B and C). [Drogari-Apiranthitou, M. 2002. Ram, S. 1999]. Capsule expression has been shown to be upregulated in response to increasing ambient temperature possibly during transition from the nasopharynx to the

blood stream, resulting in enhanced resistance against complement [Loh, E. 2013. Barnwal, R. P. 2016]. Other studies have also indicated that increased capsule expression resulting from an *IS1301* insertion in the capsule biosynthesis locus can downregulate the AP and reduce C3 binding [Uria, M. J. 2008].

Lipopolysaccharide (LPS) is a major component of the outer membrane of *N. meningitidis* which also enhances resistance of the meningococcus to complement; strains with truncated LPS have decreased serum resistance [Geoffroy, M. C. 2003]. In addition the availability of micronutrients, particularly lactate, can also facilitate complement resistance as lactate can be converted into phosphoenol pyruvate, which is a precursor of sialic acid, a constituent of the capsule in many strains, and incorporated into LPS [Exley, R. M. 2005].

#### 1.7.6.2 Acquisition of complement regulators

Factor H binding protein (fHbp) is a surface expressed lipoprotein (approximately 27 kDa) which was identified by reverse vaccinology as a candidate for meningococcal vaccines [Pizza, M. 2000. Masignani, V. 2003]. It is a highly variable protein which can be grouped into three distinct variants [Brehony, C. 2009]. fHbp binds CFH at high affinity ( $\sim 3$  nM  $K_D$ ) by mimicking host glycosaminoglycans binding of CFH [Schneider, M. C. 2009]. The atomic structure of the CFH:fHbp complex indicates that fHbp binds CFH *via* CCP domains 6 and 7 [Schneider, M. C. 2009]. All three fHbp variants can bind CFH at similar high affinities independent of serogroup and sialylation of LPS [Madico, G. 2006. Schneider, M. C. 2006. Johnson, S. 2012. van der Veen, S. 2014]. Binding of CFH on the surface of *N. meningitidis* reduces C3b deposition and increases survival of the bacterium in whole blood and resistance against complement-mediated lysis [Madico, G. 2006. Schneider, M. C. 2006]. fHbp is upregulated in response to increasing temperature. Therefore the bacterium would express more fHbp when in the blood stream than in the nasopharynx [Loh, E. 2013. Loh, E. 2016].

Some *N. meningitidis* strains have been isolated from individuals which lack expression of fHbp [Lucidarme, J. 2011]. Further research has identified other receptors for CFH on the bacterial surface. Neisserial surface protein A (NspA) was identified in a  $\Delta fHbp$  which bound CFH, and binding was enhanced by sialylation of LPS [Lewis, L. A. 2010. Lewis, L. A. 2012]. More recent evidence has also

suggested that *N. meningitidis* binds CFH *via* the abundant meningococcal porin, PorB2, although this was only shown in a strain deficient in both NspA and fHbp [Lewis, L. A. 2013]. CFH binding to both NspA and PorB2 enhances resistance to complement-mediated lysis.

C4 binding protein (C4bp) is a major negative regulator of the CP which acts by accelerating the decay of the CP C3 convertase and a co-factor for FI cleavage of C4b [Gigli, I. 1979]. Meningococcal PorA loops 1 and 4 act as receptors for C4bp on the surface of *N. meningitidis* with binding increasing resistance to complement-mediated lysis [Jarva, H. 2005]. Of note, encapsulated strains had a significant reduction in the amount of bound C4bp therefore the relevance of C4bp binding to *N. meningitidis* is unclear; furthermore experiments were performed at non-physiological NaCl concentrations [Jarva, H. 2005].

*N. meningitidis* has been shown to bind vitronectin by both the *Neisseria* opacity protein, Opc, and Msf [Hubert, K. 2012. Griffiths, N. J. 2011]. Soluble vitronectin is an inhibitor of the TP and reduces the formation and insertion of the MAC by occupying the membrane binding site of the C5b-7 complex and preventing C9 polymerisation [Millis, L. 1993]. Interestingly, circulating concentrations of vitronectin are reduced in the acute phase of meningococcal disease [Høggåsen, K. 1994]. Opc binds vitronectin by sulphated tyrosines and facilitates bacterial adherence to human brain endothelial cells, whereas Msf is a homologue of the autotransporter adhesion, Hsf, from *Haemophilus influenzae*, which binds to integrins on epithelial cells [Prince, S. M. 2002. Hubert, K. 2012. Peak, I. R. 2000]. Vitronectin interactions with Opc and Msf decrease MAC deposition and enhance *N. meningitidis* resistance to complement-mediated lysis [Hubert, K. 2012. Griffiths, N. J. 2011. Hill, D. J. 2015].

#### *1.7.6.3 Protease activity against complement components*

The autotransporter protease, NalP, has previously been shown to process proteins on the surface of *N. meningitidis*, cleaving proteins involved in biofilm formation [Roussel-Jazédé, V. 2013]. This serine protease also cleaves C3, into C3a-like and C3b-like components, with the C3b-like component rapidly degraded by CFH and FI [Del Tordello, E. 2014]. Generation of the C3b-like component also

results in reduced deposition of C3b on the bacterial surface and enhanced survival in normal human serum (NHS) [Del Tordello, E. 2014].

#### 1.7.7 *N. meningitidis* vaccines

Even with effective antibiotic treatment, meningococcal disease has a fatality rate of approximately 6-10%, with 4.3-11.2% of survivors suffering from long term sequelae [Harrison, L. H. 2001. Goldacre, M. J. 2003. Robbins, J. B. 2003. Edmond, K. 2010]. Vaccination is a key preventative strategy against meningococcal disease. Effective vaccines not only protect immunised individuals, but can also provide herd immunity [Maiden, M. C. 2002. Ramsay, M. E. 2003. Maiden, M. C. 2008]. This is particularly evident from the implementation of polysaccharide conjugate vaccines against meningococcal C strains in the UK and meningococcal A strains in Africa [Campbell, H. 2009. Sow, S. O. 2011. Sambo, L. 2015]. Vaccines available against *N. meningitidis* are reviewed below.

##### 1.7.7.1 *Correlates of protection*

The establishment of a serological correlate of protection against *N. meningitidis* was essential for the development of meningococcal vaccines [Gotschlich, E. C. 1969]. Serum bactericidal activity (SBA) is the accepted 'gold standard' for evaluating the protective efficacy of meningococcal vaccines and is correlated with natural immunity against meningococcal disease [Goldschneider, I. 1969]. There is an inverse correlation between the age-related incidence of meningococcal disease and the presence of SBA [Goldschneider, I. 1969]. In the laboratory, SBA measures the ability of antibodies in sera to lyse bacteria *via* the CP in the presence of an exogenous source of complement. The dilution of serum that results in at least a 50% reduction in bacteria is defined as the SBA titre [Goldschneider, I. 1969; Holst, J. 2003; Borrow, R. 2006]. An SBA titre of  $\geq 4$  using human serum as the complement source, or  $\geq 8$  using rabbit serum correlates with protection against meningococcal disease [Goldschneider, I. 1969. Borrow, R. 2006. Santos, G. F. 2001].

##### 1.7.7.2 *Polysaccharide vaccines*

Capsular polysaccharides of serogroups A and C were initially investigated as vaccine targets largely due to the success of pneumococcal polysaccharide vaccines [Gotschlich, E. C. 1969a. Gotschlich, E.

C. 1969b. Finland, M. 1935. Finland, M. 1931. Francis, T. 1930]. Polysaccharide vaccines are effective in adults and older children but fail to induce an immune response in children less than two years of age as polysaccharides do not induce a T-cell dependent immune responses [Artenstein, M. S. 1970. Gold, R. 1971. Wahdan, M. H. 1977. Wahdan, M. H. 1973. Gold, R. 1975]. Furthermore, polysaccharide vaccines have no effect on meningococcal carriage [Gotschlich, E. C. 1969. Artenstein, M. S.1970. Sivonen, A.1981]. Despite these drawbacks, bivalent (serogroups A and C), trivalent (serogroups A, C and W), and tetravalent (serogroups A, C, W and Y) vaccines were licensed in the 1970s to control sporadic outbreaks and immunise individuals at high risk of developing meningococcal disease [Vodopija, I. 1983. Reingold, A. L. 1985. Peltola, H. 1985. Frasch, C. E.1989].

Following the success of the *Haemophilus influenzae* type B (Hib) glycoconjugate vaccine, in which the *H. influenzae* capsular polysaccharide is conjugated to diphtheria toxoid resulting in the induction of a T-cell dependent immune response and reduction in carriage [Eskola, J. 1993; Adams, W. G. 1993. Conjugation of meningococcal polysaccharides to carrier proteins including diphtheria and tetanus toxoids were trialed and elicited higher antibody titres than observed with polysaccharide vaccines [Beuvery, E. C. 1983. Richmond, P. 1999. Southern, J. 2009. Borrow, R. 2010]. Introduction of a serogroup C conjugate vaccine in the UK during an epidemic of serogroup C disease during the 1990s reduced carriage and invasive serogroup C disease by 89-94% [Balmer, P. 2002. Maiden, M. C. 2008. Campbell, H. 2009]. Furthermore, the vaccine induces T-cell dependent responses, resulting in immunological memory [Trotter, C. L. 2004. Pollard, A. J. 2009]. Implementation of the conjugated serogroup C polysaccharide vaccines in other countries lead to similar reductions in disease [de Greeff, S. C. 2006. Cano, R. 2004. Bettinger, J. A. 2009]. The success of conjugate polysaccharide vaccines is particularly evident by the reduction of carriage, with herd immunity almost eliminating serogroup A meningococcal disease in Africa (MenAfivac) [Sow, S. O. 2011. Sambo, L. 2015. Kristiansen, P. A. 2012. Daugla, D. M. 2014]. Multivalent polysaccharide conjugate vaccines have been formulated and licensed covering serogroups A, C, W and Y [Campbell, J. D. 2002. Shao, P. L. 2009].



### 1.7.7.3 Challenges for meningococcal B vaccines

The capsular polysaccharide of serogroup B *N. meningitidis* is identical to the  $\alpha(2-8)$  *N*-acetyl neuraminic acid (polysialic acid) that is present on human tissues [Finne, J. 1983]. The capsule elicits a poor immune response even after conjugation to carrier proteins, and antibodies recognise host tissue [Finne, J. 1987. Jennings, H. J. 1981]. Therefore vaccination with the serogroup B capsule could generate auto-antibodies [Finne, J. 1987. Jennings, H. J. 1981]. Consequently, vaccines to prevent serogroup B disease must not be capsule based. Outer membrane vesicle (OMV) vaccines have been developed, and successfully used during epidemics [Kelly, C. 2007]. SBA responses from detergent extracted OMVs are mostly directed against the highly variable PorA and are not cross protective [Fredriksen, J. H. 1991. Holst, J. 2009. Tappero, J. W. 1999. Tondella, M. L. 2000]. To broaden the protection of OMV based vaccines, OMVs were extracted from multiple meningococcal strains or from strains expressing more than one PorA. However, antigenic diversity of PorA means that these vaccines are unlikely to induce broad protection [Tondella, M. L. 2000. Holst, J. 2005. Sandbu, S. 2007. van der Ley, P. 1995. Cartwright, K. 1999. Holst, J. 2007].

### 1.7.7.4 Recombinant protein vaccines against meningococcal B

To develop broadly protective vaccines against serogroup B *N. meningitidis*, new strategies were employed largely following the publication of the complete genome sequence of the serogroup B meningococcal strain, MC58 [Tettelin, H. 2000]. The open reading frames (ORF) encoding predicted surface exposed proteins were identified, amplified and expressed in *E. coli* [Pizza, M. 2000]. The recombinant proteins were purified and used to immunise mice prior to identifying antigens that elicit SBA [Pizza, M. 2000]. This approach was termed reverse vaccinology and has been utilised to identify vaccine targets in other pathogens [Talukdar, S. 2014. Chiang, M. H. 2015. Meunier, M. 2016]. Reverse vaccinology and further subsequent studies identified fHbp, Neisserial adhesin A (NadA), and *Neisseria* heparin binding antigen (NHBA) as broadly protective antigens which induce SBA in a panel of meningococcal isolates [Massignani, V. 2003. Fletcher, L. D. 2004. Giuliani, M. M. 2005. Comanducci, M. 2002. Welsch, J. A. 2003. Giuliani, M. M. 2006]. Two additional proteins, GNA1030 and GNA2091 were included in meningococcal B vaccines as fusion proteins with NHBA

and fHbp, respectively, due to these proteins inducing strong immune responses [Pizza, M. 2000. Giuliani, M. M. 2006]. The Food and Drug Administration (FDA), due to the promising immunogenicity data in phase II clinical trials, has recently approved two vaccines for the prevention of serogroup B meningococcal disease under the accelerated approval pathway [Gossger, N. 2012. Santolaya, M. E. 2014. Esposito, S. 2012. Richmond, P. C. 2012. Vesikari, T. 2016]. Bexsero® was licensed in Europe in January 2015 and contains fHbp, NadA, NHBA and OMVs from NZ98/24 which caused the New Zealand epidemic outbreak [Oster, P. 2005]. Pfizer have developed Trumenba® released in the US in November 2014, containing two lipidated fHBps. Previous studies have shown that lipidation of fHbp enhances immune responses in mice [Fletcher, L. D. 2004]. Of note, these vaccines are not restricted to serogroup B meningococcal strains, but have the potential to protect against several meningococcal serogroups. The effect of these meningococcal B vaccines on meningococcal carriage are unknown; although a reduction in carriage of all meningococcal serogroups (but not serogroup B) was observed in university students after immunisation with Bexsero® but this requires further evaluation [Read, R. C. 2014].

#### *1.7.7.5 Components of licensed meningococcal B vaccines*

##### *1.7.7.5.1 fHbp*

To date, over 1,000 unique meningococcal fHbp sequences have been identified (<http://pubmlst.org/neisseria/fHbp/>) and can be divided into three variant groups (V1, V2, and V3) [Masignani, V. 2003. Brehony, C. 2009]. Generated bactericidal responses can provide cross protective SBA across strains expressing fHbp subvariants within the same variant group but are not cross protective between variant fHbp groups [Seib, K. L. 2011]. fHbp is a surface exposed lipoprotein composed of two  $\beta$ -barrels arranged as a bi-lobed structure anchored to the bacterial membrane by a lipid group covalently bound to the initial cysteine residue [Mascioni, A. 2009. Schneider, M. C. 2009]. The C-terminal  $\beta$ -barrel is highly structured with the N-terminus having more pronounced flexibility in V2 fHbp compared to V1 and V3 fHbp [Johnson, S. 2012]. *fhbp* expression occurs under the control of two independent promoters; a bicistronic or long transcript originating from the upstream gene encoding fructose-1,6-bisphosphate (*fba*), and a monocistronic or short transcript

from its own promoter [Oriente, F. 2010]. Early investigations identified putative Fur and FNR box motifs in the *fhbp* promoter suggesting that fHbp is regulated by iron and/or oxygen concentrations [Massignani, V. 2003. Oriente, F. 2010. Sanders, H. 2012]. More recent evidence indicates that *fhbp* transcription is upregulated in iron-rich conditions [Sanders, H. 2012]. Furthermore, fHbp has been shown to be regulated by temperature involving anti-ribosomal binding sites within the ORF [Loh, E. 2013. Loh, E. 2016]. Previous analysis of fHbp determined that different *N. meningitidis* isolates express varying levels of fHbp on the surface [Donnelly, J. 2010]. Moreover, polymorphisms in the promoter region of *fhbp* can determine the level of fHbp expression, and isolates expressing V1 fHbp have significantly higher fHbp levels than V2 or V3 expressing isolates [Biagini, M. 2016]. fHbp expression levels have also been determined as affecting susceptibility of meningococcal isolates to SBA, as the amount of antigen on the bacterial surface correlates with higher SBA responses; therefore fHbp expression levels may determine the efficacy of fHbp meningococcal vaccines [Donnelly, J. 2010. Biagini, M. 2016].

#### 1.7.7.5.2 NadA

NadA is a trimeric autotransporter which is anchored to the outer membrane, and implicated in adhesion and invasion of bacteria [Comanducci, M. 2002. Malito, E. 2014. Capecchi, B. 2005. Nägele, V]. The *nadA* gene is present in approximately 50% of *N. meningitidis* isolates and is associated with hypervirulent lineages, including the ET-5 complex, the ET-37 complex, and cluster A4 [Comanducci, M. 2004]. Interestingly, NadA is rarely associated with meningococcal carriage isolates [Comanducci M, 2002. Comanducci, M. 2004]. There are currently five known variants of NadA, NadA-1 to NadA-5; NadA-1 to NadA-3 are associated with disease isolates and generate a cross-reactive SBA response whereas NadA-4 and NadA-5 are more commonly associated with carriage isolates [Comanducci M, 2002. Comanducci, M. 2004]. Moreover, SBA elicited by NadA-4 is not cross-reactive against NadA-1 to NadA-3 [Comanducci, M. 2004].

#### 1.7.7.5.3 NHBA

NHBA is a surface exposed lipoprotein which binds heparin *via* an Arg-rich repeat region and contributes to serum resistance [Pizza, M. 2000. Serruto, D. 2010] Furthermore, it is likely that NHBA

binds host glycosaminoglycans, e.g. heparin sulphate, contributing to bacterial adhesion and colonisation [Casellato, A. 2014]. Post-translational cleavage of NHBA by NalP adjacent to the Arg-rich region, releases a small NHBA fragment that has been shown to increase endothelial permeability by disrupting tight junctions, resulting in vascular leakage [Casellato, A. 2014]. *nhba* is present in all *N. meningitidis* isolates with the N- and C-termini conserved [Bambini, S. 2009. Jacobsson, S. 2009. Lucidarme, J. 2010]. Sera from meningococcal convalescent patients recognise NHBA and have SBA responses against this protein [Serruto, D. 2010. Giuliani, M. M. 2010. Plested, J. S. 2008]. Furthermore, vaccine induced antibodies can elicit cross-protective responses against strains expressing a range of NHBA peptides [Giuliani, M. M. 2006].

#### *1.7.7.6 Improving immunogenicity of meningococcal vaccines*

Further developments in meningococcal vaccination have involved the optimisation and the evaluation of new vaccine candidates. fHbp specifically binds to human CFH and is a major component of the two licensed serogroup B *N. meningitidis* vaccines [Granoff, D. M. 2009. Maignani, V. 2003. Fletcher, L. D. 2004. Giuliani, M. M. 2006]. Further studies in human CFH transgenic mouse models demonstrated that CFH binding to fHbp impairs immune responses [Beernink, P. T. 2011. Johnson, S. 2012]. Therefore non-functional fHbps have been generated by introducing amino acid substitutions that disrupt the CFH-fHbp interactions [Schneider, M. C. 2009. Johnson, S. 2012. Pajon, R. 2012. van der Veen, S. 2014]. Non-functional V1 fHbps elicit similar or higher SBA than the wild-type protein, with similar results obtained with V2 and V3 [Beernink, P. T. 2011. Johnson, S. 2012. Pajon, R. 2012. van der Veen, S. 2014].

Sequence analysis of *N. gonorrhoeae* identified a homologue of fHbp designated GHfp (gonococcal homologue of fHbp) which does not bind CFH [Hadad, R. 2012. Jongerius, I. 2013]. Interestingly, a frameshift caused by an insertion of a single base (G) at position 40 results in the loss of the lipo-box motif (LXXC) of GHfp and a further eight-base insertion after position 70 re-establishes the correct frame resulting in protein translation [Hadad, R. 2012. Muzzi, A. 2013]. Sequence analysis indicates that GHfp belongs to V3 fHbp [Jongerius, I. 2013]. Although GHfp has been postulated to bind siderophores *in vitro*, the exact function of this protein in the gonococcus has yet to be established

[Veggi, D. 2012]. Furthermore, Ghfp elicits cross-protective immune responses against strains expressing any of the three fHbp variants although SBA titres were higher for V2 and V3 fHbp [Jongorius, I. 2013]. Furthermore, GHfp engineered to express heterologous epitopes from V1 fHbp elicit higher SBA titres for V1 fHbp with similar immune responses for V2 and V3 fHbp [Rippa, V. 2015].

Other vaccine candidates are also currently under investigation including NspA, which is a well-conserved outer membrane protein that induces SBA [Lewis, L. A. 2010. Lewis, L. A. 2012. Martin, D. 1997. Cadieux, N. 1999. Martin, D. 2000]. Unfortunately, vaccination with NspA in human volunteers does not elicit bactericidal antibodies potentially due to NspA binding CFH [Beernink, P. T. 2011. Halperin, S. A. 2007]. Examination of serological responses to ferric enterobactin receptor (FetA) of convalescent patients prompted the evaluation of FetA as a vaccine antigen [Sanders, H. 2015]. Initial trials in animal models indicated that bactericidal antibodies could be generated against FetA in OMVs [Sanders, H. 2015]. Subsequent Phase I clinical trials also generated significant SBA titres [Sanders, H. 2015. Marsay, L. 2015].

#### *1.7.7.7 Conservation of meningococcal vaccine antigens*

There are high rates of genetic exchange between *Neisseria* spp. that colonise human mucosal surfaces, highlighted by the presence of multiple genes encoding meningococcal virulence factors, including FetA, Tfp, Opas, and iron transport [Stabler, R. A. 2005. Snyder, L. A. 2006. Bennett, J. S. 2009. Marri, P. R. 2010. Wörmann, M. E. 2016]. High rates of recombination between *Neisseria* spp. are likely due to the conservation of a 10 bp DNA uptake sequence (GCCGTCTGAA) which contributes to the transformation of *N. meningitidis* and *N. gonorrhoeae* [Liu, S. V. 2002. Snyder, L. A. 2007. Cehovin, A. 2013. Marri, P. R. 2010]. Therefore commensal *Neisseria* spp. might act as reservoir for virulence-associated factors [Marri, P. R. 2010]. More recent studies have evaluated the presence and conservation of antigens in the licensed meningococcal B vaccine, Bexsero® within *Neisseria* to assess the potential effect of this vaccine on the commensal flora [Muzzi, A. 2013]. The conservation of the antigens in Bexsero® is discussed below.

#### 1.7.7.7.1 fHbp

*fHbp* gene is present in *N. cinerea* and *N. polysaccharea*, but not in other commensal species [Muzzi, A. 2013]. Sequence analysis established that *N. cinerea* encodes a V1 fHbp whereas the *N. polysaccharea* fHbp belongs to V3 fHbp. Some strains of *N. polysaccharea* contain *fHbp* with a frameshift mutation [Muzzi, A. 2013]. The expression and function of fHbp from *N. cinerea* and *N. polysaccharea* has not been evaluated.

#### 1.7.7.7.2 NadA

*nadA* is not present in *N. gonorrhoeae* or other *Neisseria* spp. although some *N. cinerea* isolates harbour fragments of *nadA* with only one isolate having an intact *nadA* homologue [Comanducci, M. 2004. Hadad, R. 2012. Muzzi, A. 2013]. The *N. cinerea nadA* gene is related to alleles *nadA4* and *nadA5* from *N. meningitidis* [Comanducci, M. 2004. Muzzi, A. 2013].

#### 1.7.7.7.3 NHBA

Besides being present in all *N. meningitidis* isolates, *nhba* homologues are present in *N. gonorrhoeae*, *N. polysaccharea*, and *N. lactamica*. Conversely, *nhba* is not present in *N. cinerea* [Hadad, R. 2012. Muzzi, A. 2013]. Two functional motifs have been described for NHBA, the lipo-box, which is conserved across the *Neisseria* genus, and the Arg-rich, heparin-binding motif (RFRRSARSRRS) [Serruto, D. 2010]. The initial three amino acids (RFR) of the Arg-rich motif are were not conserved between species while the central six residues (RSARS) are conserved [Muzzi, A. 2013].

### 1.8 *Neisseria cinerea*

*N. cinerea* is a Gram negative diplococcus originally isolated in 1906 and designated *Micrococcus cinereus* [von Lingelsheim, W. 1906]. Isolates of *N. cinerea* have previously been misidentified as *Moraxella catarrhalis* and *Neisseria pseudocatarrhalis* prior to being correctly identified as *N. cinerea* in 1962 [Berger, U. 1962. Knapp, J. S. 1984].

#### 1.8.1 *N. cinerea* carriage

*N. cinerea* frequently colonises the oropharynx of adults and children [Knapp, J. S. 1988. Sheikhi, R. 2015]. Initial studies of *N. cinerea* colonisation rates in the US suggests that approximately 27% of

adults are colonised by *N. cinerea* [Knapp, J. S. 1988]. More recent studies in adults suggest that the colonisation is lower (approximately 10%) whereas estimated carriage rates of *N. cinerea* from children in Iran are approximately 1.6% [Sáez Nieto, J. A. 1998. Sheikhi, R. 2015]. Although there are limited studies, it appears that *N. cinerea* colonisation rates are lower in childhood and increase with age [Gold, R. 1978. Goldschneider, I. 1969. Cartwright, K. A. 1987. Sheikhi, R. 2015].

### 1.8.2 Infections caused by *N. cinerea*

Instances of disease caused by *N. cinerea* are mainly associated with immuno-compromised patients [Kochi, C. V. 1999]. Positive blood cultures for *N. cinerea* have been obtained from patients with meningitis, nosocomial pneumonia, peritonitis, bacteraemia and tricuspid valve endocarditis [Kochi, C. V. 1999. Kirchgerner, V. 1995. von Kietzell, M 2016. Boyce, J. M. 1985. Taegtmeier, M. 2006. Southern, P. M. 1987. Benes, J. 2003]. Furthermore, *N. cinerea* has been isolated from newborns and is associated with conjunctivitis and purulent ophthalmia [Au, Y. K. 1990].

## 2. AIMS

*N. meningitidis* is a common commensal of the human nasopharynx of between 10-40% of the population but in rare instances can cause systemic disease [Stephens, D. S. 2007]. The genetic factors which contribute to an individual's likelihood of developing systemic disease vs. asymptomatic carriage are not known, although individuals with rare defects in the AP or TP have approximately 7,000-10,000 fold increased risk of developing meningococcal disease [Figuroa, J. E. 1991. Ram, S. 2010]. GWAS have identified SNPs in *CFH* and *CFHR3* which are associated with meningococcal disease although the molecular mechanisms underlying these findings have yet to be defined [Davila, S. 2010. Biebl, A. 2015]. Understanding the function of the CFHRs has been hampered by the lack of reagents to study these proteins. Therefore, to determine a molecular mechanism for host susceptibility to meningococcal disease, novel reagents need to be generated. My initial aims are to:

1. Generate and characterise specific mAbs against CFHRs.
2. Determine if *N. meningitidis* binds CFHR3 and Investigate the biological relevance of *N. meningitidis* binding CFHR3.

Multi-component protein based vaccines, which include fHbp, have been licensed against serogroup B *N. meningitidis* [Pizza, M. 2000. Massignani, V. 2003. Fletcher, L. D. 2004]. *fHbp* has been identified in *N. gonorrhoeae* and commensal *Neisseria* spp. including *N. cinerea* [Hadad, R. 2012. Muzzi, A. 2013]. *N. cinerea* also colonises the human respiratory tract therefore implementation of protein based vaccines containing fHbp could affect the nasopharyngeal flora. My final aims were to:

3. Determine if *N. cinerea* expresses fHbp and if expressed fHbp is functionally similar to meningococcal fHbp.
4. Evaluate the immune response elicited by Bexsero® against *N. cinerea*.



### 3. MATERIALS AND METHODS

#### 3.1. Antibodies

Antibodies used throughout this study are listed in Table 3.1.

Antibodies	Description	Isotype	Reference/ Catalogue number
HSL-1*	mouse monoclonal raised against CCP domains 4-5 of CFHR3	IgG1κ	This Study
CFHR1	mouse monoclonal against Glu19-Ala328 of CFHR1	IgG2B	R&D, MAB4247
CFHR2	mouse monoclonal raised against CFHR2		Gift Paul Morgan
CFHR4	mouse monoclonal against Glu20-Glu331 of CFHR4	IgG1	R&D, MAB5980
CFHR5	goat polyclonal raised against Glu19-Glu569 of CFHR5	-	R&D, AF3845
CFHR5	mouse monoclonal raised against Glu19-Glu569 of CFHR5	IgG1	R&D, MAB3845
fHbp	mouse polyclonal sera raised against fHbp V1	-	[Johnson, S. 2012]
GHfp	mouse polyclonal sera raised against GHfp	-	[Jongorius, I. 2013]
RecA	rabbit polyclonal raised against <i>E.coli</i> RecA	-	Abcam, ab63797
OX24	mouse monoclonal which recognises an epitope in CCP domain 5 of CFH	IgG1κ	[Sim, E. 1983]
CFH	goat polyclonal raised against CFH	-	Calbiochem, 341276
HRP-mouse IgG	goat anti-mouse immunoglobulins antibody conjugated to HRP	-	Dako, P0447
HRP-Goat IgG	rabbit anti-goat immunoglobulins antibody conjugated to HRP	-	Dako, P0160
HRP-rabbit IgG	goat anti-rabbit IgG antibody conjugated to HRP	-	Santa Cruz, sc-2004
647-mouse IgG	goat anti-mouse IgG (H+L) conjugated to Alexa Fluor® 647	-	LifeTech, A-21235
488-mouse IgG	goat anti-mouse IgG (H+L) conjugated to Alexa Fluor® 488	-	LifeTech, A-11029

Table 3.1: Antibodies

## 3.2. Mouse immunisations

All animal experiments were carried out under protocols reviewed and approved by the Home Office, United Kingdom under licence number PPL 30/3194.

### 3.2.1 Generation of mAbs

Antigens used are listed in Table 3.2 and were conjugated to keyhole limpet hemocyanin (KLH) carrier (Imject™ mKLH, PBS, Pierce) via N-hydroxysuccinimide ester cross-linking according to the manufacturer's instructions. Female BALB/c mice (Charles River, Margate; >8 weeks of age) were immunised with 50 µg of antigen emulsified in an equal volume of TitreMax®Gold (Sigma, UK) and administered subcutaneously on day 1. Two subsequent immunisations were administered intraperitoneally (IP) on days 15 and 29 with 50 µg of antigen in PBS. A booster of 100 µg of antigen was given by IP in PBS three days prior to cell fusion on day 43. Mice were sacrificed by cervical dislocation and the spleens removed. Blood was collected by cardiac puncture.

CFHR	Antigen (Sequence)	Spleen number
CFHR2	Recombinant CFHR2 <sub>(3,4)</sub>	1
	Recombinant CFHR2 <sub>(3,4)</sub>	2
CFHR3	Recombinant CFHR3 <sub>(4,5)</sub>	1
	Recombinant CFHR3 <sub>(4,5)</sub>	2
CFHR4	Peptide (ENSRAKSNGM)	1
	Peptide (ENSRAKSNGM)	2
CFHR4	Recombinant CFHR4 <sub>(4,5)</sub>	1
	Recombinant CFHR4 <sub>(4,5)</sub>	2
CFHR5	Peptide (IAGVNIKTLKLSG)	1
	Peptide (IAGVNIKTLKLSG)	2
CFHR5	Recombinant CFHR5 <sub>(8,9)</sub>	1
	Recombinant CFHR5 <sub>(8,9)</sub>	2

Table 3.2: Summary of antigens used in the generation of mouse anti-human CFHR mAbs

J.C. and P.W. expressed and purified recombinant CFHR CCP domains (CCP domains in subscript)

Recombinant C-terminal CCP domains of CFHR2-CFHR5 were expressed using pET-15b vectors in *E. coli* BL21 (D3). Peptides were designed to regions with low sequence identity with other CFHRs and CFH. Both recombinant proteins and synthetic peptides were conjugated to KLH prior to immunising two BALB/c mice per antigen.

Antigens were conjugated to KLH prior to immunisation by Dr. Joseph Caesar, University of Oxford. CFHR CCP domains used for immunisations and screening were expressed and purified by Dr. Joseph Caesar and Dr. Philip Ward, University of Oxford.

### 3.2.2 Generation of immune sera

Eight female BALB/c mice (6-8 week old, Charles Rivers, Margate) were immunised with recombinant V1.1 fHbp or *N. cinerea* fHbp (20 µg) absorbed to aluminium hydroxide (final composition; Al(OH)<sub>3</sub> 0.5 mg/ml, Histidine-HCL 10 mM) by mixing overnight at 4°C, or with Bexsero® (total protein 20 µg). The antigens were given by IP on days 0, 21 and 35. Sera was collected on day 49 by terminal anaesthesia and cardiac puncture.

## 3.3. Monoclonal antibody production

### 3.3.1. Media and supplements for mAb production

Buffers and media used to prepare mAbs are listed in Table 3.3.

	Media	Foetal bovine serum	Penicillin/ Streptomycin	L-glutamine	Ultrosor G	HAT*	HT**	DMSO
Myeloma media	RPMI 1640	10%	50 units/ml / 50 µg/ml	2 mM	-	-	-	-
HEPES buffered fusion media with FBS	RPMI 1640, HEPES	10%	50 units/ml / 50 µg/ml	4 mM	-	-	-	-
HEPES buffered fusion media w/o FBS	RPMI 1640, HEPES	-	50 units/ml / 50 µg/ml	4 mM	-	-	-	-

Fusion Recovery media	RPMI 1640	10%	50 units/ml / 50 µg/ml	2 mM	1%	-	-	-
Hybridoma Selection media	RPMI 1640	10%	50 units/ml / 50 µg/ml	2 mM	1%	2%	-	-
Hybridoma Competent media	RPMI 1640	10%	50 units/ml / 50 µg/ml	2 mM	1%	1%	-	-
Freezing media	-	85%	-	-	-	-	-	15%
Hybridoma recovery media	RPMI 1640	10%	50 units/ml / 50 µg/ml	2 mM	-	-	1%	-

**Table 3.3: Media and supplements used in the generation of mAbs**

\* HAT supplement (50x) is a mixture of sodium hypoxanthine (5 mM), aminopterin (20 µM) and thymidine (0.8 mM)

\*\* HT supplement (50x) is a mixture of sodium hypoxanthine (5 mM), and thymidine (0.8 mM)

### 3.3.2. Spleen preparation prior to cell fusion

Spleens were washed in 5 ml of HEPES buffered fusion media containing 10% FBS, then placed in a petri dish containing 5 ml of fresh HEPES buffered fusion media containing 10% FBS. The spleen was dissected into two and plasma cells were dissociated from the spleen using tweezers. Plasma cells were pelleted by centrifugation at 280 x *g* for 5 minutes, then resuspended in 10 mls of HEPES buffered fusion media without FBS if immediately proceeding with fusion protocol, or in 3 mls of freezing media for long term storage. Prior to cell fusion, frozen plasma cells were thawed and immediately placed into 15ml of HEPES buffered fusion media with FBS prior to pelleting at 280 x *g* for 5 minutes. Plasma cells were resuspended in 10 mls of HEPES buffered fusion media without FBS prior to cell fusion.

### 3.3.3. Cell fusion

NS0 myeloma cells were grown in myeloma media to confluency. Three days prior to cell fusion the myeloma cells were diluted 1:40 to ensure that cells were in exponential growth at the time of fusion. Plasma cells ( $10^8$ ) and NS0 myeloma cells ( $10^7$ ), or alternatively a ratio of 1 myeloma cell to every 10

plasma cells, were combined and washed in 30 ml of HEPES buffered fusion media without FBS and pelleted for 10 minutes at 280 x *g*. The supernatant was removed and the cell pellet was dissociated by gentle tapping. All further steps were conducted in a 37°C waterbath. Cell fusion was initiated by the addition of 1.2 ml of polyethylene glycol (PEG 1500, Roche, UK) over one minute with continual agitation. While continuing to agitate the cells, 7 ml of HEPES buffered fusion media without FBS was added slowly over seven minutes; cell fusion was halted with 8 ml of HEPES buffered fusion media with FBS. Fused cells were then pelleted at 280 x *g* for 5 minutes, and the cells resuspended in 10 ml, of fusion recovery media. The fused cells were then added to 190 ml of fusion recovery media, and 1 ml plated into each well of eight 24-well plates. After 24 hours, 1 ml of hybridoma selection media was added to each of the wells. Cells were then incubated for seven to ten days prior to screening for positive clones.

#### 3.3.4. Cloning hybridomas by limiting dilution

Hybridomas were thawed a few days prior to cloning to ensure that cells were in exponential growth. Cells were enumerated and 10 µl of cells were placed in 10 ml of cloning media (hybridoma competent media with 1% or 2% Condimed, Roche, 11088947001) then cells were diluted to 5 cells/ml in a total volume of 16 ml of cloning media. 200 µl of solution containing diluted hybridomas was added to 80 wells of a 96-well plate then incubated for five to seven days. Each well was screened under a microscope to identify those containing single colonies prior to screening for specific mAbs in the media. Cells were sequentially expanded into T-75 tissue culture flasks for storage and mAb production.

#### 3.3.6. Monoclonal antibody isotyping

The isotype of mAbs was determined using a mouse isotyping kit (AbD Serotec, UK, catalogue number MMT1) according to manufacturer's instructions. In brief, microparticle beads coupled to anti-mouse kappa and anti-mouse lamda mAbs bind to murine antibodies. The isotyping strip contains immobilised goat anti-mouse antibodies specific for mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA along with a second strip with immobilised antibodies specific for mouse lamda and kappa light chains. Tissue culture supernatants containing mAbs were diluted in PBS to approximately 1

$\mu\text{g/ml}$ , and 200  $\mu\text{l}$  of mAb was incubated with the microparticle beads at room temperature for one minute. The test strips were then added to the mAb conjugated microparticle beads; blue aggregates form after 5-10 minutes incubation where the microparticle beads are bound by the specific isotyping antibodies.

### 3.3.7. Monoclonal antibody production and purification

Hybridoma cell cultures were transferred to serum free or ultra-low bovine IgG prior to antibody production. Cells were grown in hybridoma competent media without antibiotics or Ultrosor G serum substitute. Cells were then serially diluted into hybridoma recovery media and subsequently serially diluted into myeloma media without antibiotics. Hybridoma cells were then adapted to growth in either RPMI-1640 with L-glutamine with either 10% ultra-low IgG FBS (Life Technologies, 16250078) or 5% FreeAdd serum replacement (BioWest, S6000) prior to harvesting tissue culture supernatant for mAb purification. Cell debris was removed by centrifugation at 3,500  $\times g$  and the supernatant passed through a 0.22  $\mu\text{m}$  filter prior to antibody purification. Antibodies were purified using Pierce™ Protein G Agarose resin (ThermoScientific, 20398) according to manufacturer's instructions. Antibodies were eluted with 0.1 M glycine, pH 2.5 and dialysed overnight into Tris pH 7.5, then further purified by size exclusion chromatography (Superdex HiLoad 16/600 200pg, GE Healthcare, Little Chalfont, UK, buffer 50 mM Tris pH 7.5, 150 mM NaCl).

## 3.4. Enzyme-linked immunosorbent assay (ELISA)

Primary antibodies were used at the following concentrations: hybridoma tissue culture supernatants, neat; anti- fHbp polyclonal sera, 1:100; anti-CFH, 1:1,000 (Calbiochem). Goat anti-mouse HRP (Dako, UK) and rabbit anti-goat HRP (Dako, UK) secondary antibodies were used at a final concentration of 1:5,000.

### 3.4.1 mAb screening

To identify positive hybridomas wells, ELISA plates (F96 maxisorp, Nunc) were coated with 50  $\mu\text{l}$  of unconjugated immunising antigen in PBS (5  $\mu\text{g/ml}$ ) for 1 hour at room temperature. Plates were washed with PBS 0.05% Tween 20 prior to blocking with 2% skimmed milk in PBS 0.05% Tween 20 overnight at 4°C. Plates were stored at -20°C prior to screening. To determine the specificity of mAbs

produced by hybridomas, ELISA plates were coated with the two C-terminal CCP domains of CFH or CFHR1-CFHR5 (5 µg/ml, 50 µl per well) prior to blocking and stored as described for the immunising antigen. To evaluate the immunogenicity of whole IgG and antibody Fab fragments, ELISA plates were coated with CFHR3<sub>(4,5)</sub> (1 µg/ml, 50 µl per well) overnight at 4°C. Plates were washed with PBS 0.05% Tween 20 prior to blocking with 2% skimmed milk in PBS 0.05% Tween 20 for 1 hour at 37°C. Blocking buffer was removed prior to washing with PBS 0.05% Tween 20 and labelling protocol followed as below.

For ELISAs, plates were incubated with neat hybridoma culture supernatant, or purified Fab fragment for 1 hour at room temperature prior to washing with PBS-0.05% Tween 20. Plates were incubated with HRP-conjugated goat anti-mouse for a further hour. After washing, antibody recognition was detected using ELISA substrate and stop solution (Roche) according to manufacturer's instructions. Plates were read at OD<sub>A450nm</sub> using SpectraMax M5 spectrophotometer (Molecular Devices)

#### 3.4.2. Evaluation of CFH binding

To determine CFH binding, 96 well microplates (F96 maxisorp, Nunc) were coated with recombinant fHbp (3 µg/ml, 50 µl per well) overnight at room temperature. All further incubation steps were performed for one hour at 37°C. Plates were washed with PBS 0.05% Tween 20 prior to blocking with 3% bovine serum albumin (BSA) in PBS with 0.05% Tween 20. Plates were incubated with full length recombinant CFH (Sigma) in two fold dilutions from 10 µg/ml, and binding was detected with anti-CFH antibody (Calbiochem) and HRP-conjugated rabbit anti-goat antibody (Dako). CFH binding was visualised with substrate and stop solution (Roche) according to manufacturer's instructions, and the OD<sub>A450nm</sub> measured (SpectraMax M5, Molecular devices). Statistical significance was tested using a unpaired student t-test (GraphPad Prism v.6.0) to compare means ± S.D using a p<0.05 cutoff for significance.

#### 3.4.3. Evaluation of immunogenicity sera

To analyse the immunogenicity of sera generated from mice immunized with either recombinant fHbp or Bexsero®, ELISA plates were coated with recombinant V1.1 fHbp, V1.110 fHbp and BSA (2.5 µg/ml, 50 µl per well) overnight at room temperature. Pooled mouse sera was used as primary

antibody with three-fold dilutions from a starting dilution of 1:300. All incubation steps were performed for one hour at 37°C and plates were blocked with 3% bovine serum albumin (BSA) in PBS with 0.05% Tween 20. Antibody binding was detected with secondary HRP-conjugated goat anti-mouse antibody (Dako) and visualised as above.

### 3.5. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE analysis of proteins was performed according to the method described by Sambrook *et al.* [Sambrook, J. 1989]. Reagents used to prepare both 12% and 16% polyacrylamide gels are shown in Table 3.4.

		Stacking Gel		
		Resolving Gel		
		4%	12%	16%
30% Acrylamide (ml)	Severn Biotech, 20-2600-05	1.3	4.0	5.3
0.5 M Tris pH 6.8 (ml)	Sigma, T1503	2.5	-	-
1.5 M Tris pH 8.8 (ml)	Sigma, T1503	-	2.5	2.5
10% SDS (μl)	Sigma, L3771	100.0	100.0	100.0
dH <sub>2</sub> O (ml)	-	6.1	3.4	2.1
TEMED (μl)	Sigma, T9281	10.0	5.0	5.0
10% APS (μl)	Sigma, A3678	50.0	50.0	50.0
Total volume (ml)		10.0	10.0	10.0

Table 3.4: Reagents for the preparation of polyacrylamide gels

SDS-PAGE was performed using the Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (BioRad) and polyacrylamide gels were run in SDS buffer (200 mM glycine, 248 mM Tris, 34 mM SDS) at 100 V until the gel front reached the bottom of the gels.



### 3.6. Western Blotting

Primary antibodies were used at the following concentrations: hybridoma tissue culture supernatants, 1:2; anti-fHbp V1.1 polyclonal sera, 1:1,000 [Johnson, S. 2012]; anti-GHfp polyclonal sera, 1:10,000 [Jongerijs, I. 2013]; anti-fHbp polyclonal sera, 1:1,000 (this study); anti-RecA, 1:5,000 (Abcam, Cambridge); anti-CFH, 1:10,000 (Calbiochem). Secondary antibodies were used at a final concentration of 1:10,000; goat anti-mouse HRP (Dako, UK), rabbit anti-goat HRP (Dako, UK) and goat anti-rabbit HRP (Santa Cruz Biotechnology, Germany).

After SDS-PAGE, proteins were transferred to immobolin P polyvinylidene fluoride (PVDF) membranes (Millipore, USA) using the Trans-Blot® SD semi-dry transfer system (Biorad, USA). PVDF membranes were activated in methanol prior to equilibrating membranes and Whatman paper in Transfer buffer (48 mM Tris base, 39 mM Glycine, 20% methanol (v/v), pH8.3). The transfer sandwich was assembled into the Trans-Blot® drawer with four sheets of Whatman paper, activated PVDF membrane, polyacrylamide gel, and finally four further sheets of Whatman paper. Protein transfer was performed at 15V for 30 minutes. Membranes were blocked in 3% (w/v) dry milk/PBS with 0.05% (v/v) Tween-20 overnight, then incubated with primary antibodies for 60 minutes. Membranes were washed three times with PBS with 0.05% (v/v) Tween-20 and incubated for a further hour with HRP-conjugated secondary antibodies. Binding was detected with ECL Western Blotting Detection kit (Amersham, USA) or ECL Prime Western Blotting Detection kit (Amersham, USA). All antibodies were diluted in 1% (w/v) dry milk/PBS.

#### 3.6.1. mAb specificity screening

To determine specificity of the mAbs, the two C-terminal CCP domains of CFH and CFHR1-CFHR5 (10 µg/ml), and NHS (1 in 200) diluted in 1x SDS-PAGE sample buffer (100mM Tris-HCl, pH6.8, 20µM β-mercaptoethanol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) were separated on 16% polyacrylamide gels with PageRuler Plus Prestained protein marker (ThermoScientific) or All blue protein ladder (BioRad) prior to western blot analysis. Membranes were probed with hybridoma tissue culture supernatant and detected with HRP conjugated goat anti-mouse secondary antibody (Dako, 1/10,000).

### 3.6.2. Detection of fHbp

For the preparation of whole cell lysates, bacteria were grown overnight on BHI and resuspended in PBS. The concentration of the bacterial suspension was quantified by measuring the A<sub>260nm</sub> of 20 µl of the bacterial suspension mixed with 980 µl of P2 lysis buffer (0.1 M NaOH, 1% SDS; Qiagen) using a UVVIS spectrophotometer (Shimadzu). The bacterial suspension was adjusted to 10<sup>9</sup> colony-forming units (c.f.u.) ml<sup>-1</sup>, centrifuged at 16,000 x g for 5 minutes, and resuspended in 100 µl of SDS-PAGE sample buffer, and boiled for 10 min prior to loading 10 µl for analyses by SDS-PAGE.

For the detection of fHbp, bacterial whole cell lysates or recombinant fHbp (10 µg/ml in SDS-PAGE sample buffer) were separated on 12% polyacrylamide gels. After transferring to a PVDF membrane, membranes were probed with anti-fHbp V1.1 sera [Johnson, S. 2012], anti-Ghfp [Jongerijs, I. 2013] or anti-fHbp polyclonal sera (this study) prior to detection with a HRP conjugated goat anti-mouse secondary antibody. For whole cell lysates, RecA was used as a loading control and detected with HRP conjugated goat anti-rabbit secondary antibody. Alternately, polyacrylamide gels were stained with Coomassie blue (0.2% Coomassie Blue R-250, 40% ethanol (v/v), 10% acetic acid (v/v), 50% d H<sub>2</sub>O) for 10 minutes then de-stained overnight in dH<sub>2</sub>O.

### 3.6.3. Evaluation of CFH binding

For far western blot analysis, whole cell lysates or recombinant proteins were separated on 12% polyacrylamide gels as for the detection of fHbp prior to transferring to a PVDF membrane. Membranes were then initially incubated with NHS (1:200 diluted in 1% (w/v) dry milk/PBS) for 60 minutes and then washed with PBS with 0.05% (v/v) Tween-20. CFH binding was then detected with anti-CFH pAb and HRP conjugated rabbit anti-goat secondary antibody as described above.

## 3.7. Bacterial strains and growth

*N. meningitidis* and *N. cinerea* (Table 3.5) were grown on brain heart infusion (BHI) agar (Oxoid) or BHI broth supplemented with Levinthal's base (500 ml defibrinated horse blood, autoclaved with 1 l BHI broth). Bacteria grown on solid media were incubated overnight at 37°C in the presence of 5% CO<sub>2</sub>. Liquid culture were inoculated with 10<sup>9</sup> bacterial per 10 ml and grown with shaking at 180 r.p.m. at specified temperatures to OD A<sub>600</sub> ~0.5 unless otherwise specified.

*E. coli* was grown in Luria-Bertani (LB) broth or on LB agar. Liquid cultures were grown in 5ml of media inoculated from a single colony overnight at 37°C with shaking at 180 r.p.m. Bacteria grown overnight in liquid cultures were diluted the next day, 1 in 100 into fresh media for protein expression.

<i>N. meningitidis</i>							
Strain	fHbp variant group	fHbp sub-variant	Year	Country	cc	Serogroup: serotype: serosubtype	Reference
H44/76	1	1	1976	Norway	32	B:15:P1.7,16	[Maiden, M. C. 1998]
H44/76Δ <i>fhbp</i>	-	-	-	-	32	B:15:P1.7,16	[Lucidarme, J. 2011]
M1239	3	28	1994	USA	41/44	B:14:P1.23,14	[Masignani, V. 2003]
M1239Δ <i>fhbp</i>	-	-	-	-	41/44	B:14:P1.23,14	[Jongorius, J. 2013]
M1239Δ <i>fhbp</i> :: <i>fhbp</i> V1.1	1	1	-	-	41/44	B:14:P1.23,14	[Jongorius, J. 2013]
M1239Δ <i>fhbp</i> :: <i>fhbp</i> V1.14	1	14	-	-	41/44	B:14:P1.23,14	This study
M1239Δ <i>fhbp</i> :: <i>fhbp</i> V2.22	2	22	-	-	41/44	B:14:P1.23,14	This study
M1239Δ <i>fhbp</i> :: <i>fhbp</i> V3.28	3	28	-	-	41/44	B:14:P1.23,14	This study
M1239Δ <i>fhbp</i> :: <i>fhbp</i> V3.28 <sup>T286A</sup>	3	28	-	-	41/44	B:14:P1.23,14	This study
NZ98/254	1	14	1998	New Zealand	41/44	B:4:P1.4	[Martin, D. R. 1998]
FAM18	2	22	1983	USA	11	C:2a:P1.5,2	[Feavers, I. M. 1999]

<i>N. cinerea</i>							
CCUG 346 T	1	110	Unknown	Unknown	ND	ND: P1.ND,ND	[Bennett, J. S. 2012]
CCUG 346 T $\Delta$ fhbp	-	-	-	-	ND	ND: P1.ND,ND	This study
CCUG 346 T $\Delta$ fhbp:: <i>N.c. fhbp</i>	1	110	-	-	ND	ND: P1.ND,ND	This study
CCUG 25879	1	564	1989	Sweden	ND	ND: P1.ND,ND	[Bennett, J. S. 2012]
CCUG 27178 A	1	110	1983	Unknown	ND	ND: P1.ND,ND	[Bennett, J. S. 2012]
CCUG 53043	1	534	2006	Sweden	ND	ND: P1.ND,ND	[Bennett, J. S. 2013]
CCUG 5746	1	563	1977	Sweden	ND	ND: P1.ND,ND	[Bennett, J. S. 2012]

Table 3.5: *N. meningitidis* and *N. cinerea* strains

Year and country of isolation, clonal complex (cc), and serogroup:serotype:serosubtype classification along with fhbp variant family and subvariant group are shown.

### 3.8. Antibiotics

Antibiotics were used at the concentrations listed in Table 3.6. Stock solutions of kanamycin (1 mg/ml) and carbenicillin (1 mg/ml) were prepared in dH<sub>2</sub>O passed through a 0.22  $\mu$ m filter prior to storage at -20°C. Erythromycin (100  $\mu$ g/ml) was prepared in ethanol prior to storage at -20°C.

	Kanamycin ( $\mu$ g ml <sup>-1</sup> )	Erythromycin ( $\mu$ g ml <sup>-1</sup> )	Carbenicillin ( $\mu$ g ml <sup>-1</sup> )
<i>N. meningitidis</i>	100	2	-
<i>N. cinerea</i>	100	10	-
<i>E. coli</i>	50	-	100

Table 3.6: Concentration of antibiotics

### 3.9. Molecular Cloning

The genome sequences of the *Neisseria* strains analysed in this work are publicly available online in the PubMLST *Neisseria* BIGSdb (<http://pubmlst.org/neisseria/>).

#### 3.9.1. Genomic DNA preparation

Genomic DNA was isolated from *Neisseria* strains using the Wizard® Genomic DNA Purification Kit (Promega). Briefly, bacteria were grown overnight on BHI agar prior to resuspending in 600 µl Nuclei lysis solution. Bacteria were incubated at 80°C for 5 minutes and allowed to cool to room temperature. Protein was precipitated with the addition of 200 µl of Protein precipitation solution prior to centrifuging at 15,000 x *g* for 3 minutes. The supernatant was then added to 600 µl isopropanol, and the DNA pelleted at 15,000 x *g* for 5 minutes, then further washed in 600 µl of 70% ethanol. DNA was again pelleted and allowed to dry. DNA was resuspended in 100 µl of dH<sub>2</sub>O with 1 µl of RNAase.

#### 3.9.2. Polymerase Chain Reaction (PCR)

For *fhbp* amplification from *Neisseria* genomic DNA, reaction mixtures contained 1 pmol/ µl of each primer (Primers listed in table 3.7 and synthesized by Sigma), 1.5 mM MgCl<sub>2</sub> (Roche), 200 µM dNTPs (New England Biolabs), 2 U Expand High fidelity DNA polymerase (Roche), and 0.1 volume polymerase buffer (Roche) in a total volume of 25 µl. The conditions for thermal cycling were an initial denaturation step of 94°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 80 seconds (Expand High fidelity DNA polymerase extension time 1 kb/min) and a final extension step at 72°C for 7 minutes. Taq DNA polymerase (1 kb/min extension time; Sigma) was used for the amplification of *fHbp* prior to sequencing.

To generate *N. cinerea*  $\Delta fhbp$ , the PCR volume was increased to 50 µl; Herculase II Fusion DNA polymerase (Agilent) was used for amplification and extension times adjusted accordingly (15 sec/min).

Primer	fHbp variant group	fHbp amplified (strain)	Primer sequence (restriction site underlined)	Restriction site	Reference
pgcc4V1.1 F	1.1	H44/76	CGG <u>TAAATTA</u> AGGAGTAATTTTG TGAATCGAACTGCCTTCTGCT	<i>PacI</i>	[Jongerijs, I. 2013]
pgcc4V1.1 R	1.1	H44/76	CGG <u>TAAATTA</u> ATTATTGCTTGGCG GC	<i>PacI</i>	
pgcc4V1.14 F	1.14	NZ98/254	CGG <u>TAAATTA</u> AGGAGTAATTTTG TGAACCGAACTGCC	<i>PacI</i>	
pgcc4V1.14 R	1.14	NZ98/254	CGG <u>TAAATTA</u> ATTATTGCTTGGCG GCAAGAC	<i>PacI</i>	
pgcc4V2.22F	2.22	FAM18	CGG <u>TAAATTA</u> AGGAGTAATTTTG TGAACCGAACTGCCTTCTGCT	<i>PacI</i>	
pgcc4V2.22R	2.22	FAM18	CGG <u>TAAATTA</u> ACTACTGTTTGCCG GCGATGC	<i>PacI</i>	
pgcc4V3.28 F	3.28	M1239	CGG <u>TAAATTA</u> AGGAGTAATTTTG TGAATCGAACTGCCTTCTGCT	<i>PacI</i>	
pgcc4V3.28 R	3.28	M1239	CGG <u>TAAATTA</u> ACTACTGTTTGCCG GCGATGC	<i>PacI</i>	
pncc1V1.110 F	1.11	CCUG 346 T	CGG <u>TAAATTA</u> AGGAGTAATTTTG TGAACC	<i>PacI</i>	
pncc1V1.110 R	1.11	CCUG 346 T	CGG <u>TAAATTA</u> ATTATTGCTTGGCG GC	<i>PacI</i>	
<i>F<sub>fhbpV1.110</sub></i>	1.11	CCUG 346 T	GCC <u>ATATGAT</u> GGCCGCCGACAT	<i>NdeI</i>	
<i>R<sub>fhbpV1.110</sub></i>	1.11	CCUG 346 T	GCCTCGAGTTGCTTGGCGGCAAG GCCGATAT	<i>XhoI</i>	
<i>F N.cΔfhbp</i>	-	H44/76Δ <i>fhbp</i>	GCC <u>ATATGG</u> CTGAAAGTCATCAA CGAAT	<i>NdeI</i>	
<i>R N.cΔfhbp</i>	-	H44/76Δ <i>fhbp</i>	GCCTCGAGGTGCAATACAAAATG CCGTCCGAACGGTAA	<i>XhoI</i>	

## Mutagenesis primers

Primer name	fHbp		Primer sequence	Reference
	variant	Modification		
	group			
V3.28 <sup>T286A</sup> F	3.28	T286A	AGCGAAGAAAAAGGCgctTACCA CCTCGCCCTT	[van der Veen, S. 2014]
V3.28 <sup>T286A</sup> R	3.28	T286A	AAGGGCGAGGTGGTAagcGCCTT TTTCTTCGCT	[van der Veen, S. 2014]
V1.1 <sup>I311A</sup> F	1.1	I311A	AAAACCGTAAACGGCgcaCGCCA TATCGGC	[Johnson, S. 2012]
V1.1 <sup>I311A</sup> R	1.1	I311A	GCCGATATGGCGtgcGCCGTTTAC GGTTTT	[Johnson, S. 2012]

**Table 3.7: Primers**

Lower case bases indicate target site for site-directed mutagenesis

Plasmids were purified from *E. coli* using the GenElute Plasmid Miniprep kit (Sigma) according to manufacturer's instructions and were analysed by gel electrophoresis. DNA was combined with gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol) and resolved on 1% agarose (Sigma) gels with 1x Tris Acetate EDTA (TAE) buffer (Tris 0.4 M, EDTA 0.01 M, acetic acid 0.2 M, Syber Safe DNA stain (1:10,000). DNA was visualised by UV illumination using a Safe Imager™ 2.0 Blue Light Transilluminator.

### 3.9.3. Site directed mutagenesis

Alanine substitution mutants, V1.1<sup>I311A</sup> and V3.28<sup>T286A</sup> fHbp, were generated from either the complementation or expression plasmids using QuikChange II Site-Directed Mutagenesis Kit (Agilent) according to manufacturer's instructions. Briefly, reaction mixtures contained 35 ng DNA template, 100 ng/μl of both forward and reverse primers (Primers listed in Table 3.6 and synthesised by Sigma), 800 μM dNTPs (New England Biolabs), 2.5 U *PfuUltra* HF DNA polymerase, and 0.1 volume reaction buffer in a total volume of 50 μl. Conditions for thermal cycling were an initial denaturation step of 95°C for 30 seconds; followed by 18 cycles of 95°C for 30 seconds, 55°C for 60 seconds and

68°C for either 11 minutes or 7 minutes for PGCC4/ PNCC1 or pET-21b, respectively (extension time 1 kb/min) and a final extension step at 68°C for 10 minutes. Following amplification, reaction mixtures were incubated with *DpnI* for 1 hour at 37°C to digest methylated DNA prior to transforming into *E. coli* DH5 $\alpha$ .

#### 3.9.4. Bacterial transformation

PCR products or linearised plasmid DNA was transformed into *Neisseria* by spot transformation as follows. *Neisseria* were harvested from overnight growth on BHI agar into PBS and a 10  $\mu$ l aliquot was placed onto BHI agar and allowed to dry. DNA (approx. 500 ng) was added to the bacteria and allowed to dry. Bacteria were incubated at 37°C, 5% CO<sub>2</sub> for 5 h for meningococcal strains or overnight for *N. cinerea*. Transformants were selected by streaking bacterial growth onto BHI agar containing antibiotics. Transformants were verified PCR and sequenced.

For *E. coli* transformations, ligation reactions were added to 50  $\mu$ l of DH5 $\alpha$  or BL21(DE3)Lys (Promega) competent cells and incubated on ice for 30 minutes. Cells were heat shocked for 45 seconds at 37°C and immediately incubated on ice for 2 minutes, then 800  $\mu$ l of LB broth was added to the cells and incubated at 37°C with continuous agitation (180 r.p.m) for one hour prior to plating on selective media.

#### 3.9.5. Construction of complemented *N. meningitidis* and *N. cinerea* strains

Complemented *Neisseria* strains were constructed by amplifying *fhhp* using Expand High Fidelity DNA polymerase (Roche, UK) from genomic DNA isolated from *Neisseria* strains listed in Table 3.4 using primers listed in Table 3.6. PCR products were ligated into either pGCC4 for *N. meningitidis* or pNCC1 for *N. cinerea* with T4 DNA ligase [Mehr, I. J. 1998. Wörmann ME. 2016]. In brief, ligations were performed using a 1:5 ratio of insert to vector with 4  $\mu$ l 5x T4 ligase buffer and 1  $\mu$ l T4 DNA ligase (1 U/ $\mu$ l) (LifeTechnologies, 15224017) in 20  $\mu$ l. Ligation reactions were incubated at 16°C overnight prior to transformation into *E. coli* DH5 $\alpha$ . Complementation plasmids are listed in Table 3.8. Prior to transforming *Neisseria* strains, plasmids were linearised by *ClaI* digestion to remove plasmid-encoded kanamycin resistance, the construct was gel extracted and then the linearised fragments were then transformed into *N. meningitidis* strain or *N. cinerea* strain.



<i>E. coli</i> strain	Plasmid	Insert	Modification	Antibiotic resistance cassette	Reference
DH5α	pGCC4	V1.1	-	kan/ erm	[Jongerijs, I. 2013]
DH5α	pGCC4	V1.1 <sup>I311A</sup>	I311A	kan/ erm	This study
DH5α	pNCC1	V1.110	-	kan/ erm	This study
DH5α	pGCC4	V1.14	-	kan/ erm	This study
DH5α	pGCC4	V2.22	-	kan/ erm	This study
DH5α	pGCC4	V3.28	-	kan/ erm	This study
DH5α	pGCC4	V3.28 <sup>T286A</sup>	T286A	kan/ erm	This study

Table 3.8: *Neisseria* complementation plasmids

### 3.9.6. Generation of fHbp expression constructs

V1.1 and V1.1<sup>I311A</sup> fHbp expression constructs were generated in a previous study [Johnson, S. 2012]. *fHbp* was amplified from *N. cinerea* CCUG 346 T using primers, F<sub>fHbpV1.110</sub> and R<sub>fHbpV1.110</sub> (Table 3.7). The resulting product was ligated into a pET-21b expression vector using containing a C-terminal His-Tag using T4 ligase, prior to transformation into *E. coli* BL21 (DE3) (Agilent). *fHbp* expression constructs are listed in Table 3.9.

pET-21b expression vector containing *N. cinerea* CCUG 346 T fHbp V1.110 was generated by Katy Poncin under my direct supervision.

Strain	Plasmid	Insert	Modification	Antibiotic resistance cassette	Reference
BL21(DE3)Lys	pET21b	V1.1 fHbp		carb	[Johnson et al. 2012]
BL21(DE3)Lys	pET21b	V1.1 <sup>I311A</sup> fHbp	I311A	carb	[Johnson et al. 2012]
BL21(DE3)Lys	pET21b	V1.110 fHbp		carb	This study

Table 3.9: Plasmids used for protein expression in *E. coli*

### 3.9.7. Generation of *N. cinerea* CCUG 346 T $\Delta$ *fhbp*

*N. cinerea* strain CCUG 346 T $\Delta$ *fhbp* was constructed by insertional inactivation of *fhbp* with a cassette encoding kanamycin resistance. N.c  $\Delta$ *fhbp* forward primer and N.c  $\Delta$ *fhbp* reverse primer (Table 3.6) were used to amplify *fhbp* and flanking sequences from *N. meningitidis* strain H44/76 $\Delta$ *fhbp*. *N. cinerea* CCUG 346 T was then transformed with a linear PCR fragment as described previously for *N. meningitidis* [Wörmann, M. E. 2016].

*N. cinerea* strain CCUG 346 T $\Delta$ *fhbp* was generated and constructed by Katy Poncin under my direct supervision.

### 3.10. Sequence analysis and model prediction of *N. cinerea* fHbp

*N. cinerea* *fhbp* sequences were extracted from whole genome sequences available in the PubMLST BIGSdb database (<http://pubmlst.org/neisseria/>). Alignments of *N. cinerea* *fhbp* sequences against *fhbp* from *N. meningitidis* H44/76 were generated with Clustal OMEGA (<http://www.ebi.ac.uk/>); residues were numbered according to V1.1 fHbp sequence [Johnson, S. 2012]. A model of *N. cinerea* fHbp was generated using the iTASSER server [Zhang Y. 2008]; V1.1 fHbp (Schieder, PDB: 2W80) was used as the threading template and the resulting model illustrated in PyMol (<https://www.pymol.org/>). The resulting model has a high confidence with a C-score of 1.68.

### 3.11. fHbp purification

For protein expression, *E. coli* with expression plasmids (Table 3.8) were grown in liquid medium to an OD A<sub>600</sub> of 0.4–0.8, then isopropyl- $\beta$ -d-thiogalactopyranoside (IPTG) (Sigma) was added to a final concentration of 1 mM. After four hours, bacteria were harvested by centrifugation at 5,000  $\times$  *g* for 30 min at 4°C and suspended in NPI-20 (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 20 mM imidazole, pH 8.0) with EDTA-Free protease inhibitors (Roche). Cells were lysed in an EmulsiFlex-C5 homogenizer (Avestin) at 15,000 lb/in<sup>2</sup>. Lysates were centrifuged at 50,000  $\times$  *g* for 30 min at 4°C and recombinant proteins purified by affinity chromatography with a 1 ml HisTrap column (GE Healthcare) and eluted with NPI-200 (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 300 mM imidazole, pH 8.0). Proteins were dialysed against 25 mM Tris, pH 8.5 at 4°C overnight prior to further purification by anion exchange

chromatography using an AKTApurifier (GE Healthcare). Proteins were loaded onto a HiTrapQ HP column (GE Healthcare) and were eluted in salt gradient against 25 mM Tris, pH 8.5, 1 M NaCl prior to dialysing overnight against PBS. Protein concentrations were estimated using a Nanodrop 2000c spectrophotometer (Thermo Scientific).

### 3.12. Surface Plasmon Resonance (SPR)

SPR was performed using a BiaCore 3000 instrument (GE Healthcare). Purified fHbp was immobilised on a CM5 sensor chip to approximately 800 reaction units (RU) by amine coupling (GE Healthcare). Unreacted groups on the CM5 sensor chip were inactivated with ethanolamine. HBS-EB (HBS buffer 0.01 M Hepes, 150 mM NaCl, 3 mM EDTA, pH7.4) was used throughout. Increasing concentrations of CFH<sub>(6,7)</sub> (0.5 nM-32 nM) were injected over the chip surface at a flow rate of 40  $\mu$ l /min using the KINJECT command with a dissociation time of 300 seconds. Flow cells in CM5 sensor chip were washed with 1 M glycine pH 3.0 between injections.  $K_D$  values were calculated and analysed with BIAevaluation software. Kinetic data was referenced against both a blank cell and subtraction of a blank injection (HBS-EB). Binding data was collected in triplicate from two independent experiments.

### 3.13. Flow cytometry

*N. meningitidis* ( $1 \times 10^9$ ) were fixed in 1 ml of 3% paraformaldehyde for two hours then washed with PBS prior to staining whereas *N. cinerea* were fixed in 1 ml of 3% paraformaldehyde for 15 minutes after staining to prevent bacterial aggregates from forming.

Primary antibodies were used at the following concentrations: HSL-1 hybridoma tissue culture supernatant, neat; anti-fHbp V1.1 polyclonal sera, 1:1,000 [Johnson, S. 2012]; anti-GHfp polyclonal sera, 1:10,000 [Jongerijs, I. 2013]; anti-CFH, 1:10,000 (OX24). Alexa Fluor<sup>®</sup>-conjugated secondary antibodies were used at a final concentration of 1:1,000: goat anti-mouse Alexa Fluor<sup>®</sup> 647 (Molecular probes, Life technologies) and goat anti-mouse Alexa Fluor<sup>®</sup> 488 (Molecular probes, Life technologies).

fHbp (*N. meningitidis* and *N. cinerea*) was detected using anti-fHbp V1.1 sera and goat anti-mouse IgG-Alexa Fluor 647 conjugated antibody. Samples were run on a FACSCalibur (BD Biosciences), and

at least  $10^4$  events recorded before results were analysed by calculating the geometric mean FL-4 in FlowJo vX software (Tree Star). Statistical significance was tested using the unpaired student t-test comparison as implemented in GraphPad Prism v.6.0 (GraphPad Software Inc.) to compare means  $\pm$  S.D using a  $p < 0.05$  cutoff for significance.

#### 3.13.1. Evaluation of CFHR3 and CFH binding

To evaluate CFHR3 binding,  $5 \times 10^7$  CFU/ml were resuspended in 10  $\mu$ l of NHS or 10  $\mu$ l of  $\Delta$ CFHR3/CFHR1 sera for 30 min at room temperature. After two washes in 0.05% BSA, binding was detected following incubation with 50  $\mu$ l mAb anti-CFHR3 antibody mAb (HSL-1) for 30 min at 4°C in of PBS. Cells were washed twice in 0.05% BSA, then resuspended in 50  $\mu$ l of goat anti-mouse IgG-Alexa Fluor 647 conjugate and incubated for 30 min at 4°C.

To evaluate CFH binding,  $5 \times 10^7$  CFU of bacteria were resuspended in 10  $\mu$ l of heat inactivated NHS (HI-NHS) or CFH depleted sera for 30 min at room temperature. NHS was heat inactivated for 1 hour at 56°C. Binding was detected with an anti-CFH mAb (OX24) and goat anti-mouse IgG-Alexa Fluor 647 conjugate antibody and analysed as for CFHR3 binding.

#### 3.13.2 Competition assay

To evaluate the influence of CFHR3 on CFH,  $5 \times 10^7$  CFU/ml of *N. meningitidis* M1239 were incubated with 10  $\mu$ l of  $\Delta$ CFHR3/CFHR1 sera and two-fold dilutions of recombinant full length CFHR3 (from 1  $\mu$ M) for 30 min at room temperature. CFHR3 binding was detected with HSL-1 or an anti-human CFH mAb (OX24), followed by incubating with goat anti-mouse IgG-Alexa Fluor 647 conjugated antibody prior to analysis by flow cytometry. Maximum fluorescence for CFHR3 was normalised to the results obtained following addition of 1  $\mu$ M exogenous CFHR3 while the maximum fluorescence for CFH was normalised to results without CFHR3. Statistical significance was tested using the one-way ANOVA multiple comparisons as implemented in GraphPad Prism v.6.0 (GraphPad Software Inc.) to compare means  $\pm$  S.E.M. using a  $p < 0.01$  cutoff for significance

### 3.14. Bacterial serum survival assay

NHS was obtained by collecting venous blood and allowing it to coagulate at room temperature for 60 minutes before centrifuging at 3,000 x g for 20 minutes at 4°C. Sera was heat inactivated at 56°C for 30 minutes prior to use.

*N. meningitidis* and *N. cinerea* were grown overnight on BHI agar; media was supplemented with 1 mM IPTG (final concentration) for complemented strains. 10<sup>4</sup> CFU of bacteria were incubated in dilutions of NHS (0-70% NHS as specified) for 30 minutes at 37°C in the presence of CO<sub>2</sub>. Bacterial survival was determined by plating onto BHI agar in triplicate. The percentage survival was calculated by comparison with results of samples containing no sera. Statistical significance was tested using an unpaired student t-test (GraphPad Software Inc). To analyse AP activation, NHS was preincubated with 5 mM MgCl<sub>2</sub> and 10 mM EGTA to inhibit the CP and LP [Des Prez, R. M. 1975]. Percentage bacterial survival and significance was analysed as described above.

#### 3.14.1. Evaluating the effect of CFHR3 on bacterial survival

*N. meningitidis* was grown overnight on BHI agar, and resuspended in PBS then diluted to 1 x 10<sup>5</sup> CFU/ml in DMEM (Sigma). A total of 1x 10<sup>4</sup> CFU of bacteria were pre-incubated with 1 μM CFHR3, 1 μM CFHR3 domains or PBS for 10 minutes prior to incubation with 5% NHS or 5% HI-NHS for 30 minutes at 37°C in the presence of CO<sub>2</sub>. Bacterial survival was determined by plating onto BHI agar. Relative survival was calculated from samples containing no CFHR3 and incubated with heat-inactivated sera and expressed as percentage survival. Statistical significance was tested using the unpaired student t-test comparison as implemented in GraphPad Prism v.6.0 (GraphPad Software Inc.) to compare means ± S.D using a p<0.05 cutoff for significance. Serum concentration and incubation times are as specified unless otherwise stated.

For assays of the AP, bacteria were grown on BHI agar supplemented with 1mM IPTG. The other complement pathways were inhibited with 5mM MgCl<sub>2</sub>/ 10mM EGTA, and bacteria were incubated with 25% NHS. Analysis was performed as before. Serum concentrations and incubation times are as specified unless otherwise stated.

### 3.15. Serum Bactericidal Assay (SBA)

For serum bactericidal assays,  $5 \times 10^4$  CFU/ml of *N. cinerea* were mixed with an equal volume of baby rabbit complement (Cedarlane) diluted 1:10 in SBA buffer (Dulbecos phosphate buffered saline containing 0.1% (wt/vol) glucose, 1 mM  $\text{CaCl}_2$ , 0.5 mM  $\text{MgCl}_2$ ). Sera was pooled from all mice and heat inactivated for 1 hour at  $56^\circ\text{C}$  prior to being added to wells in two-fold dilutions starting at 1:8. Control wells contained either no serum or no complement. Following incubation for one hour at  $37^\circ\text{C}$ , 10  $\mu\text{l}$  from each well was plated onto solid BHI media in triplicate, and the number of surviving bacteria determined after overnight growth. The SBA was expressed as the reciprocal of the highest dilution of sera required to kill more than 50% of bacteria.

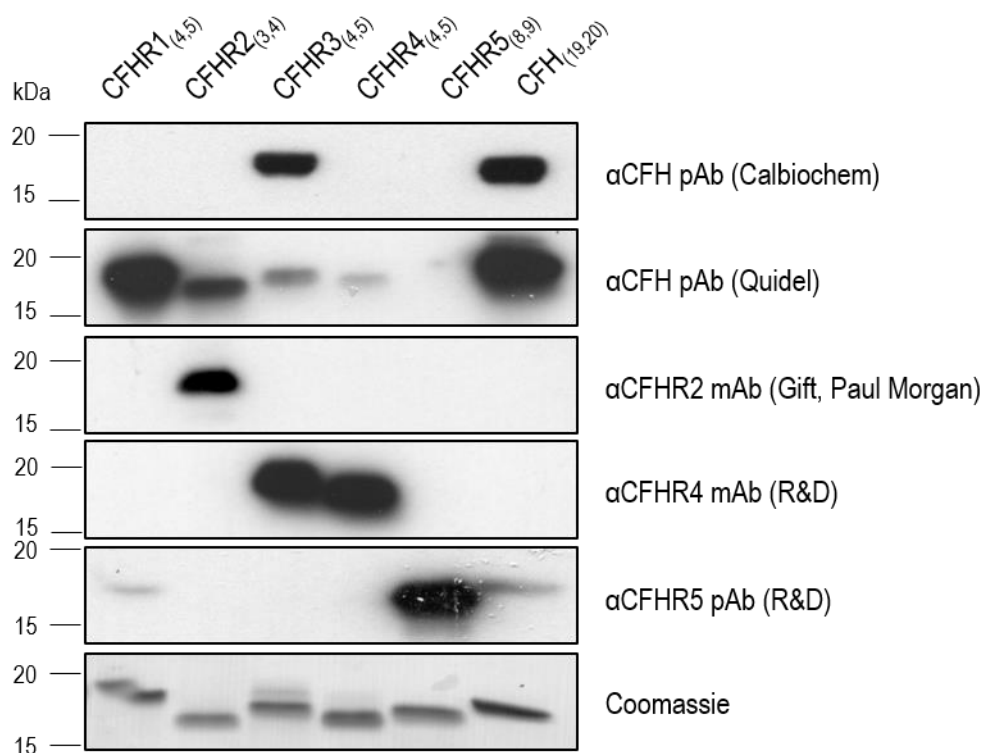
## 4. GENERATION AND CHARACTERISATION OF CFHR MONOCLONAL ANTIBODIES

### 4.1 Introduction

The CFH family of proteins consists of CFH, the major regulator of the AP, and five CFHR proteins, CFHR1-CFHR5 [Skerka, C. 2013]. The CFHRs are closely related to CFH and composed of between 4-9 CCP domains. These CCP domains have a high degree of amino acid sequence identity (between 36-100%) with CFH and other CFHRs suggesting the CFHRs CCP domains have related functions (Figure 1.4) [Díaz-Guillén, M. A. 1999. Skerka, C. 2013]. Whereas the role of CFH in regulating complement by distinguishing self from non-self surfaces and down regulating the AP C3 activation is well established, the function of CFHRs is poorly understood [Jokiranta, T. S. 2005. Weiler, J. M. 1976. Whaley, K. 1976. Pangburn, M. K. 1977]. Recent evidence suggests that contrary to CFH, the CFHRs lack co-factor activity for FI and decay accelerating activity, which have been attributed to CCP domains 1-4 of CFH; all the CFHRs lack homologous CCP domains [Heinen, S. 2009. Hellwage, J. 1999. Hebecker, M. 2012. Eberhardt, H. U. 2013]. Furthermore, CFHR1, CFHR2 and CFHR5 have been shown to act as antagonists of CFH by competing with CFH for C3b, promoting complement activation [Goicoechea de Jorge, E. 2013. Tortajada, A. 2013]. Therefore the CFHRs are thought to act by fine-tuning complement activation on surfaces.

The importance of CFHRs is highlighted by genetic variation across the CFH locus as polymorphisms are associated with several human diseases. For example, individuals homozygous for a *CFHR3/CFHR1* deletion ( $\Delta CFHR3/CFHR1$ ) are susceptible to aHUS yet are protected from AMD and IgA nephropathy [Holmes, L. V. 2013. Zipfel, P. F. 2007. Moore, I. 2010. Dragon-Durey, M. A. 2009. Francis, N. J. 2012. Hughes, A. E. 2006. Gharavi, A. G. 2011]. Susceptibility to C3GN is associated with a *CFHR3/CFHR1* fusion or an internal duplication of *CFHR1*, while duplications of *CFHR5* are also associated with DDD [Tortajada, A. 2013. Francis, N. J. 2012. Malik, T. H. 2012. Abrera-Abeleda, M. A. 2006]. A GWAS has also identified SNPs in *CFHR3* which are associated with protection against meningococcal disease [Davila, S. 2010].

To further understand the pathogenesis of these human diseases and how CFHRs contribute to disease susceptibility and progression, the physiological role of each CFHR needs to be defined.



**Figure 4.1: Available antibodies cross-react with other CFH family members**

Western blot analysis of the two C-terminal CCP domains of CFH and CFHR1-CFHR5 using available mAbs and pAbs. Coomassie blue stain was used to determine protein loading.

Studies of CFHRs have been hampered by the lack of specific reagents. Antibodies raised against either CFH or the CFHRs often cross-react with other family members due to their high amino acid and structural identity (Figure 4.1) [Skerka, C. 2013]. This is highlighted by the finding that auto-antibodies against CFH also recognise CFHRs [Kopp, A.2012. Blanc, C. 2012]. Cross-reactivity has limited purification of individual CFHRs from serum and measurements of their levels in circulation. Estimated levels of CFHRs in plasma have been reported to be equimolar to CFH (116-562 ug/ml, 0.7-3.6 uM); CFHR1 levels have been estimated to range between 70-100 ug/ml (1.6-2.4 uM) [Esparza-Gordillo, J. 2004. Heinen, S. 2009]. However, CFHR1 can form homo- and heterodimers with CFHR2 and CFHR5 which may affect protein estimations. Dimerisation and sequence similarity may have led to overestimation of CFHR concentrations [Goicoechea de Jorge, E. 2013]. Recent data using two cross reactive antibodies indicates that CFHR3 is approximately 132-fold lower than CFH levels,



ranging between 0-2.7 µg/ml (0-0.05 µM) whereas previously the circulating plasma concentration was estimated to range between 50-80 µg/ml (1-1.6 µM) [Pouw, R. 2016. Fritsche, L. G. 2010].

Several strategies can be used to generate specific reagents that distinguish between CFHRs and CFH. The first is to generate polyclonal antibodies (pAbs); serum from immunised animals contains a heterogeneous mixture of antibodies that recognise multiple epitopes on the target molecule. The heterogeneity of epitopes recognised by pAbs offers an increased chance of recognising the native protein even following post-translational modification. The disadvantage of pAbs is cross-reactivity with related proteins. In contrast, the generation of mAbs provides an unlimited supply of a clonal antibody often with reproducible specificity for a single antigen, which is ideal for identifying CFHRs.

The generation of an effective immune response is essential for producing mAbs. Therefore antigens must be capable of stimulating both T and B cell responses [MacLennan, I. C. 1997]. Poor immune responses are often observed when immunising with proteins that are closely related to those found in mice, therefore the immunising protein or peptide needs careful consideration [Stollar, B. D. 1970. Stollar, B. D. 1978]. Human CFH and murine CFH (mCFH) share approximately 63% amino acid identity, while mice also have five predicted CFHR proteins, CFHRa-CFHRe [Vik, D. P. 1988. Vik, D. P. 1990. Hellwage, J. 2006]. CFHRb and CFHRc are homologues of CFHR1 and/or CFHR2 and CFHR5 respectively, based on the organisation of CCP domains, whereas CFHRa and CFHRd are predicted to be pseudogenes [Hellwage, J. 2006]. There is evidence that CFHRe is transcribed but no protein has been detected [Vik, D. P. 1990]. The amino acid sequence identity of CCP domains between CFHRb and CFHR1/ CFHR2 (34-72%), and CFHRc and CFHR5 (36-63%) are sufficiently different to enable the generation of specific antibodies [Hellwage, J. 2006].

The main aim of this chapter was to generate specific mAbs against human CFHRs to allow further understanding of the role of CFHRs and to estimate circulating CFHR levels, detect their tissue localisation, and determine the effect of polymorphisms and copy number variation (CNV) on disease. Furthermore, mAbs could be used to purify CFHRs from complex mixtures such as sera.

## 4.2 Results

mAbs generated in this study were analysed for their specificity against recombinant CFH and CFHRs and were further characterised to determine recognition of proteins in NHS. The mAb production and screening strategy is shown in Figure 4.2.

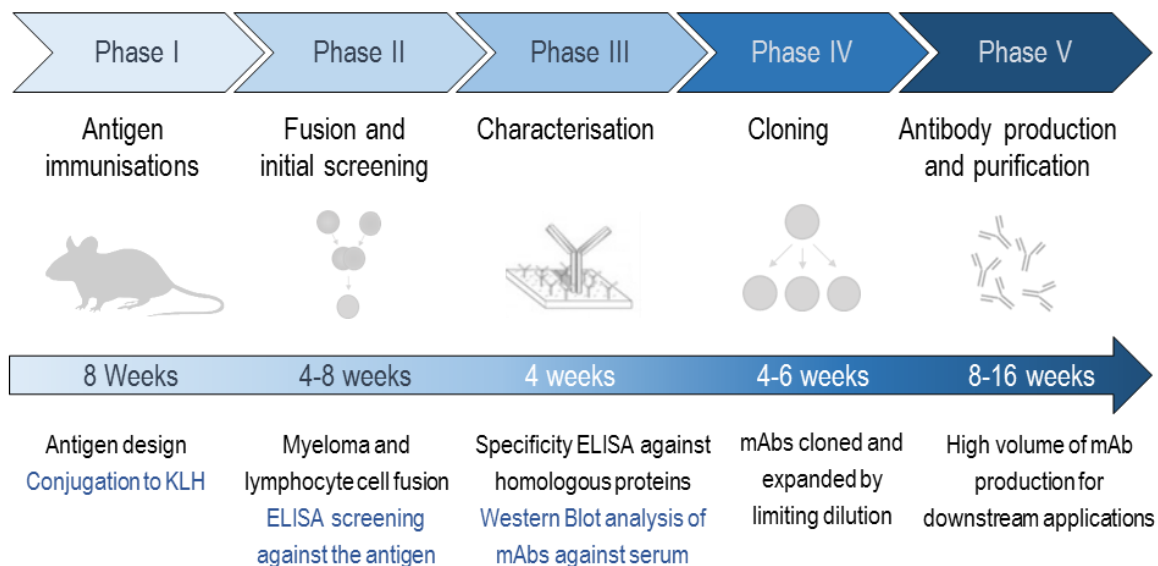


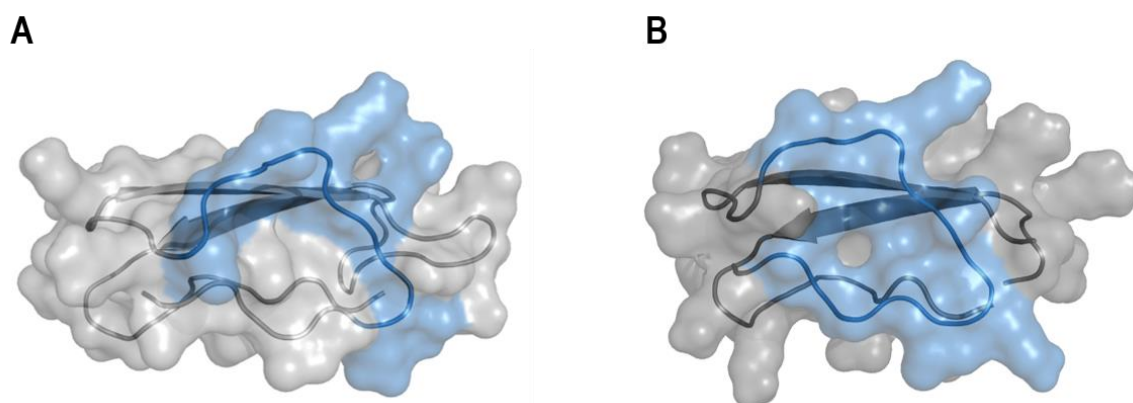
Figure 4.2: Schematic representation of mAb generation and identification

Approximate time frames for the completion of each phase is indicated, with mAb development taking approximately 28-42 weeks.

### 4.2.1 Generation of anti-human CFHR antibodies

Currently available antibodies against CFH and CFHRs have been shown to cross-react with other family members due to their high amino acid and structural identity hampering the further study of CFHR function (Figure 4.1). Therefore, the initial aim of this thesis was to generate a panel of mAb reagents which specifically recognise CFHRs. The two C-terminal CCP domains of CFHR2-CFHR5 were chosen as antigens for the generation of mAbs due to their lower sequence similarity with the related CCP domains in CFH and CFHRs (Figure 1.4). Peptides were also designed to surface epitopes of CFHR4<sub>(3)</sub> and CFHR5<sub>(6)</sub> as an alternative strategy: CCP domain 3 of CFHR4 is homologous to CCP domain 9 of CFH (amino acid identity of 57%) with no homologous domains in other CFHRs, whereas CFHR1-CFHR4 do not have a homologous CCP domain to domain 6 of CFHR5 which has 47% sequence identity with CCP domain 13 of CFH (Figure 4.3). The following peptides were used for immunisation; ENSRAKSNGM for CFHR4 and IAGVNIKLLKLSG for CFHR5. Of note, the chosen

peptide sequences have no similarity to murine sequences using BLAST searches against the mouse genome. The protein and peptide antigens used here were conjugated to the carrier molecule, KLH, to enhance immune responses. The immunogenicity of the antigens was also enhanced by the addition of adjuvant (TitreMax Gold™), which slowly releases the antigen at the site of immunisation (subcutaneous) and induces a strong inflammatory response during the primary immunisations [Bennett, B. 1992].



**Figure 4.3: Peptides used to generate mAbs against CFHR4 and CFHR5 are surface localised**

Structural prediction of (A) CFHR4<sub>(3)</sub> and (B) CFHR5<sub>(6)</sub> (space filled models) with CCP domain 9 and 13 of CFH, respectively, used as a threading template (grey, ribbon model). The peptides used to generate mAbs are shown in blue. Models generated using the I-TASSER server and figure drawn using Pymol

For each antigen (Table 3.2), two female BALB/c were immunised according to the schedule shown in Figure 4.4a, then plasma cells were isolated from each mouse. Cells were either directly fused with the mouse myeloma cell line, NS0, according to the protocol summarised in figure 4.4b, or stored in liquid nitrogen prior to fusion. A single fusion was performed to generate antibodies specific for CFHR2 and CFHR3. Few specific antibodies were generated in initial fusions following immunisation with CFHR4 and CFHR5 peptides. Therefore, cell fusions were also performed from mice immunised with recombinant C-terminal CCP domains of CFHR4 and CFHR5. The second fusion for CFHR4 yielded no specific antibodies, whereas the second fusion for CFHR5 resulted in specific mAbs. The results of screening are summarised in Table 4.1.

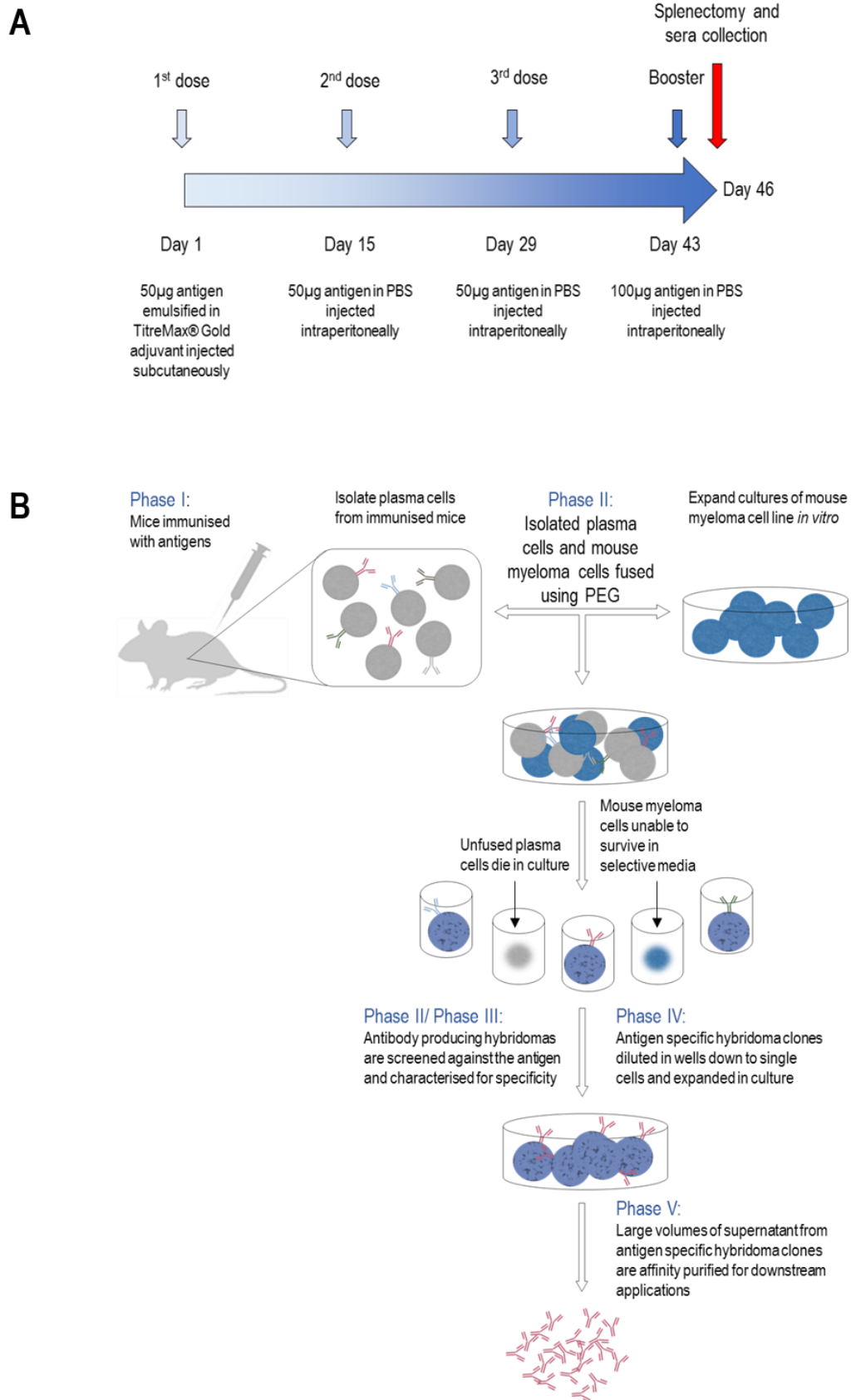


Figure 4.4: Schematic showing the generation of mAbs

(A) BALB/c mice were immunised with antigen conjugated to KLH subcutaneously in TitreMax® gold, then twice intraperitoneally followed by a booster immunisation three days prior to cell fusion. (B) Splenic plasma cells were isolated and fused with mouse myeloma cells and resulting hybridomas were selected from unfused plasma cells and mouse myeloma cells with HAT prior to screening for mAb specificity.

Spleen number	Fusion antigen	Phase II-a Initial well screening	Phase II-b Initial colony screening	Phase III-a Specificity screening by ELISA	Phase III-b Specificity screening by Western blotting	Phase III-C Specificity screening by Western blotting of NHS	Phase IV Cloning by limiting dilution	Phase V Antibody purification
1	CFHR2 <sub>(3,4)</sub>	43/192	46/219	30/46	2/30*	2/2	CFHR2-63a <sup>†</sup> , CFHR2-157b	Pre-Phase V
1	CFHR3 <sub>(4,5)</sub>	51/192	41/153	17/98	2/17*	1/2	CFHR3-144a	CFHR3-144a-H7 (HSL-1)
1	CFHR4	10/192	5/21	-	-	0/5	-	-
2	CFHR4	23/192	2/53	-	-	1/2	CFHR4-33, CFHR4-55a	Pre-Phase V
1	CFHR4 <sub>(4,5)</sub>	42/192	10/81	5/10	0/5	-	-	-
1	CFHR5	14/192	5/66	1/5	-	0/1	CFHR5-80b <sup>††</sup>	-
2	CFHR5	15/192	5/29	-	-	0/5	-	-
1	CFHR5 <sub>(8,9)</sub>	24/192	9/39	3/9	0/3	-	-	-
2	CFHR5 <sub>(8,9)</sub>	61/192	64/292	50/64	2/2*	4/6**	CFHR5-68a, CFHR5-86a	Pre-Phase V

**Table 4.1: Summary of the selection of mouse anti-human CFHR mAbs in each phase of production**

\* Not all positive clones from Phase III-a were analysed for their specificity although all were stored.

\*\* For CFHR5<sub>(8,9)</sub>, six clones identified in Phase III-a were tested for specificity in Phase III-b and -c. Recombinant C-terminal CCP domains of all CFHRs and CFH showed some protein degradation. Therefore only clones that potentially recognised CFHR5 in NHS were screened again in Phase III-b.

<sup>†</sup> CFHR2-63a was not successfully cloned by limiting dilution after two attempts under different conditions.

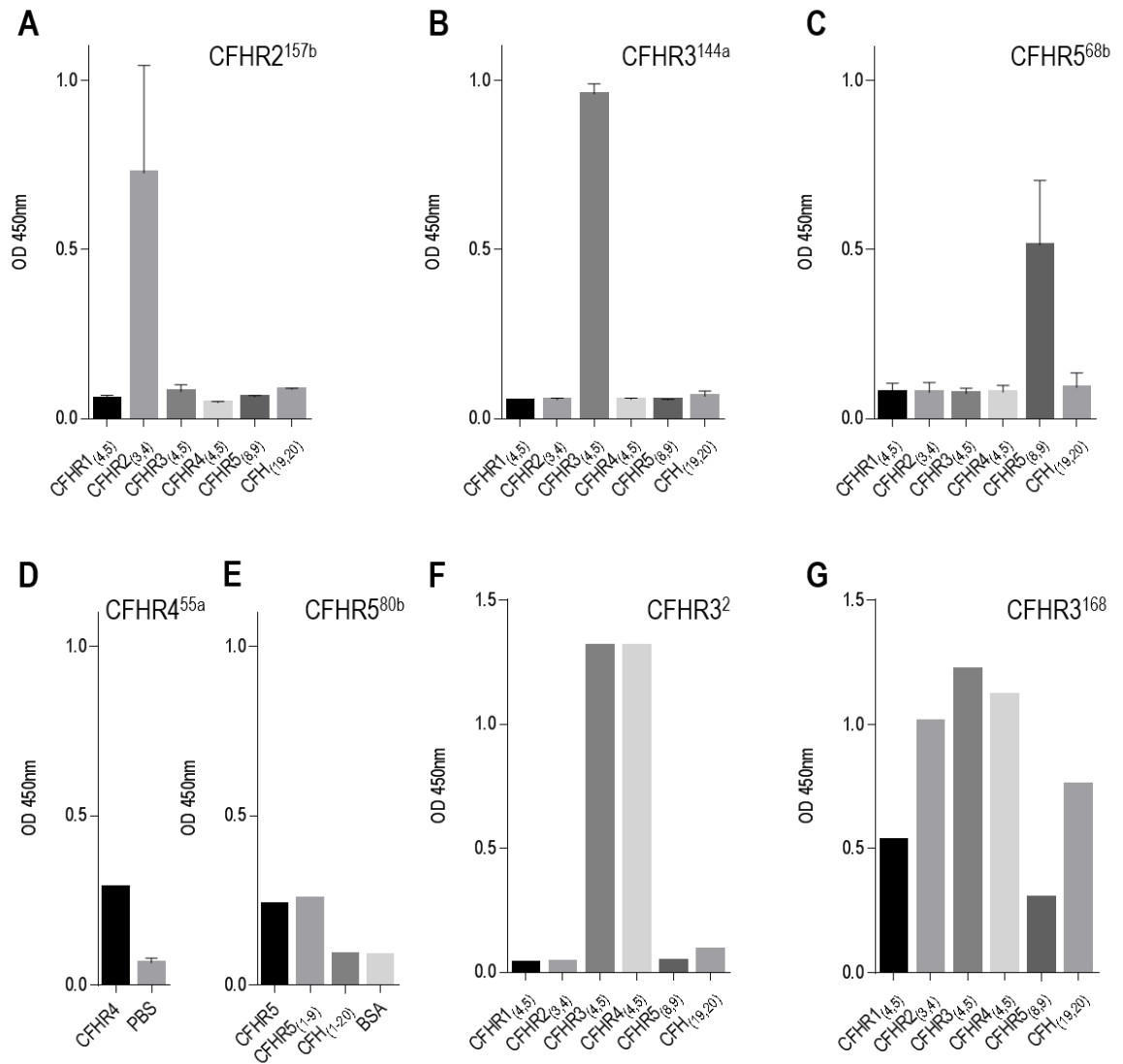
<sup>††</sup> CFHR5-80b was identified as having an IgM<sub>k</sub> isotype was not pursued further due to instability and inconsistent results.

#### 4.2.2 Phase II screening: Cell fusion and screening of mAbs against the immunising antigen

Hybridomas were initially selected by examining the ability of cell culture supernatant, to detect the immunising antigen by ELISA (Phase II-a). Initial Phase II-a screening identified: 43 CFHR2; 51 CFHR3; 75 CFHR4; and 114 CFHR5 positive wells from 1, 1, 3 and 4 cell fusions, respectively. Individual colonies of hybridoma cells were picked from positive wells and placed into separate wells containing hybridoma competent media. Supernatants from wells containing individual colonies were again screened by ELISA against the immunising antigen once cells became confluent (Phase II-b). From the Phase II screening: 46/219 CFHR2 clones, 41/153 CFHR3 clones, 17/155 CFHR4 clones and 83/426 CFHR5 clones were taken forward into Phase III screening to determine mAb specificity.

#### 4.2.3 Phase III screening: Determining the specificity of mAbs against CFH and CFHRs

Due to the high sequence identity between CFH and the CFHRs, and the demonstrated cross reactivity of available polyclonal antibodies and mAbs (Figure 4.1), additional screening by both ELISA and western blotting was used to determine the specificity of mAbs produced by each clone [Skerka, C. 2013]. Initially, culture supernatants from all Phase II-b positive hybridoma clones were screened by ELISA against the C-terminal CCP domains of CFH and CFHR1-CFHR5, when the immunising antigen was composed of C-terminal CCP domains. Screening of anti-peptide mAbs against CFHR4 was performed by western blotting against NHS as no full length recombinant CFHR4 was available for screening (Phase III-c screening), while anti-peptide CFHR5 mAbs were analysed for recognition of full length recombinant CFHR5 (provided by Matthew Pickering). Plates were coated with 5 µg/ml of either recombinant protein or peptide, and blocked with 3% skimmed milk with 0.05% Tween 20 prior to screening with hybridoma supernatants. From the Phase III-a screening: 30/46 CFHR2 clones, 17/41 CFHR3 clones, 5/10 CFHR4 clones (raised against CFHR4<sub>(4,5)</sub>), 1/5 CFHR5 clone (anti-peptide) and 53/73 CFHR5 clones (raised against CFHR5<sub>(8,9)</sub>) were specific for the immunising antigen by ELISA. Representative examples of the specificity of mAbs raised against CFHR2-CFHR5 are shown in Figure 4.5. CFHR3<sup>2</sup> (*i.e.* clone 2 generated against CFHR3) cross-reacted with CFHR4 (Figure 4.5f) while CFHR3<sup>168</sup> cross-reacted with CFH and all CFHRs (Figure 4.5g). Cross-reactive mAbs were further characterised as they can be valuable for certain applications.

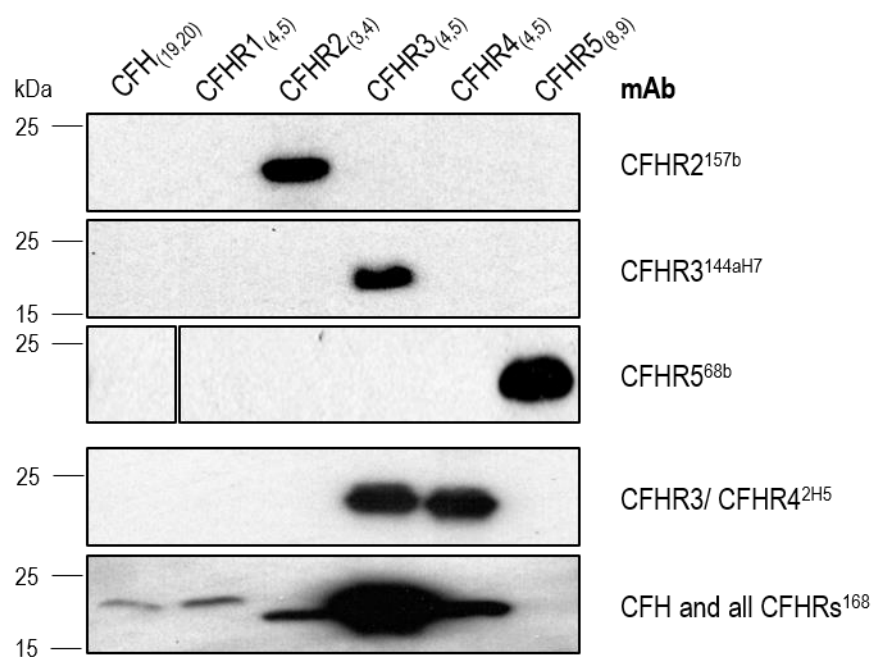


**Figure 4.5: Specificity of mAbs during Phase III-a screening**

ELISA plates were coated with the two C-terminal CCP domains of CFH and CFHR1-CFHR5 or peptides (ENSKAKNSGM for CFHR4 and IAGVNIKTLKLSG for CFHR5) at a concentration of 5 µg/ml. Results of ELISA analysis of hybridoma clones (A) CFHR2<sup>157b</sup>, (B) CFHR3<sup>144a</sup> and (C) CFHR5<sup>68b</sup> which specifically recognise the immunising antigen. Anti-peptide mAbs (D) CFHR4<sup>55a</sup>, and (E) CFHR5<sup>80b</sup> recognise the peptide used for immunisation. Hybridoma clones (F) CFHR3<sup>2</sup> and (G) CFHR3<sup>168</sup> cross-react with CFHR4, and CFH/CFHR1-CFHR5, respectively. Error bars are SEM of two independent experiments.

To confirm the specificity of the hybridomas, mAbs were also assessed for their ability to recognise recombinant C-terminal CCP domains of CFH and CFHRs by western blotting when generated by immunising with their two C-terminal CCP domains (*i.e.* CFHR2-CFHR5, Figure 4.6, Phase III-b). Proteins were diluted to a final concentration of 10 µg/ml in 2x sample buffer and resolved on 16% polyacrylamide gels, prior to transferring proteins to PVDF membranes by semi-dry transfer. Proteins were detected with mAbs in hybridoma culture supernatants diluted 1:2 with 1% skimmed milk/ PBS.

From the Phase III-b screening; 2 of 4 CFHR2 clones (CFHR2<sup>63a</sup> and CFHR2<sup>157b</sup>), 2 of 17 CFHR3 clones (CFHR3<sup>144a</sup> and CFHR3<sup>184c</sup>), and both CFHR5 clones (CFHR5<sup>68a</sup> and CFHR5<sup>86a</sup>) specifically detected the C-terminal CCP domains of CFHR2, CFHR3 and CFHR5 respectively (Figure 4.6). In western blot Phase III-b analysis, CFHR3<sup>2</sup> cross-reacted with CFHR4, and CFHR3<sup>168</sup> recognised CFH and CFHR1-CFHR5 (Figure 4.6). Not all positive clones identified in Phase II-b were screened for their specificity. Once hybridomas producing CFHR-specific mAbs were identified, no further hybridomas were analysed for that CFHR.



**Figure 4.6: Specificity of novel mAbs during Phase III-b screening**

Western blot analysis showing the recognition of recombinant two C-terminal CCP domains of CFH and CFHR1-CFHR5 by mAbs from generated hybridoma clones. CFHR2<sup>63a</sup>, CFHR2<sup>157b</sup>, CFHR3<sup>144a</sup>, CFHR3<sup>184c</sup>, CFHR5<sup>68b</sup> and CFHR5<sup>86a</sup> specifically recognise the immunising antigen whereas, CFHR3<sup>2</sup> and CFHR3<sup>168</sup> cross-reacts with CFHR4, and CFH/ CFHR1-CFHR5, respectively.

Hybridoma clones were also analysed by western blotting for recognition of CFHRs in NHS (Figure 4.7, Phase III-c). Previous analysis of CFHRs in serum with non-specific antibodies indicated that CFHRs are often detected as multiple bands by western blot analysis, consistent with post-translational modification of CFHRs [Skerka, C. 2013]. Samples of NHS were examined by western blot analyses using the mouse anti-human CFH mAb, OX24. Results confirmed that this mAb recognises both CFH (approximately 155kDa) and CFHL, an alternative splice variant of CFH (of



approximately 37kDa) (Figure 4.7a) [Sim, E. 1983. Ripoche, J. 1988]. Epitope mapping has demonstrated that CFH<sup>OX24</sup> recognises CCP domain 5 of CFH [Sim, E. 1983]. Therefore it is unlikely that CFH<sup>OX24</sup> will cross-react with CFHR1-CFHR5, as the related proteins do not have CCP domains related to CCP domain 5 of CFH.

CFHR2<sup>157b</sup> recognised two bands in NHS by western blotting consistent to the molecular weight of two glycosylation forms of CFHR2 previously detected; a single glycosylation form of approximately 24kDa, CHFR2, and a double glycosylation form of approximately 29kDa, CHFR2 $\alpha$  that have been described (Figure 4.7b) [Skerka, C. 1992]. A similar staining pattern was observed for CFHR2<sup>63a</sup> (data not shown). Four glycosylation variants of CFHR3 have been previously described, CFHR3, CFHR3 $\alpha$ , CFHR3 $\beta$  and CFHR3 $\gamma$  [Skerka, C. 1993]. CFHR3<sup>144a</sup> recognised three bands between the molecular weights of 35-46kDa in NHS consistent with the glycosylation forms CFHR3 $\alpha$ , CFHR3 $\beta$  and CFHR3 (Figure 4.7c). Of note, no protein band was detected with CFHR3<sup>144a</sup> using sera from an individual with a homozygous *CFHR3/CFHR1* deletion, indicating that this hybridoma produces a mAb which is specific for CFHR3.

Two isoforms of CFHR4 have previously been described; the long isoform (CFHR4A) has nine CCP domains resulting from an internal duplication of CCP domains 1-3 (CCP domains 5-7 of CFHR4A), whereas the alternative splice variant, CFHR4B, has five CCP domains [Józsi, M. 2005]. Bands consistent with both isoforms of CFHR4 were detected in NHS using CFHR4<sup>55a</sup> at approximately 86 and 42kDa for CFHR4A and CFHR4B, respectively (Figure 4.7d). A third band of unknown identity was also detected at 240kDa (Figure 4.7d). Two potential glycosylation forms of CFHR5, CFHR5 (62kDa) and CFHR5 $\alpha$  (55kDa) were detected with CFHR5<sup>68a</sup> (Figure 4.7e) with limited cross-reactivity of other proteins in NHS with the exception of bands at 200 and 240kDa [Murphy, B. 2002. Goicoechea de Jorge, E. 2013].

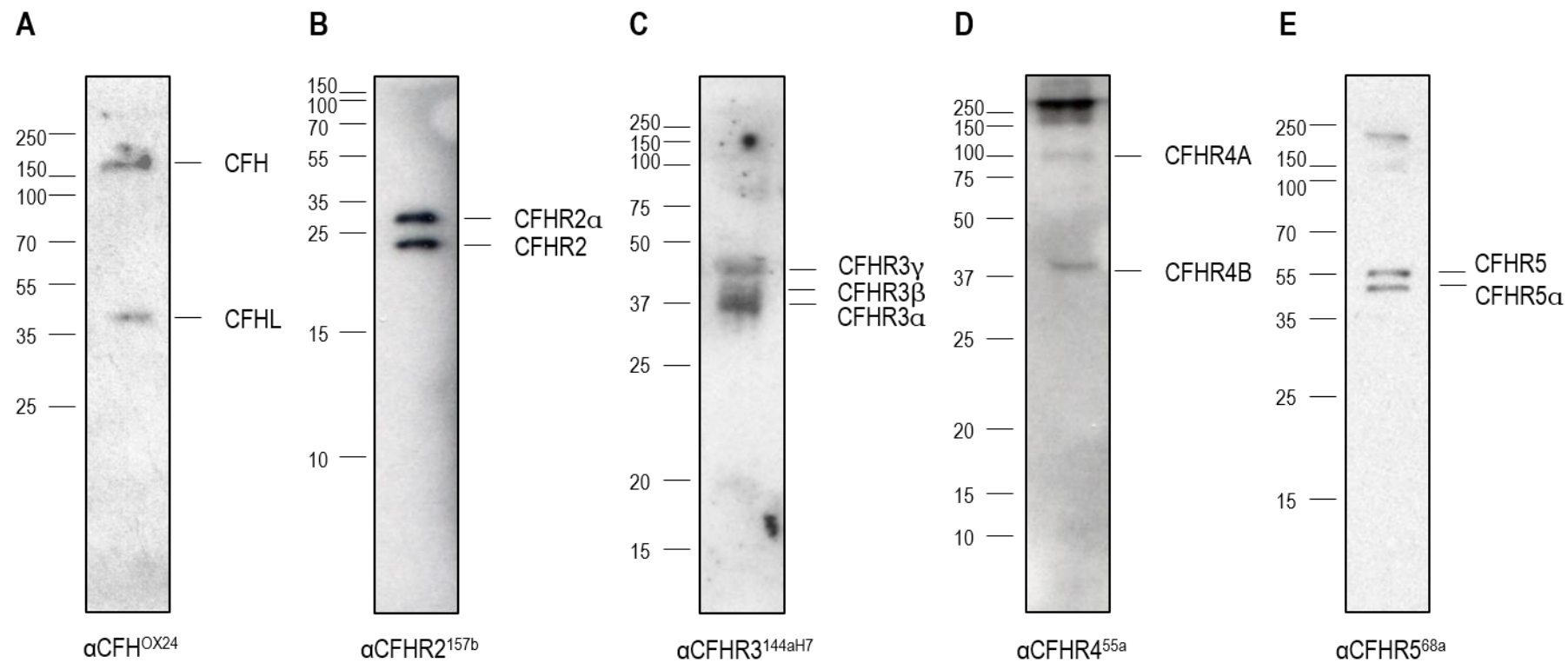


Figure 4.7: Recognition of CFH and CFHR2-CFHR5 in NHS

(A) CFH<sup>OX24</sup> specially recognises CFH and CFHL, (B) while two potential glycosylation forms of CFHR2 were recognised by anti-CFHR2<sup>157b</sup>, (C) and three of the four potential glycosylation forms of CFHR3 are recognised by anti-CFHR3<sup>144a</sup>. (D) Anti-peptide mAb CFHR4<sup>55a</sup> detected two bands corresponding to the molecular weight of CFHR4A and CFHR4B but also a third band of approximately 240kDa. A similar sized band was also detected by (E) CFHR5<sup>68a</sup> which also detects two potential glycosylation forms of CFHR5.

#### 4.2.4 Phase IV: Screening of anti-human CFHR mAbs cloned by limiting dilution

Specific hybridoma mAb clones for each antigen identified in Phase III screening was further characterised in Phase IV analysis. Therefore, hybridomas producing specific mAbs were cloned by limiting dilution to ensure that the mAbs were derived from a single hybridoma rather than a mixed population. Hybridoma cell cultures were diluted to 5 cells/ml and plated at a concentration of approximately 1 cell per well in 96-well tissue culture plates. Plates were then screened by light microscopy for the growth of single colonies; wells containing multiple colonies were discarded. Single colonies were expanded into 24-well plates containing hybridoma competent media prior to reanalysing antibody specificity by ELISA. Two colonies for each hybridoma were then expanded in T-25 flasks, and the cell lines stored. A summary table of hybridomas that were successfully cloned by limiting dilution is shown in Table 4.2.

An anti-peptide mAb CFHR5<sup>80b</sup> showed weak reactivity against full length recombinant CFHR5 by ELISA (Figure 4.5e); however this mAb did not recognise any proteins in NHS by western blot analysis. Serum concentrations of CFHR5 have been estimated to range between 0.05-0.09  $\mu$ M, Therefore CFHR5 could be below the detection limit of the assay [McRae, J.L. 2005; Goicoechea de Jorge, E. 2013]. CFHR5<sup>80b</sup> was successfully cloned by limiting dilution and found to have an IgM <sub>$\kappa$</sub>  isotype; mAb isotype was determined using a mouse isotyping kit (AbD Serotec, UK). Therefore, further anti-CFHR5 mAbs were generated against CFHR5 using CFHR5<sub>(8,9)</sub> as the antigen; this yielded the specific mAbs, CFHR5<sup>68a</sup> and CFHR5<sup>86a</sup>, which recognise CFHR5 by ELISA and western blot analysis of NHS (Figures, 4.5c, 4.6 and 4.7e), indicating the CFHR5 is not below the limit of detection. Both, CFHR5<sup>68a</sup> and CFHR5<sup>86a</sup> were successfully cloned by limiting dilution using 1% condensed in the cloning media.

In summary, novel mouse monoclonals, CFHR2<sup>157b</sup>, CFHR3<sup>144a</sup>, CFHR4<sup>55a</sup> and CFHR5<sup>68a</sup>, which are specific for CFHR2-CFHR5 respectively, were generated and characterised in this study (summarised in Table 4.1). All four mAbs recognise the corresponding CFHR by ELISA (Figure 4.5) and western blotting (Figure 4.6), and potentially recognise different glycosylation forms in NHS (Figure 4.7). CFHR2<sup>157b</sup>, CFHR3<sup>144a</sup> and CFHR5<sup>68a</sup> have an IgG1 <sub>$\kappa$</sub>  isotype whereas CFHR4<sup>55a</sup> has an IgM <sub>$\kappa$</sub>  isotype as determined using the mouse isotyping kit (AbD Serotec, UK). Two cross-reactive IgG1 <sub>$\kappa$</sub>  antibodies

with known specificity *i.e.* CFHR3<sup>2-H5</sup> and CFHR3<sup>168-B5</sup> were also cloned by limiting dilution. These two mAbs were isolated as controls and for purifying CFH and CFHR1-CFHR5 from NHS.

Phase IV mAbs					
Spleen number	Fusion antigen	Hybridoma clone number	Specificity	Isotype	Applications tested
1	CFHR2 <sub>(3,4)</sub>	157b-F7	CFHR2	IgG1 <sub>k</sub>	ELISA, WB
1	CFHR2 <sub>(3,4)</sub>	157b-H12	CFHR2	IgG1 <sub>k</sub>	ELISA, WB
<b>1</b>	<b>CFHR3<sub>(4,5)</sub></b>	<b>144a-H7</b>	<b>CFHR3</b>	<b>IgG1<sub>k</sub></b>	<b>ELISA, WB, Flow cyt</b>
1	CFHR3 <sub>(4,5)</sub>	144a-C5	CFHR3	IgG1 <sub>k</sub>	ELISA, WB
1	CFHR3 <sub>(4,5)</sub>	2-H5	CFHR3 and CFHR4	IgG1 <sub>k</sub>	ELISA, WB
1	CFHR3 <sub>(4,5)</sub>	2-G8	CFHR3 and CFHR4	IgG1 <sub>k</sub>	ELISA, WB
1	CFHR3 <sub>(4,5)</sub>	168-B6	All CFHRs and CFH	IgG1 <sub>k</sub>	ELISA, WB
1	CFHR3 <sub>(4,5)</sub>	168-F6	All CFHRs and CFH	IgG1 <sub>k</sub>	ELISA, WB
2	CFHR4	33-C6	-	IgG2a <sub>k</sub>	ELISA
2	CFHR4	33-G8	-	IgG2a <sub>k</sub>	ELISA
2	CFHR4	55a-H6	CFHR4	IgM <sub>k</sub>	ELISA, WB
2	CFHR4	55a-H9	CFHR4	IgM <sub>k</sub>	ELISA, WB
2	CFHR5 <sub>(8,9)</sub>	68a-E4	CFHR5	IgG1 <sub>k</sub>	ELISA, WB
2	CFHR5 <sub>(8,9)</sub>	68a-G8	CFHR5	IgG1 <sub>k</sub>	ELISA, WB
2	CFHR5 <sub>(8,9)</sub>	86a-D3	CFHR5	IgG1 <sub>k</sub>	ELISA, WB
2	CFHR5 <sub>(8,9)</sub>	86a-F8	CFHR5	IgG1 <sub>k</sub>	ELISA, WB
1	CFHR5	80b	-	IgM <sub>k</sub>	ELISA
1	CFHR5	80b	-	IgM <sub>k</sub>	ELISA

**Table 4.2: Summary of characterised mouse anti-human CFHR mAbs cloned by limiting dilution**

All mAbs that were analysed through to Phase IV were examined by ELISA and western blotting to determine their specificity. mAbs cloned by limiting dilution were further characterised by confirmation of mouse isotype. CFHR4<sup>33a</sup> and CFHR5<sup>80b</sup> (grey) do not recognise any serum proteins. CFHR3<sup>144aH7</sup> (blue) has been published under clone name HSL-1 [Caesar, J. J. 2014], and recognises CFHR3 by flow cytometry.

#### 4.2.5 Production and purification of HSL-1 (Phase V)

GWAS identified SNPs in both CFH and CFHR3 that are associated with susceptibility to meningococcal disease [Davila, S. 2010]. CFH is the major fluid phase negative regulator of the AP whereas the function of CFHR3 has not been defined due to the lack of reagents [Weiler, J. M. 1976. Whaley, K. 1976. Pangburn, M. K. 1977. Skerka, C. 2013]. The novel anti-CFHR3 mAb, CFHR3<sup>144a-H7</sup> (designated HSL-1) with specificity for CFHR3 was analysed further, due to the association of CFHR3 with meningococcal disease.

Initially, the genes encoding the variable heavy chain (V<sub>H</sub>) and variable light chain (V<sub>L</sub>) of HSL-1 were amplified by V-region PCR and sequenced using conventional Sanger sequencing; data were analysed using DNASTAR Lasergene software (performed by Absolute Antibodies Ltd.). The sequence of V<sub>H</sub> and V<sub>L</sub> is shown in Figure 4.8a and is consistent with functional V<sub>H</sub> and V<sub>L</sub> chains. Sequencing also confirmed the isotype of this mAb (IgG1κ). Furthermore, the Fab fragment of HSL-1 was generated by Absolute Antibodies, by transient transfection of HEK293 cells and purification of the mAb by nickel chromatography.

The activity and specificity of the recombinant FAB fragment of HSL-1 was compared with the mAb from culture supernatants. ELISA plates were coated with 1 µg/ml of CFH<sub>(19,20)</sub>, CFHR3<sub>(4,5)</sub>, CFHR4<sub>(4,5)</sub> or BSA then incubated with two-fold dilutions of whole mAb or the Fab fragment (Figure 4.8b-c) from 10 µg/ml. Both the mAb and FAB fragment of HSL-1 specifically recognised CFHR3<sub>(4,5)</sub> by ELISA (Figure 4.8b-c). Detection of CFHR3<sub>(4,5)</sub> by HSL-1 FAB fragment was significantly reduced, compared to the mAb, (OD<sub>450</sub> 0.72 and 0.95 respectively, at 5 µg/ml). Potential explanations include differences in detection of mouse IgG by the secondary antibody, as the pAb secondary potentially has a higher avidity for full length IgG due to more binding epitopes, or lower affinity of the Fab fragment for the antigen. This has not been confirmed using secondary antibodies raised against mouse Fab fragments.

For further downstream applications, HSL-1 was purified from large volumes (2-4 litres) of cell culture supernatant. Cells expressing HSL-1 were adapted for purification by sequential dilution into hybridoma recovery media prior to transferring hybridomas into RPMI-1640 with L-glutamine

supplemented with either FreeAdd (serum substitute) or 10% ultra-low bovine FBS. Antibodies were purified using Pierce™ Protein G Agarose resin, and size exclusion chromatography.

**A**

**Heavy chain sequence:**

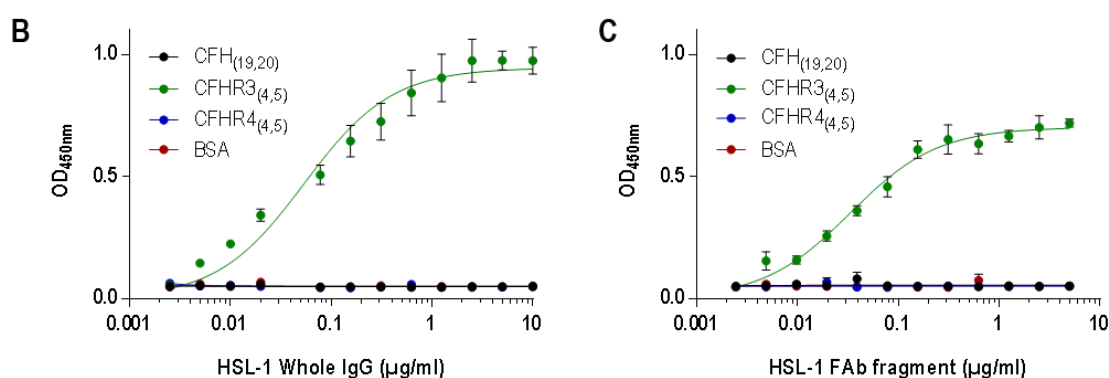
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QVQLQQPGAELLKPGASVKLSCKASGYFTFSYWMHWVKQRPGQGLEWIGEINPSNGRTNYNEKFKTKATLTVDKSSSTAYMQL
SSLISEDSAVYYCANFNYYGPPYTMDYWGQGTSTVTVSSAKTTPPSVYPLAPGSAQAQNSMVTGLCLVKGYFPEPVTVTWNSGS
LSSGVHTFPAVLQSDLYTLSSSVTVPSSTWPSQTVTCNVAHPASSTKVDKIKHHHHHHHHHH
  
```

**Light chain sequence:**

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DIVLTQSPASLAVSLGQRATISCKASQSIDYDGDSYMNHQQKPGQPPKLLIYGASILQSGIPARFSGSGSGTDFTLNIHPVEEED
AATYYCQQSNEDPYTFGGGTKLEIKRADAAPTVSIFPPSSEQLTSGGASVVCFLNRFYPKDINVKWKIDGSRQNGVLNSWTDQ
DSKDYSTYMSSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC
  
```



**Figure 4.8: Recombinant Fab fragment of HSL-1 retains specificity for CFHR3**

Sequence of the variable heavy ( $V_H$ ) and variable light ( $V_L$ ) domains of HSL-1 (A); sequencing was performed by Absolute Antibody. ELISA analysis of HSL-1 whole IgG from tissue culture supernatant (B) or the purified Fab fragment of (C) against recombinant C-terminal CCP domains of CFH (black), CFHR3 (green) and CFHR4 (blue); BSA (red) was used as a negative control. ELISA plates were coated with proteins at a concentration of  $1\mu\text{g/ml}$ . Error bars are SEM of three independent experiments.

### 4.3 Discussion

This chapter describes the generation of a panel of mAbs against human CFHR2-CFHR5, which specifically recognise each of the closely related proteins. To validate antibodies produced here, the reactivity against both the immunising protein (CFHR2-CFHR5) and serum proteins was determined. Initially, high throughput screening of hybridomas was performed by ELISA and identified 46 CFHR2, 41 CFHR3, 17 CFHR4 and 83 CFHR5 positive clones. However, many antibodies that recognise the immunising antigen have limited reactivity against the endogenous protein, or, in the case of closely related proteins, recognise more than one member of a protein family [Pulford, K. 2006]. Cross-reactivity can often be predicted due to high sequence identity, as for the CFHRs, but antibodies can also recognise cross-reactive epitopes or structural conformations of proteins which are difficult to predict from the amino acid sequence [Pulford, K. 2006. Martins S. 2007]. Therefore all positive hybridomas were also tested for their ability to bind against recombinant C-terminal CCP domains of CFH and CFHR1-CFHR5 by ELISA and western blot analysis, whereas CFHR5 anti-peptide mAbs were screened with full length CFHR5 and CFH by ELISA. Screening of mAbs identified two CFHR2, two CFHR3, no CFHR4 and four CFHR5 clones that specifically recognised the respective CFHR.

The specific recognition of the C terminal CCP domains from the corresponding CFHR does not guarantee that mAbs only bind the protein of interest. Therefore subsequent validation was performed using NHS, as the mAbs generated in this study will be used to determine levels of CFH and CFHRs in serum. Western blotting analysis was performed to demonstrate the molecular weight of proteins detected, to identify cross reaction with other proteins, and determine if mAbs can recognise known post-translational modifications *e.g.* glycosylation of CFHRs [Skerka, C. 1992. Skerka, C. 1993. Józsi, M. 2005. Murphy, B. 2002. Goicoechea de Jorge, E. 2013. Skerka, C. 2013].

CFHR2<sup>157b</sup> detected two bands in NHS consistent with the described glycosylation forms of CFHR2; with the glycosylation site at Asn<sup>126</sup> residing in CCP domain 2 [Skerka, C. 1992. Chen, R. 2009]. While CFHR3<sup>144a</sup> potentially detects 3 of 4 known glycosylation forms of CFHR3 [Skerka, C. 1993. Chen, R. 2009]. The known glycosylation sites for CFHR3 are at positions Asn<sup>108, -205, -309</sup>, with another predicted glycosylation site at Asn<sup>185</sup>; three of these sites are within CCP domains 2-3, while Asn<sup>309</sup> is within

CCP domain 5 [Chen, R. 2009]. As CFHR3<sup>144a</sup> may only detect three of the described glycosylation forms of CFHR3, the mAb binding site may overlap with the Asn<sup>309</sup> glycosylation site. Alternatively, the Asn<sup>185</sup> glycosylation site may not be modified in sera under these conditions. Furthermore, CCP domain 5 of CFHR3 and CFHR4 have a lower amino acid identity (93%) compared to CCP domain 4 of CFHR3 and CFHR4 (98%). Therefore the CFHR3<sup>144a</sup> binding epitope might reside within CCP domain 5 of CFHR3 [Skerka, C. 1993. Chen, R. 2009. Skerka, C. 2013].

CFHR5<sup>68a</sup> detected a second band by western blot analysis consistent with a predicted post translational modifications of CFHR5 at Asn<sup>126, -400</sup> which reside in CCP domains 2 and 7 respectively [Murphy, B. 2002. Goicoechea de Jorge, E. 2013]. CFHR4<sup>55a</sup> detected two predicted isoforms of CFHR4 as determined by the molecular weight of proteins identified in western blotting of NHS [Józsi, M. 2005].

The mAbs generated in this study will be used in the development of assays, both sandwich ELISA and Bio-Plex, to estimate the relative circulating concentrations of CFHRs in serum in the future. Therefore the mAb binding site will need to be characterised by epitope mapping [Engvall, E. 1971; Houser, B. 2012]. Determining the mAb epitope on CFHRs will elucidate the mechanism of binding and predict the effect of glycosylation on protein recognition and therefore on protein concentration estimation in NHS. The effect of glycosylation on mAb recognition could also be assessed by the removal of *N*-linked glycans by glycosidases to determine if protein estimations and detection remain the same.

Antibodies raised by immunisation with peptides generally have high specificity for the immunising antigen. However, synthesised peptides are generally not post-translationally modified and may not adopt their native conformations. Therefore the mAbs elicited by peptides may fail to recognise endogenous proteins [Forsström, B. 2015]. This may explain why peptides of CFHR4 and CFHR5 yielded few positive hybridomas (13 in total), of which only one (CFHR4<sup>55a</sup>) recognised endogenous CFHR4. The number of initial positive wells found for fusions after the immunisation with CFHR4 or CFHR5 peptides was lower than the number of positive wells generated with recombinant proteins (Table 4.1). Additionally, a significant number of Ig secreting hybridomas displayed no specificity for



the peptide or protein (Table 4.1), and two anti-peptide mAb clones, CFHR4<sup>55a</sup> and CFHR5<sup>80b</sup>, have an IgM isotype. IgM antibodies are secreted by B2-lymphocytes and are part of the initial humoral response to exogenous antigens [Henry, C. 1968. Baumgarth, N. 1999]. After antigen stimulation, B2-lymphocytes undergo clonal expansion, and in the presence of T-helper cells in splenic germinal centres, are subject to an isotype switch to IgG and the production of memory B cells [Bretscher, P. 1970. Lopes-Carvalho, T. 2004. Carsetti, R. 2004]. IgM occurs predominately as cyclic pentamers (960kDa) or hexamers (1150kDa) and are more susceptible to degradation than IgGs [Randall, T. D. 1990]. These properties make IgM antibodies more challenging to purify and unstable [García-González, M. 1988. Middaugh, C. R. 1977]. IgM antibodies generated in response to an antigenic stimulus are generally monospecific but have lower affinities ( $10^{-7}$ - $10^{-9}$  mol<sup>-1</sup> s<sup>-1</sup>) for antigens compared to IgG; *in vivo* this lower affinity is compensated by higher avidity [Baumgarth, N. 1999]. For these reasons and the lack of recognition of CFHR5 in NHS, it was decided to not characterise the IgM CFHR5<sup>80b</sup> further. Analysis of CFHR4<sup>55a</sup> showed specificity for CFHR4A and CFHR4B in NHS; therefore CFHR4<sup>55a</sup> will be further characterised to establish its specificity. If appropriate, the IgM heavy chain could be engineered to express an IgG heavy chain. Additionally, the CFHR4 peptide, ENSRAKSNGM, can be used to help determine mAb specificity by absorbance assays. Of note, subsequent CFHR5 fusions, obtained after immunisation with recombinant protein, successfully generated CFHR5 specific IgG mAbs, CFHR5<sup>68a</sup> and CFHR5<sup>86a</sup>.

CFHR4<sup>55a</sup> recognises bands corresponding to the predicted molecular weights of both previously described isoforms of CFHR4 (CFHR4A and CFHR4B) while CFHR5<sup>68a</sup> recognises bands corresponding to two predicted glycosylation forms of CFHR5 [Skerka, C. 1997. Józsi, M. 2005. Murphy, B. 2002]. However, mAbs CFHR4<sup>55a</sup> and CFHR5<sup>68b</sup> also recognise a non-specific band at approximately <250kDa and 200kDa respectively. Serum is a complex mixture consisting of an estimated 325-490 distinct circulating proteins with albumin, transferrin, haptoglobin, and immunoglobulins being some of the most abundant [Pieper, R. 2003. Tirumalai, R. S. 2003]. It is therefore possible that proteins aggregate during SDS-PAGE if not fully denatured [Marshall, T. 1984]. Samples were boiled at 100°C

for 10 minutes in sample buffer prior to loading. Reducing the denaturation temperature may reduce aggregate formation.

Specificity screening of CFHR2<sup>157b</sup>, CFHR4<sup>55a</sup> and CFHR5<sup>68a</sup> by western blot analysis of NHS (Figures 4.7b, d and e) was performed prior to mAbs being cloned by limiting dilution; therefore screening should be repeated with tissue culture supernatants from cloned hybridomas, or preferably purified mAbs to reduce potential background recognition of serum proteins from other antibodies. Initial western blots of NHS using CFHR3 had higher background staining with an additional band, not corresponding to the predicted molecular weight of the different glycosylation forms of CFHR3, detected at approximately 230kDa. After cloning the mAbs by limiting dilution subsequent western blots of NHS had reduced non-specific binding therefore cross-recognition of serum proteins by other antibodies in FCS or antibodies in the supernatant would be the most likely explanation for recognition of non-specific bands. It was not possible to clone CFHR2<sup>63a</sup> by limiting dilution. During clonal expansion, hybridomas are diluted to the single cell level and media is supplemented with BM Condimed H1; this hybridoma cloning supplement contains a mixture of growth factors and cytokines from Phorbol 12-myristate 13-acetate (PMA) stimulated mouse lymphoma cell lines [Rathjen, D. A. 1986]. Although clones were identified following dilution on two occasions, none of the clones generated supernatants that recognise the immunising antigen by ELISA. This indicates that CFHR2<sup>63a</sup> was not a clonal hybridoma. It is possible that CFHR2-negative hybridoma(s) outcompeted any positive CFHR2<sup>63a</sup> clones during growth. However, testing of further CFHR2 hybridomas identified CFHR2<sup>157b</sup> which specifically recognises CFHR2 and was successfully cloned by limiting dilution.

Recognition of the recombinant CFHRs and the presence of multiple bands of the molecular weight corresponding to different post-translational modifications or splice variants of CFH and CFHR1-CFHR5 are initial steps in validating CFHR mAbs. However, the specificity of mAbs has not been demonstrated unequivocally or in other applications, such as flow cytometry and immunohistochemistry. The binding affinity of mAb for CFH and CFHR1-CFHR5 can be determined by surface plasmon resonance (SPR), and specificity could be further demonstrated by flow

cytometry by conjugating recombinant CFH and CFHR1-CFHR5 to labelled polystyrene beads to analyse mAb reactivity. Ideally, the specificity of mAbs could be demonstrated using sera from patients with defined deletions, *e.g.*  $\Delta CFHR3/CFHR1$  and  $\Delta CFHR1/CFHR4$ , although deletions of *CFHR2* and *CFHR5* have not been described [Skerka, C. 2013]

Antibodies generated in this chapter will be used to study the biological function of the CFHRs and to determine the circulating concentrations of these proteins in NHS. For assay development and further downstream applications, mAbs need to be purified from tissue culture supernatants. Both mouse IgG and bovine IgG have high affinity for Protein G. Therefore contaminating bovine IgG needs to be removed from the tissue culture supernatants prior to mAb purification [Richman, D. D. 1982]. In the first instance HSL-1 was adapted to chemically defined, serum free tissue culture media. HSL-1 hybridoma cells did not grow well in serum replacement media, and had reduced mAb production (from 15 mg/L to 3 mg/L). To increase the concentration of mAbs produced by this hybridoma, cells were transferred into high expression system Integra™ flasks (CELLine CL350, IBS Integra Biosciences, Switzerland). Integra™ CELLine flasks enable cells to reach high density for maximum mAb secretion [Trebak, M. 1999].

Currently, there are no available antibodies which specifically recognise CFHR1, and none were generated during this study. This lack of reagents is most likely due to the high nucleotide identity of CCP domains 1-2 of CFHR1 with the N-terminal two domains of CFHR2 and CFHR5 (nucleotide sequence identity ranges from 85-100%, Figure 1.4). Furthermore the C terminal CCP domains 3-5 of CFHR1 are 97-100% identical to CCP domains 18-20 of CFH (Figure 1.4). Analysis of the residues which are different between CFHR1 with CFHR2, CFHR5 and CFH are predicted to not be surface exposed, therefore antibodies would have to recognise a structural epitope or only be effective against the denatured protein.

In summary the high degree of amino acid sequence identity between CFH and CFHR proteins has hampered the study of CFHR function. Cross reactivity of reagents has meant that purification of CFHRs from human serum and determining their physiological concentrations have been challenging. This study generated four, novel mAbs, CFHR2<sup>157b</sup>, CFHR3<sup>144a</sup>, CFHR4<sup>55a</sup> and CFHR5<sup>68a</sup>,

which are specific for CFHR2-CFHR5 respectively. These mAbs are likely to recognise multiple, glycosylation forms of the CFHRs, and can be utilised to study CFHR function in the general population as well as their role in human disease.

## 5. COMPETITION BETWEEN HOST MOLECULES INFLUENCES

### SUSCEPTIBILITY TO MENINGOCOCCAL DISEASE

#### 5.1 Introduction

*N. meningitidis* is a major cause of bacterial meningitis and sepsis worldwide [Stephens, D. S. 2007]. The annual number of cases is estimated to be 1.2 million, with an overall incidence of 1-3 per 100,000 of the population in Europe and North America [Rouphael, N. G. 2012. Yazdankhah, S. P. 2004]. Invasive disease incidence rates can rise to 500-1,000 per 100,000 of the population during epidemics in sub-Saharan Africa [Yazdankhah, S. P. 2004. Maiden, M. C. 1998. Greenwood, B. M. 1987]. However, for most individuals *N. meningitidis* is a commensal of the respiratory tract with carriage rates estimated to vary from 10 to 35% in the general population [Cartwright, K. A. 1987. Caugant, D. A. 1994. Stephens, D. S. 1999. Claus, H. 2005]. High rates of meningococcal carriage have been reported in closed or semi-closed populations, particularly among military recruits and university students where carriage rates can be higher than 70% [Caugant, D. A. 1992. Neal, K. R. 2000. Caugant, D. A. 2009].

The mechanisms underlying why some individuals develop invasive disease while others become asymptomatic carriers are incompletely understood. Individuals with rare, inherited deficiencies in components of the AP and TP have a markedly increased risk of developing invasive meningococcal disease, with complement deficiencies of C5 to C9 associated with a 7,000 to 10,000 fold increased risk compared to the general population [Figueroa, J. E. 1991. Ram, S. 2010]. The association of complement and invasive meningococcal disease is also highlighted in those receiving Eculizumab which inhibits C5 cleavage, and is associated with an elevated risk of developing meningococcal disease [Dmytrijuk, A. 2008. Kelly, R. J. 2011. Bouts, A. 2011]. Furthermore, a GWAS demonstrated that SNPs in *CFH* and the downstream gene, *CFHR3* are associated with meningococcal disease [Davila, S. 2010. Biebl, A. 2015].

CFH is the major fluid phase negative regulator of the AP recruited by *N. meningitidis*, inhibiting host complement activation and promoting survival in human blood [Weiler, J. M. 1976. Whaley, K. 1976. Pangburn, M. K. 1977. Madico, G. 2006. Schneider, M. C. 2006]. Almost all *N. meningitidis* isolates express the major CFH receptor, fHbp, which binds CFH at high affinity [Masignani, V. 2003. Lucidarme, J. 2011. Schneider, M. C. 2009]. fHbp is a variable protein, (<http://pubmlst.org/neisseria/fHbp/>), which can be divided into three variant groups that have >85% amino acid sequence identity within groups, but lower sequence identity between groups [Masignani, V. 2003. Brehony, C. 2009]. fHbps from all variant groups bind CFH at similar affinities with  $K_{Ds}$  in the nanomolar range [Johnson, S. 2012. Caesar, J. J. 2014].

While the role of CFH is well understood, the function of *CFHR3* has yet to be elucidated [Madico, G. 2006. Schneider, M. C. 2006]. The CFHRs are a group of proteins that are structurally similar to CFH but, unlike CFH, lack complement regulatory domains (CCP 1-4) that mediate co-factor for FI and decay accelerating activity [Díaz-Guillén, M. A. 1999. Skerka, C. 2013]. Recent structural studies have identified a conserved dimerisation motif in CFHR1, CFHR2, and CFHR5 which are required for the formation of homo- and hetero-dimers, whereby dimerisation increases the avidity of CFHRs for C3b [Goicoechea de Jorge, E. 2013]. CFHR1, CFHR2 and CFHR5 can compete with CFH for tissue-bound complement fragments and act as antagonists to modulate complement activation [Goicoechea de Jorge, E. 2013. Tortajada, A. 2013].

The aim of this chapter was to investigate the interaction between *CFHR3* and *N. meningitidis*, and to characterise the biological consequences of this interaction to determine how *CFHR3* might influence an individual's likelihood of developing meningococcal disease.

## 5.2 Results

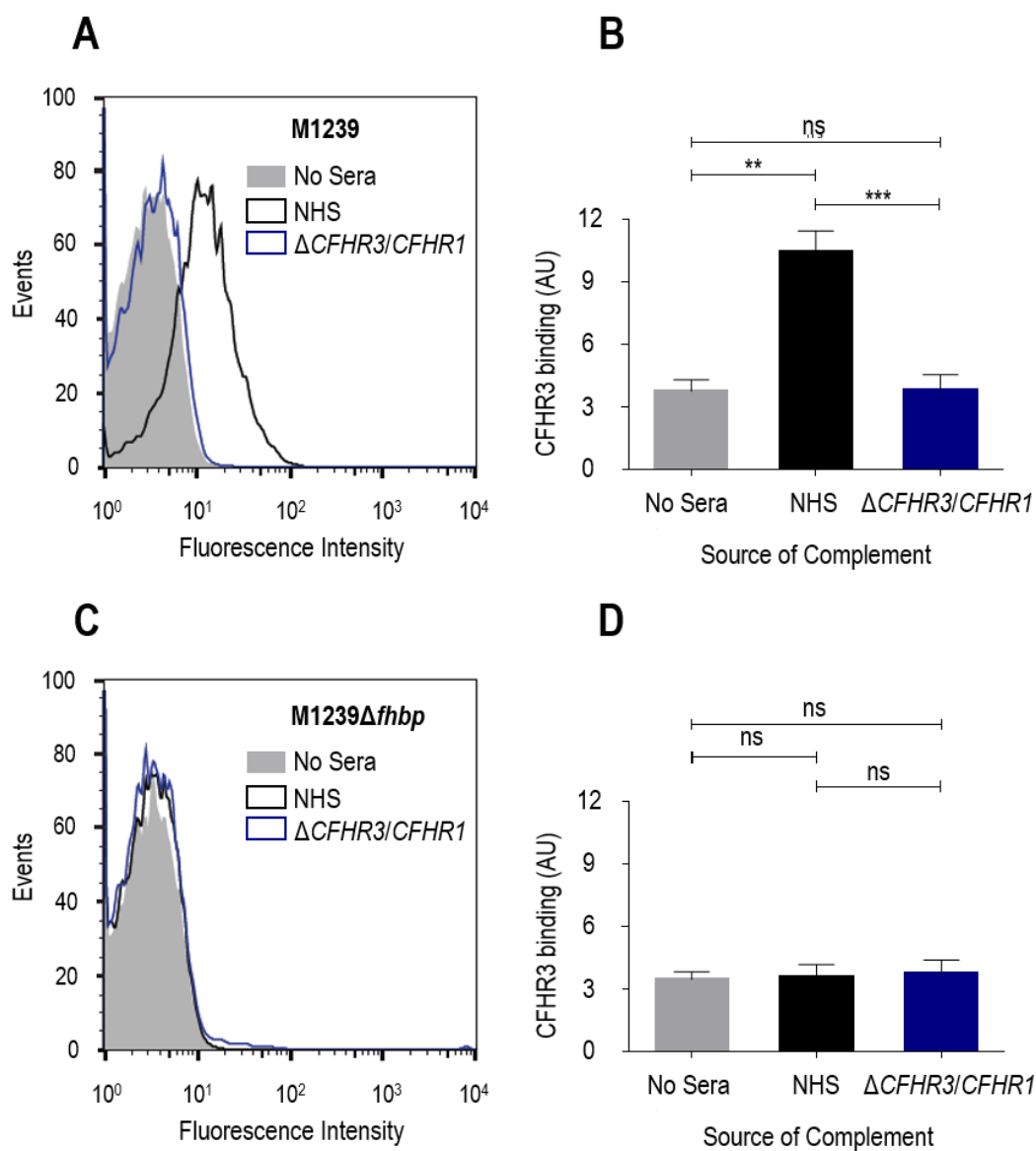
### 5.2.1 CFHR3 binds *N. meningitidis* in an fHbp-dependent manner

fHbp binds to CFH *via* CCP domains 6 and 7 (CFH<sub>(6, 7)</sub>) leading to high affinity interactions [Schneider, M. C. 2009]. CFH<sub>(6, 7)</sub> binds to fHbp by mimicking GAG binding *via* charged chains at positions H337 and R341 of CFH CCP domain 6; substitution of either of these amino acids with Ala leads to a ten-fold reduction of CFH binding affinity to fHbp ( $K_D$  2.7  $\mu$ M) [Prosser, B. E. 2007. Schneider, M. C. 2009]. The amino acid sequence analysis of the first two CCP domains of CFHR3 (CFHR3<sub>(1,2)</sub>), are 91% and 87% identical to CFH<sub>(6, 7)</sub>, respectively (Figure 1.4). Sequence alignments of CFHR3 CCP domain 1 and CFH domain 6 show that the residues important for fHbp interactions with CFH, R337 and H341, are conserved in CFHR3, suggesting that CFHR3 might bind fHbp. Therefore, binding of CFHR3 to *N. meningitidis* M1239 and M1239 $\Delta$ *fhbp* was analysed by flow cytometry using mAb HSL-1, which specifically recognises CFHR3 (Chapter 4). *N. meningitidis* M1239 expresses V3.28 fHbp [Masignani, V. 2003]. CFHR3 levels in serum are estimated to be between 50–80  $\mu$ g/ml (1–1.6  $\mu$ M); therefore NHS was initially used as a source of CFHR3 [Fritsche, L. G. 2010]. Following incubation with NHS, CFHR3 was detected on the surface of *N. meningitidis* M1239, but not M1239 $\Delta$ *fhbp* ( $p=0.005$ ) (Figure 5.1). Furthermore, no CFHR3 binding was detected following incubation of M1239 or M1239 $\Delta$ *fhbp* in sera from an individual homozygous for a combined deletion of *CFHR3* and *CFHR1* ( $\Delta$ *CFHR3/CFHR1*) (Figure 5.1). Taken together these results indicate that CFHR3 binds to the surface of *N. meningitidis* in an fHbp-dependent manner.

### 5.2.2 Development of an assay to investigate the effect of CFHR3 on bacterial survival

Having demonstrated that CFHR3 binds to fHbp on the surface of *N. meningitidis*, the next step was to determine the functional consequences of CFHR3 binding by examining the effect of surface bound CFHR3 on complement-mediated lysis. Previously, CFHR1 was reported to prevent formation of the AP C5 convertase (C3bBbC3b) by inhibiting binding of C3b to the AP C3 convertase (C3bBb) [Heinen, S. 2009]. However, recent evidence demonstrates that CFHR1, CFHR2 and CFHR5 act as antagonists of CFH by competing with CFH for tissue-bound C3b, promoting complement activation [Goicoechea de Jorge, E. 2013]. Therefore to investigate the effect of CFHR3 on complement-

mediated bacterial lysis, a serum assay was established so that after incubation in NHS approximately 80% of *N. meningitidis* survived. With these conditions, any potential reduction in bacterial survival after addition of exogenous CFHR3 should be detectable.



**Figure 5.1: *N. meningitidis* binds CFHR3 on its surface in an fHbp-dependent manner**

Flow cytometry analysis of CFHR3 binding to (A, B) *N. meningitidis* M1239, and (C, D) M1239Δ*fhbp*. Shaded area indicates results from bacteria incubated with PBS, solid black lines show bacteria incubated in NHS, and solid blue lines show bacteria incubated in CFHR3 deficient sera (Δ*CFHR3/CFHR1*). (B, D) Data presented as the mean fluorescence intensity in arbitrary units (AU). Error bars indicate ± SEM of three independent experiments and *p*-values were calculated using a two-tailed unpaired *t*-test; \*\*\*, *p* < 0.001; \*\*, *p* < 0.01; and ns > 0.05.

Initially, *N. meningitidis* M1239 was incubated in NHS (Figure 5.2 A) for increasing lengths of time (0-60 minutes) over a range of serum concentrations (0-75%) to determine the effect on bacterial



survival. The number of viable bacteria were measured by plating onto solid media and counting the number of colony forming units (CFU). A reduction in *N. meningitidis* survival was observed with increasing length of incubation of bacteria in the presence of NHS up to 60 minutes (Figure 5.2 A). Of note, no difference in survival of bacteria was observed following incubation in heat-inactivated NHS (HI-NHS) (Figure 5.2 B). For all subsequent serum survival assays, bacteria were incubated in the presence of serum for 30 minutes, as beyond this time there were limited differences in bacterial survival.

As approximately 20% of bacteria survived following incubation in 35% NHS over 30 minutes (Figure 5.2 A), *N. meningitidis* M1239 and M1239 $\Delta$ *fhhb* were incubated in serum concentrations ranging from 0% to 35% (Figure 5.2 C). In the presence of higher serum concentrations, there was decreased *N. meningitidis* survival as observed in Figure 5.1 A, while M1239 $\Delta$ *fhhb* was more susceptible to complement-mediated lysis than M1239 as expected (Figure 5.2 C). Approximately 80% bacterial survival was observed following 30 minute incubation in 5% NHS for *N. meningitidis* M1239; these conditions were used in subsequent experiments.

To ascertain the effect of CFHR3, the survival of *N. meningitidis* M1239 and M1239 $\Delta$ *fhhb* was evaluated after pre-incubating bacteria in a range of CFHR3<sub>(1,2)</sub> concentrations (0.01  $\mu$ M to 5  $\mu$ M) for ten minutes prior to exposing bacteria to NHS. Binding of CFHR3 to the meningococcus is mediated by CFHR3<sub>(1,2)</sub> which are homologous to CFH<sub>(6,7)</sub> [Caesar, J. J. 2014]. Therefore the effect of CFHR3<sub>(1,2)</sub> on bacterial survival was analysed. Pre-incubation in increasing concentrations of CFHR3<sub>(1,2)</sub> enhanced the susceptibility of *N. meningitidis* M1239 to complement-mediated lysis relative to bacteria incubated with no protein (Figure 5.2 D). Of note, the response of *N. meningitidis* M1239 to complement-mediated lysis after pre-incubation with CFHR3<sub>(1,2)</sub> was biphasic, indicating that there are potentially two subpopulations of bacteria even though the original population was clonal. The first subpopulation is rapidly killed whereas a second population is more resistant to complement-mediated lysis until higher CFHR3<sub>(1,2)</sub> concentrations enhance bacterial lysis. This phenomenon is typically observed in the emergence of 'persistence' after exposure to antibiotic treatment [Balaban, N. Q. 2004]. Moreover, pre-incubating M1239 $\Delta$ *fhhb* with CFHR3<sub>(1,2)</sub> had no effect on bacterial

survival (Figure 5.2 E) and CFHR3<sub>(1,2)</sub> did not affect the survival of M1239 or M1239Δ*fHbp* in the absence of complement (Figure 5.2 D-E). Therefore the addition of CFHR3<sub>(1,2)</sub> reduces *N. meningitidis* resistance to complement in a dose-dependent and fHbp-dependent manner.

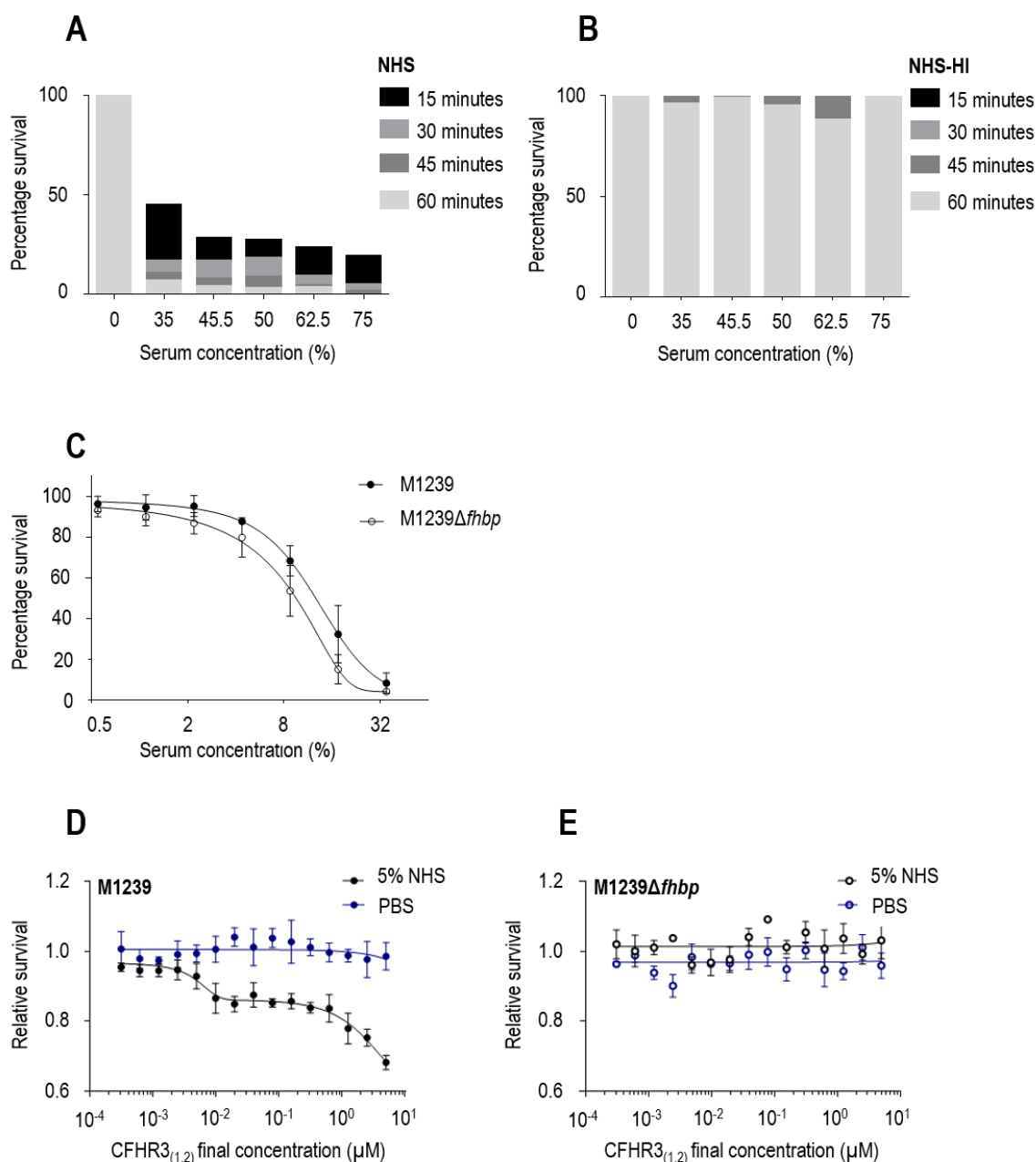
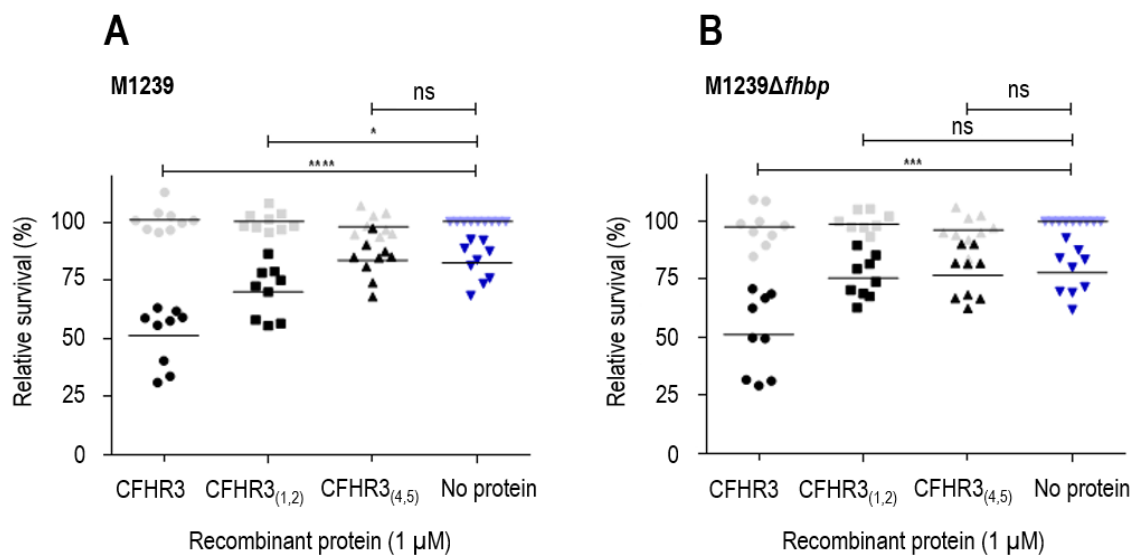


Figure 5.2: Optimisation of serum assays

Serum survival of *N. meningitidis* M1239 analysed after different incubation times (indicated) in (A) NHS or (B) HI-NHS over a range of serum concentrations (0-75%). Data are expressed as survival relative to the number of CFU without NHS in PBS at time point 0. (C) Survival of *N. meningitidis* M1239 (filled circles) and M1239Δ*fHbp* (unfilled circles) after incubation with a range of NHS concentrations (0-35%) for 30 minutes, approximately 80% of bacteria survive following a 30 minute incubation in 5% NHS. Survival of *N. meningitidis* (D) M1239 and (E) M1239Δ*fHbp* in the presence of 5% NHS (black circles and line) or PBS (blue circles and line) following pre-incubation in different concentrations of CFHR3<sub>(1,2)</sub>. Data are presented as percent survival relative to bacteria incubated without serum and CFHR3<sub>(1,2)</sub>. Error bars are ±SEM of three independent experiments.

### 5.2.3 *N. meningitidis* susceptibility to complement mediated lysis is altered by the acquisition of CFHR3

To further examine the biological consequences of *N. meningitidis* binding CFHR3, the effect of pre-incubating *N. meningitidis* with full length or domains of CFHR3 was analysed in serum assays. Subsequent experiments were performed with an exogenous protein concentration of 1  $\mu$ M, as this concentration of CFHR3<sub>(1,2)</sub> had a significant impact on *N. meningitidis* survival ( $p = 0.0118$ ) compared with no CFHR3. Pre-incubation of full length CFHR3 or CFHR3<sub>(1,2)</sub> resulted in significantly reduced survival of *N. meningitidis* M1239 in serum (two tailed  $t$ -test  $p = < 0.0001$  and  $p = 0.0161$ , respectively) (Figure 5.3 A); In contrast, CFHR3<sub>(3,4)</sub> had no effect on bacterial survival compared with no protein control ( $p = 0.8191$ ). Furthermore, the addition of CFHR3 or domains of CFHR3 had no effect on bacterial survival in the presence of HI-NHS (Figure 5.3 A). Furthermore, CFHR3<sub>(1,2)</sub> or CFHR3<sub>(3,4)</sub> did not affect the survival of *N. meningitidis* M1239 $\Delta$ *fhbp* after incubation in NHS or HI-NHS but the addition of full length CFHR3 resulted in significantly reduced bacterial survival in the absence of fHbp (two tailed  $t$ -test  $p = 0.001$ ) (Figure 5.3 B).



**Figure 5.3: CFHR3 increases susceptibility of *N. meningitidis* to complement-mediated lysis**

Sensitivity of *N. meningitidis* strains (A) M1239 and (B) M1239 $\Delta$ *fhbp* to complement-mediated lysis after pre-incubation with 1  $\mu$ M CFHR3, CFHR3<sub>(1,2)</sub>, CFHR3<sub>(4,5)</sub>, or PBS. (A, B) Faded symbols are bacteria incubated in HI-NHS. Data presented as percentage survival relative to bacteria incubated in HI-NHS without exogenous CFHR3. Line shows the mean of three independent experiments, each with three technical replicates with individual results indicated as symbols. Significance

was calculated using a two-tailed unpaired *t*-test when compared to the no protein control; \*\*\*\**p* < 0.0001; \*\*\**p* < 0.001; \**p* < 0.05; ns *p* > 0.05.

#### 5.2.4 Generation of *N. meningitidis* isogenic strains expressing different fHbp variants

fHbp is a highly variable lipoprotein [Masignani, V. 2003. Brehony, C. 2009]. To determine if sequence variation of fHbp affects binding of CFHR3 and consequently alters the serum sensitivity of *N. meningitidis*, M1239Δ*fhbp* was complemented with different *fhbp* alleles (*i.e.* V1.1, V1.14, V2.22, V3.28 and V3.28<sup>T286A</sup> fHbp). The gene encoding *fhbp* was ligated into PGCC4 complementation vector prior to transformation into M1239Δ*fhbp* at an ectopic site, in the intergenic region between NEIS0480 and NEIS0481, under the control of an IPTG inducible promoter; positive clones were selected on plates containing erythromycin [Mehr, I. J. 1998]. V3.28<sup>T286A</sup> fHbp has decreased CFH binding compared to the wild-type protein. The threonine residue at position 286 of V3.28 fHbp is located at the CFH:fHbp interface and so V3.28<sup>T286A</sup> fHbp was consequently used as a control [Johnson, S. 2012]. Expression and surface localisation of *fhbp* alleles after induction with IPTG was examined by flow cytometry analysis (Figure 5.4). For strains expressing the introduced V1.1 and V1.14 *fhbp* alleles, fHbp expression was detected with an anti-V1.1 fHbp pAb (Figure 5.4 A-B), whereas V2.22, V3.28 and V3.28<sup>T286A</sup> expressing strains were examined with anti-Ghfp pAb (Figure 5.4 C-D) [Johnson, S. 2012. Jongerius, I. 2013]. fHbp was expressed and localised on the surface of all strains with an introduced *fhbp* allele but not detected on the surface of M1239Δ*fhbp* (Figure 5.4). Apparent differences in detected expression levels of fHbp on the surface of complement M1239 strains are likely to be caused by differences in the recognition of fHbp sequence by the antibodies used.

#### 5.2.5 Development of Alternative Pathway serum assays to assess the functional consequences of CFHR3 binding

To further examine the biological consequences of binding CFHR3 on *N. meningitidis* and determine if fHbp sequence is likely to influence CFHR3 binding at the bacterial surface, an AP assay was established to exclude the potential influence of antibodies against different fHbps in sera. The CP and LP pathways were inactivated in sera by the addition of 10 mM EGTA, which specifically chelates

calcium, which is necessary for the formation of the C1 complex by the association of C1q and C1r<sub>2</sub>s<sub>2</sub>; Ca<sup>2+</sup> is also necessary for the association of MBL with the MASPs [Des Prez, R. M. 1975]. The AP is activated in the presence of 5mM MgCl<sub>2</sub> causing spontaneous hydrolysis of C3 and is non-specific as C3b deposition can occur on any surface unprotected by complement regulators [Des Prez, R. M. 1975].

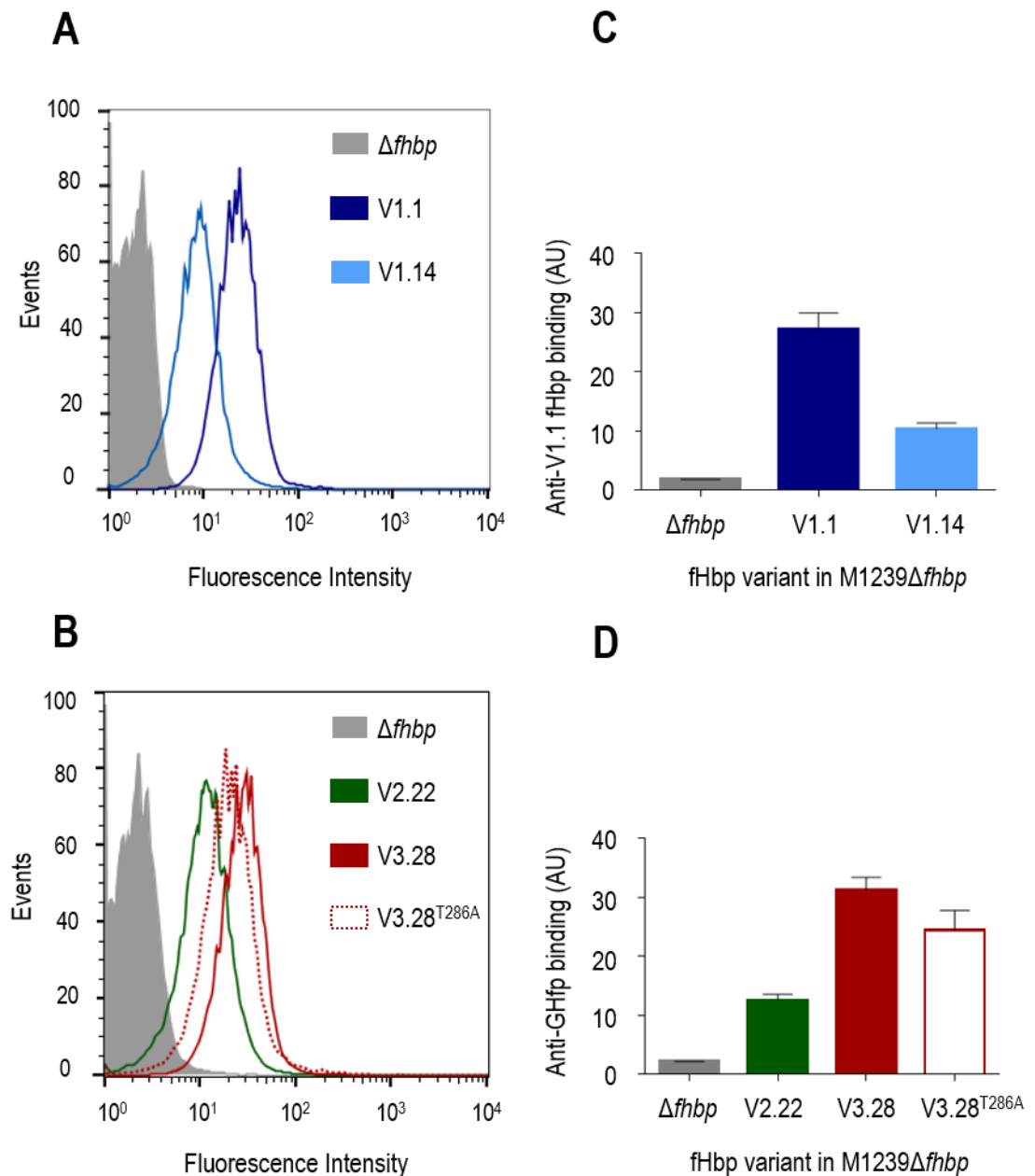
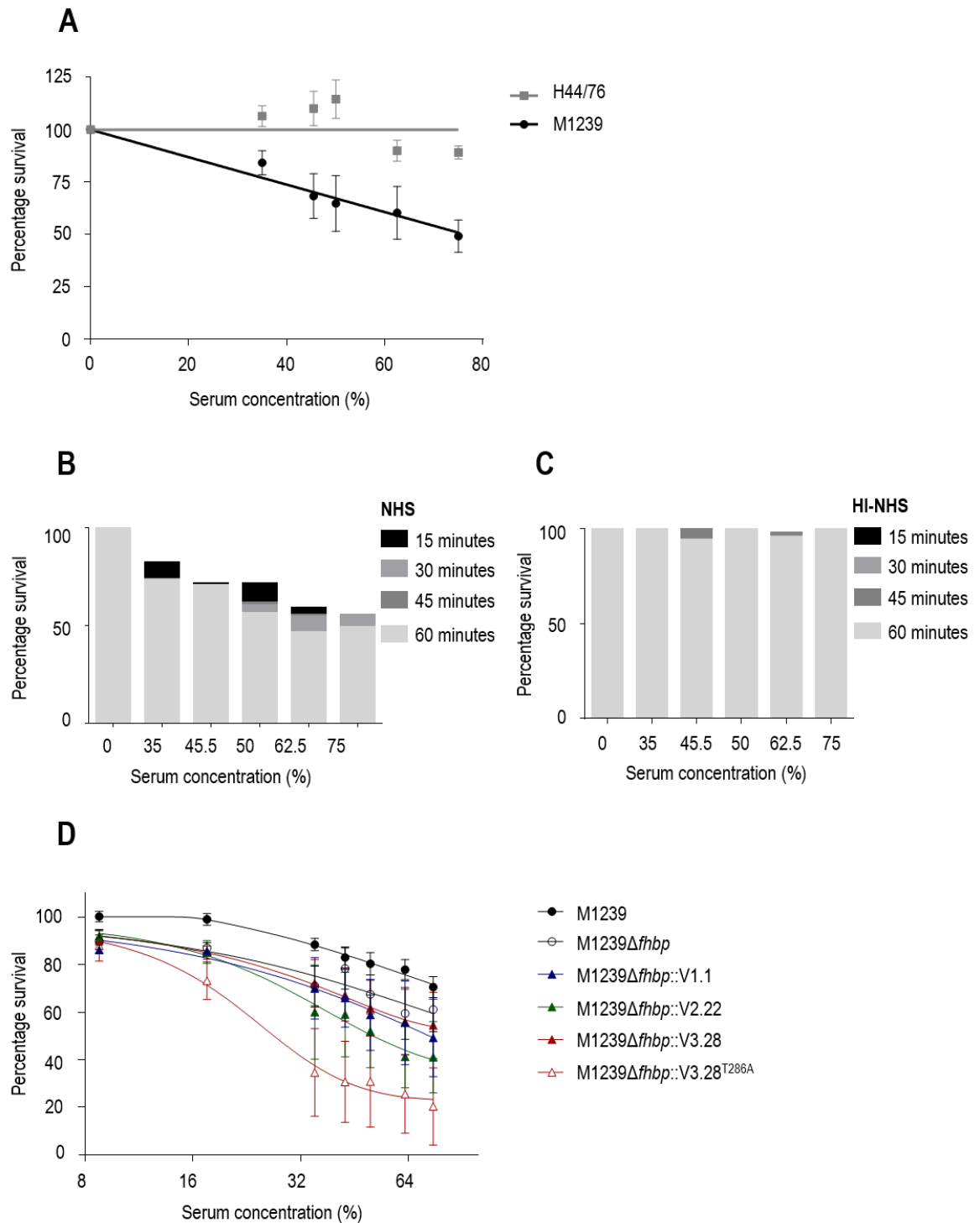


Figure 5.4: Characterisation of isogenic *N. meningitidis* expressing different fHbps

Flow cytometry analysis of *N. meningitidis* M1239, and isogenic strains with either (A, B) anti-V1.1 fHbp sera or (C, D) anti-GHfp sera. Detection of surface localised V1.1 (A-B, dark blue), V1.14 (A-B, light blue), V2.22 (C-D, green), V3.28 (C-D red) and V3.28<sup>T286A</sup> (C-D unfilled red) fHbp. Grey shaded area shows the plot for M1239Δfhbp. (B, D) Data presented as arbitrary units from the mean fluorescence of FACS Calibur FL-4 channel. Error bars are ± SEM of three independent experiments.

To evaluate the susceptibility of *N. meningitidis* to AP mediated lysis, *N. meningitidis* H44/76 (V1.1 fHbp) and M1239 (V3.28 fHbp) were incubated in NHS concentrations ranging from 0% to 75% with the addition of EGTA/ MgCl<sub>2</sub> (Figure 5.5 A). The percentage survival was calculated by comparing the number of CFU recovered after incubation in NHS compared with the number of CFU obtained after incubation without serum. The percentage survival of both *N. meningitidis* M1239 and H44/76 reduced with increasing serum concentration as expected (Figure 5.5 A). Furthermore, M1239 was more sensitive to AP-mediated lysis than H44/76, with percentage survival of, 49.1% and 89.2% respectively in 75% NHS (Figure 5.5 A). Of note, H44/76 was more resistant to AP-mediated lysis in the presence of NHS at all NHS concentrations examined, therefore M1239 was used in subsequent AP serum assays.

*N. meningitidis* M1239 was incubated in NHS (Figure 5.5 B) or HI-NHS (Figure 5.5 C) for increasing lengths of time (0-60 minutes) over a range of serum concentrations (0-75%) with only the AP active. In contrast to assays with all the complement pathways active, (Figure 5.2 A), there was no significant decrease in bacterial survival in AP assays beyond 15 minutes incubation (Figure 5.5 B). Moreover, there was no difference in the number of bacteria recovered after incubation in HI-NHS over 60 minutes (Figure 5.5 C). Next, the survival of *N. meningitidis* M1239, M1239 $\Delta$ fHbp and M1239 $\Delta$ fHbp isogenic strains (V1.1, V1.14, V2.22, V3.28 and V3.28<sup>T286A</sup>) was assessed in the AP assay with a range of serum concentrations (0-75%) in the presence of EGTA/Mg<sup>2+</sup> to determine the concentration of NHS required for 80% bacterial survival (Figure 5.2 D). As expected, there was a decrease in *N. meningitidis* survival observed with increasing NHS concentrations, and M1239 $\Delta$ fHbp was more susceptible to AP mediated lysis than M1239 (Figure 5.5 D). Interestingly, *N. meningitidis* M1239 fHbp complemented isogenic strains were more sensitive to AP mediated lysis than both M1239 and M1239 $\Delta$ fHbp. Furthermore, M1239 $\Delta$ fHbp::V3.28<sup>T286A</sup>, which expresses a non-functional fHbp, had significantly reduced survival in AP serum assays compared to M1239 $\Delta$ fHbp (Figure 5.5 B,  $p = 0.0493$ ). Of note, approximately 80% bacterial recovery of *N. meningitidis* M1239 was seen in AP assays following a 30 minute incubation in 25% NHS. Subsequent experiments to investigate the effect of sequence variants of fHbp on *N. meningitidis* survival were performed under these conditions.



**Figure 5.5: Optimisation of AP assays**

(A) Survival of *N. meningitidis* M1239 (black line) and H44/76 (grey line) in AP survival assays using a range of NHS concentrations (0-75%) for one hour. Survival of *N. meningitidis* M1239 in AP assays analysed at different incubation times (indicated) in either (B) NHS or (C) HI-NHS over a range of serum concentrations (0-75%); data expressed as the percent survival compared with the number of CFU in the absence of NHS at time point 0. (D) Survival of *N. meningitidis* M1239 (black), M1239ΔfHbp and fHbp isogenic strains (indicated) in AP assays over a range of NHS concentrations (0-70%). Approximately 80% bacterial survival is seen following a 30 minute incubation in 25%-35% NHS. Data presented as percent survival relative to bacteria incubated in HI-NHS for 30 minutes. Error bars are ± SEM of three independent experiments.

## 5.2.6 fHbp sequence affects strain sensitivity to complement mediated lysis after incubation with CFHR3

Binding affinities of CFH and CFHR3 with fHbp have been determined for 25 naturally occurring fHBps from clinical isolates of *N. meningitidis*. The results demonstrate that V2 and V3 fHbp have no selectivity for CFH or CFHR3 (average  $K_D$ s 1.8 nM and 2.4 nM, respectively) [Caesar, J. J. E. 2014]. However, V1 fHbp had lower affinities for CFHR3 compared with CFH (average  $K_D$  for CFHR3, 8.9 nM) [Caesar, J. J. E. 2014]. To determine if the functional consequences of CFHR3 binding to *N. meningitidis* is affected by fHbp sequence, the effect of CFHR3, CFHR3<sub>(1,2)</sub> and CFHR3<sub>(4,5)</sub> on bacterial survival of *N. meningitidis* fHbp isogenic strains was examined in AP assays (Figure 5.6 A-C). *N. meningitidis* expressing V3.28 fHbp was more sensitive to AP-mediated lysis following pre-incubation of CFHR3 (Figure 5.6 A) or CFHR3<sub>(1,2)</sub> (Figure 5.6 B) compared with bacteria expressing V1.1 fHbp ( $p = < 0.0001$  and 0.9974, respectively) consistent with the preferential affinity of V1.1 fHbp for CFH compared with CFHR3. Bacteria expressing either V1.14 or V2.22 fHbp had intermediate survival (Figure 5.6 A-B). Irrespective of the fHbp sequence, pre-incubation of *N. meningitidis* M1239 with full length CFHR3 (Figure 5.6 A) led to decreased bacterial survival compared with CFHR3<sub>(1,2)</sub> (Figure 5.6 B), [ $p$  values, 0.9996 to  $< 0.0001$ ]. Of note, bacteria expressing V3.28<sup>T286A</sup>, which has previously been shown to have decreased binding affinity for CFH, were unaffected after pre-incubation with CFHR3<sub>(1,2)</sub> (Figure 5.6 B) [Johnson, S. 2009]. Interestingly, pre incubation of full length CFHR3 with *N. meningitidis* expressing V3.28<sup>T286A</sup> led to an increase in sensitivity ( $p = 0.0019$ ) to AP-mediated lysis (Figure 5.6 A). Furthermore, pre-incubation of bacteria expressing any fHbp variant with CFHR3<sub>(4,5)</sub> had no significant impact on bacterial survival compared to no protein (Figure 5.6 C). Taken together, the results demonstrate fHbp sequence influences the survival of *N. meningitidis* in the presence of CFHR3 in AP assays.



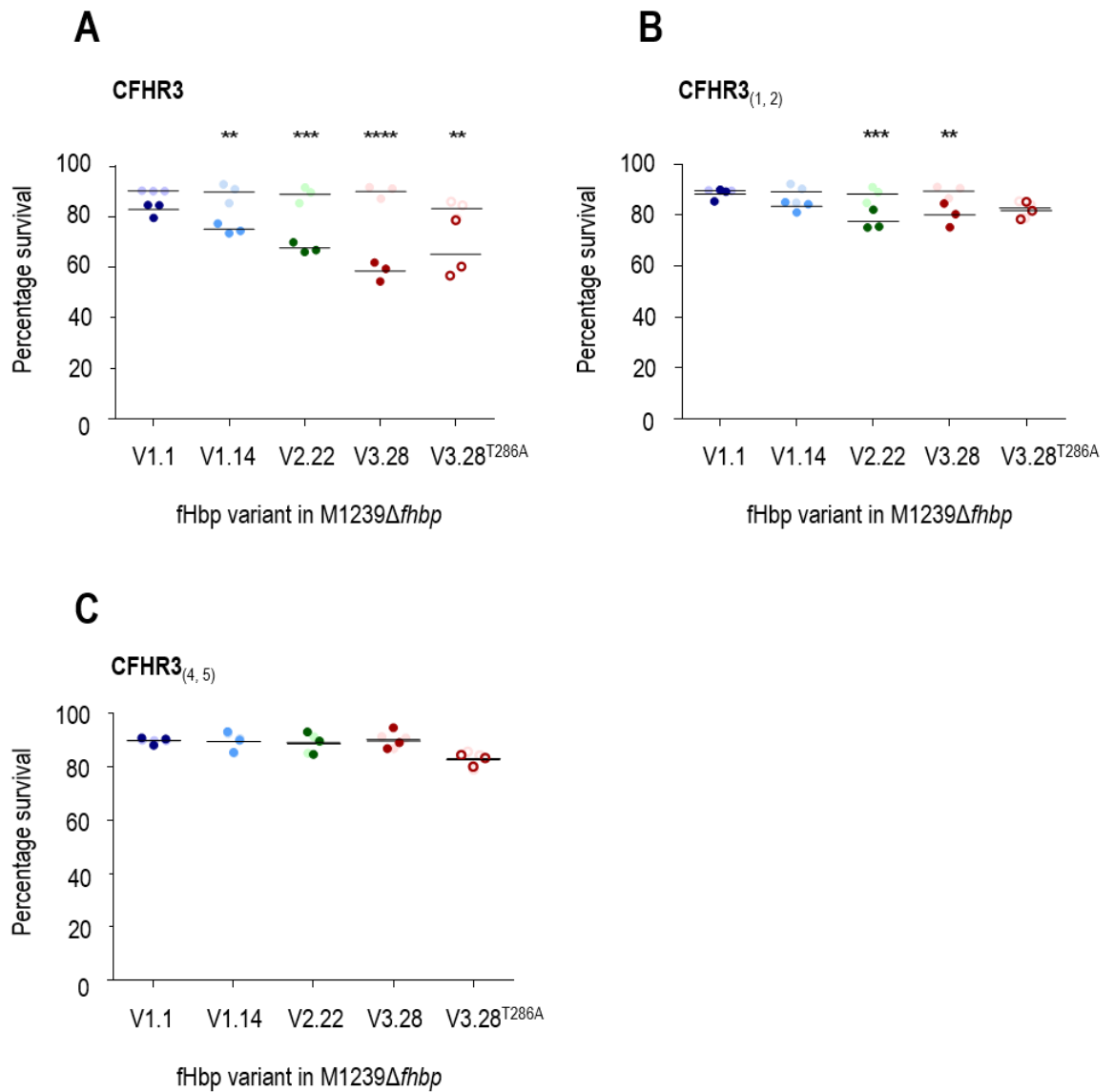


Figure 5.6: Survival of *N. meningitidis* in AP assays is affected by the fHbp sequence

AP-mediated bacterial lysis of *N. meningitidis* M1239 isogenic strains expressing V1.1 (dark blue), V1.14 (light blue), V2.22 (green), V3.28 (red) and V3.28<sup>T286A</sup> (unfilled red) fHbp in the presence of 1 μM of (A) CFHR3, (B) CFHR3<sub>(1,2)</sub> or (C) CFHR3<sub>(4,5)</sub>. Faded symbols show results of survival of bacteria incubated with no exogenous protein. Line shows mean of three independent biological experiments. Data presented as percent survival relative to bacteria incubated for 30 minutes in HI-NHS without exogenous protein. Significance was calculated by comparison with no protein control (faded symbols) using two-way ANOVA multiple comparisons; *p* values; \*\*\*\*, *p* < 0.0001; \*\*\*, *p* < 0.001; \*\*, *p* < 0.01; ns *p* > 0.05.

### 5.2.7 CFHR3 competes with CFH for binding of *N. meningitidis* fHbp

As CFHR3 binds to *N. meningitidis* in an fHbp-dependent manner (Figure 5.1) and potentially occupies the same binding sites as CFH (Figure 5.6 B), the impact of CFHR3 on CFH binding *N. meningitidis* was assessed by flow cytometry. *N. meningitidis* M1239 incubated in ΔCFHR3/CFHR1

sera binds high levels of CFH on the bacterial surface (Figure 5.7 A, C). However, addition of increasing concentrations (up to 1  $\mu$ M) of CFHR3 results in a significant, dose-dependent reduction in bound CFH (Figure 5.7 A, C) and a dose-dependent increase in surface bound CFHR3 (Figure 5.7 B, C). With the addition of 1  $\mu$ M of CFHR3 the level of bound CFH on the surface of *N. meningitidis* is approximately 50% lower than observed without CFHR3.

In summary, CFHR3 competes with CFH for fHbp on the meningococcal surface, and increases the sensitivity of *N. meningitidis* to complement-mediated lysis. Furthermore, the sequence of fHbp can influence sensitivity to AP-mediated lysis, with V1 fHbp expressing strains the least sensitive to AP-mediated lysis. Therefore, an individual's risk of developing meningococcal disease is likely to be affected by the relative concentrations of circulating CFH and CFHR3, and the sequence of fHbp on the meningococcal surface.

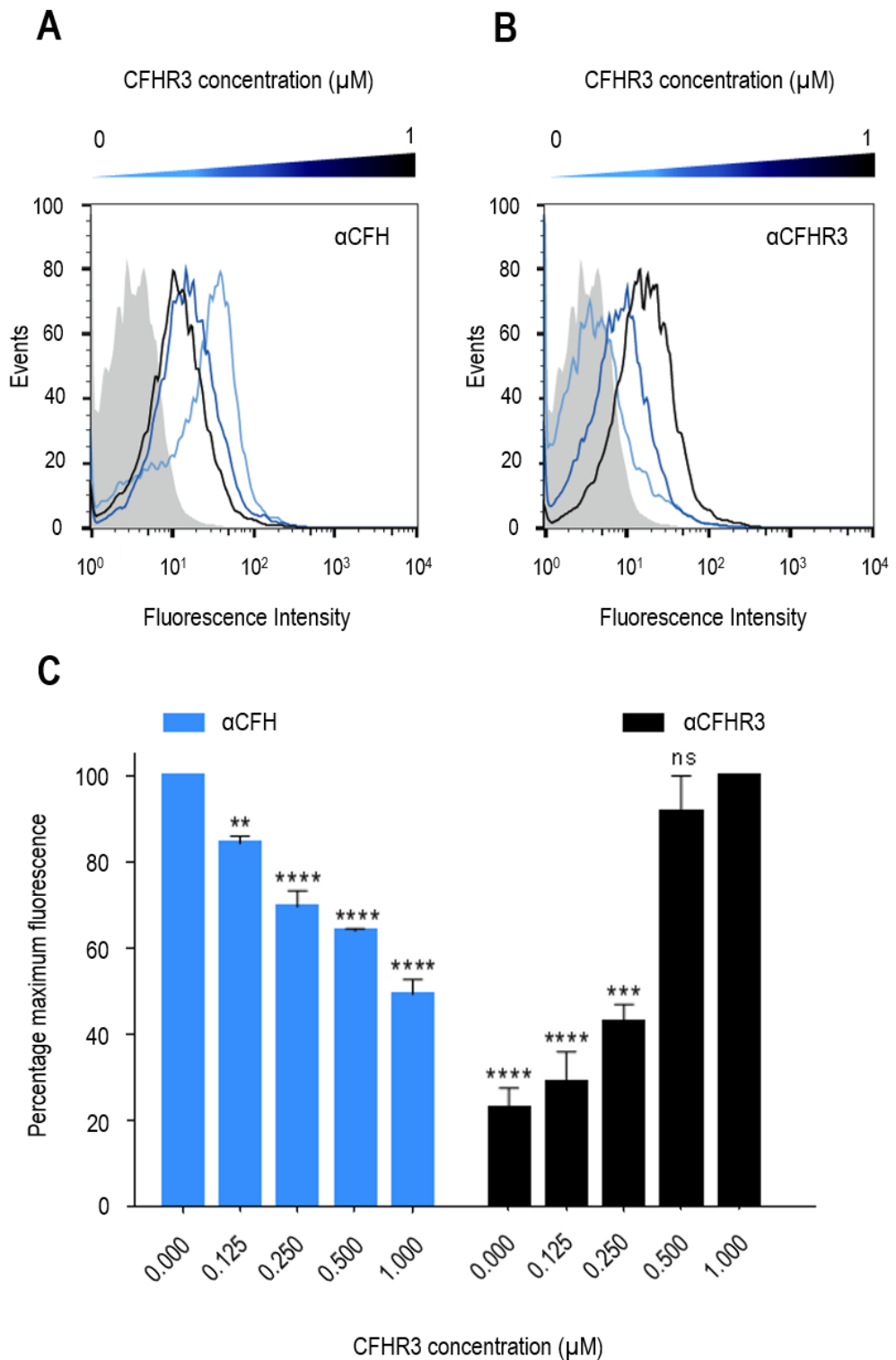


Figure 5.7: CFH and CFHR3 compete for binding to fHbp

Flow cytometry analysis of *N. meningitidis* M1239 demonstrates that addition of increasing concentrations of CFHR3 (from 0  $\mu\text{M}$  - light blue to 1  $\mu\text{M}$  - black) to CFHR3-deficient sera ( $\Delta\text{CFHR3}/\text{CFHR1}$ ) leads to decreasing levels of CFH (A and C) and increasing levels of CFHR3 (B and C) on the bacterial surface. Binding was detected using (A) anti-CFH mAb (OX24) or (B) anti-CFHR3 mAb (HSL-1). Grey shaded area indicates bacteria incubated with PBS. (C) Data presented as a percentage of the maximum fluorescence in arbitrary fluorescence units for sera without CFHR3 for CFH, and with 1  $\mu\text{M}$  additional CFHR3 for CFHR3. Statistical significance analysed with one-way ANOVA multiple comparisons;  $\pm$  S.E.M are shown,  $p$ -values; \*\*\*\*,  $p < 0.0001$ ; \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ ; ns  $p > 0.05$ .

### 5.3 Discussion

Work in this chapter demonstrates that CFHR3 binds to *N. meningitidis* in an fHbp-dependent manner, and that CFHR3 can compete with the negative complement regulator, CFH, for the same molecule on the bacterial surface. Higher levels of surface bound CFHR3 are likely to promote complement activation by acting as an antagonist of CFH and competing for surface bound C3b, leading to increased sensitivity of *N. meningitidis* to complement mediated lysis (Figure 5.3) [Caesar, J. J. E. 2014]. Sequence differences in fHbp variants also affect the susceptibility of *N. meningitidis* to AP-mediated lysis; bacteria expressing V1 fHbp were less susceptible to lysis than bacteria expressing V2 or V3 fHbp. Therefore host susceptibility to meningococcal disease may be influenced by the relative circulating serum concentrations of CFH and CFHR3, and by variation in fHbp sequence in infective strain.

Understanding the biological function of CFHR proteins has been hampered by the lack of available reagents as many antibodies cross-react with other members of the protein family (Figure 4.1). Flow cytometry analysis with mAb HSL-1, generated and characterised in Chapter 4, demonstrates that CFHR3 in NHS binds to the surface of *N. meningitidis* in an fHbp-dependent manner (Figure 5.1 A-B). This interaction is mediated by high affinity binding between CFHR3<sub>(1,2)</sub> and fHbp ( $K_D$  4nM), similar to the values obtained with CFH and fHbp [Caesar, J. J. 2014]. This finding is consistent with the high amino acid sequence identity between CFHR3<sub>(1,2)</sub> and CFH<sub>(6,7)</sub> (91% and 85% identity for the CCP domains, respectively). Of note, Ala substitutions in V1, V2 and V3 fHbp that reduce CFH-fHbp binding also impact CFHR3-fHbp interaction, indicating that CFH and CFHR3 share a common, overlapping binding site on fHbp [Caesar, J. J. 2014]. Two other meningococcal proteins, NspA and PorB2 have been suggested to bind CFH by CCP domains 6 and 7, but binding to these proteins has only been shown in the absence of fHbp [Lewis, L. A. 2010. Lewis, L. A. 2013]. Further work could investigate whether CFHR3 can bind to these other proteins. However, there is no evidence to indicate that CFHR3 binds to any protein other than fHbp on the surface of *N. meningitidis* as the  $\Delta fHbp$  did not bind CFHR3 (Figure 5.1)

Initial studies proposed that CFHRs regulate the TP, by inhibiting formation of the C5 convertase, C3bBbC3b, by binding to C3b [Heinen, S. 2009]. Other studies have suggested that CFHRs act as regulators of the AP, but were performed under non-physiological conditions [Hellwage, J. 1999. Fritsche, L. G. 2010. McRae, J. L. 2005]. However, recent findings demonstrate that CFHR1, CFHR2, and CFHR5 are antagonists of CFH, and compete with CFH for tissue bound C3b, promoting complement activation [Goicoechea de Jorge, E. 2013]. Work here also shows that CFHR3 is a competitive antagonist of CFH at the meningococcal surface.

Pathogens bind both CFH (Table 1.1) and CFHRs (Table 1.2), with *B. burgdorferi* selectively binding CFH or CFHRs *via* different receptors on the bacterial surface [Haupt, K. 2007. Siegel, C. 2010]. Here the effect of CFHR3, or domains of CFHR3 on survival of *N. meningitidis* in the presence of NHS was examined. Addition of CFHR3 or CFHR3<sub>(1,2)</sub> decreased survival of *N. meningitidis* in a dose-dependent and fHbp-dependent manner (Figure 5.2 D-E). Furthermore, the addition of CFHR3<sub>(4,5)</sub> did not affect bacterial survival. Potential explanations for this include the inability of CFHR3<sub>(4,5)</sub> to compete with CFH or to localise to the bacterial surface without the CFHR3-fHbp interaction *via* CFHR3<sub>(1,2)</sub> (Figure 5.3 B). However, full length CFHR3 decreased survival of *N. meningitidis* significantly more than CFHR3<sub>(1,2)</sub> suggesting that CFHR3<sub>(4,5)</sub> may compete for surface bound C3b when attached to the bacterial surface. Therefore CFHR3 might have two roles influencing survival of *N. meningitidis* in serum; competition with CFH for fHbp *via* CFHR3<sub>(1,2)</sub> and competition with C3b *via* CFHR3<sub>(4,5)</sub>. However, addition of CFHR3 to M1239 $\Delta$ fHbp reduced bacterial survival to a similar extent as M1239 (Figure 5.3 B) which may be due to an increased opsonisation of M1239 $\Delta$ fHbp resulting in high deposition of C3b on the bacterial surface; consequently higher concentrations of CFHR3 are bound on the bacterial surface by CFHR3:C3b interactions [Madico, G. 2006].

fHbp is expressed by almost all clinical isolates of *N. meningitidis* and is a variable protein, which can be categorised into three variant groups [Brehony, C. 2009. Lucidarme, J. 2011]. fHbps belonging to the same variant group have approximately 85% amino acid identity, with approximately 60% identity between different variant groups [Brehony, C. 2009]. Previous investigations have highlighted that fHbps from different variant groups bind CFH with affinities in the nanomolar range

[Johnson, S. 2012]. However, isogenic *N. meningitidis* strains expressing different fHbp variants had different sensitivities to AP-mediated lysis after the addition of CFHR3 or CFHR3<sub>(1,2)</sub> (Figure 5.6 A-B) consistent with evidence that sequence variation of fHbp affects the binding affinity for CFHR3 [Caesar, J. J. 2014]. Examination of the relative binding affinities of fHbp variants for both CFH and CFHR3 showed that V1 fHbp can partially discriminate between CFH and CFHR3, with V1 fHbps displaying between 5-20 fold increased affinity for CFH than CFHR3; V2 and V3 fHbp had equal binding affinities for both proteins [Caesar, J. J. 2014]. Of note, isogenic *N. meningitidis* expressing V1, V2 and V3 fHbps were more susceptible to complement mediated lysis after addition of CFHR3 (Figure 5. 6 A). The difference in the ability of fHbps to discriminate between CFH and CFHR3 might explain why V1 fHbps are the most prevalent fHbp in clinical isolates, accounting for approximately 54-70% of cases [Maignani, V. 2003. Fletcher, L. D. 2004. Jacobsson, S. 2006. Beernink, P. T. 2007. Murphy, E. 2009. Brehony, C. 2009].

Variation in the *CFH/CFHR* gene locus on human chromosome 1q32 has been linked to susceptibility to meningococcal disease [Davila, S. 2010. Biebl, A. 2015]. A deletion of  $\Delta CFHR3/CFHR1$  is also associated with the development of anti-factor H auto-antibodies in haemolytic uremic syndrome (DEAP HUS) [Holmes, L. V. 2013. Zipfel, P. F. 2007]. However the molecular mechanisms underlying host susceptibility to meningococcal disease in the general population is not well understood and the causative polymorphisms/deletions associated with the SNPs identified in the GWAS are not known. Therefore, further work will focus on examining the relative levels of CFH and CFHR3 between individuals with meningococcal disease and control groups and whether the relative levels are different in cases where the infective strains expresses a V1 fHbp compared with V2 and V3 fHbps.

Work presented here shows that CFHR3 and CFH directly compete for fHbp on the bacterial surface, therefore it is likely that relative serum levels of CFH and CFHR3 affect meningococcal survival in serum and influence disease susceptibility. Individuals with increased CFH or decreased levels of CFHR3 in the circulation may have increased CFH bound on the bacterium and may be at increased risk of meningococcal disease. In contrast, decreased CFH or increased CFHR3 levels would result in

higher concentrations of bound CFHR3, and may decrease susceptibility to meningococcal disease. Of note, the frequency of  $\Delta CFHR3/CFHR1$  is highest in Africa (54.7% in Nigeria) where there is a high incidence of meningococcal disease and lowest (0%) in individuals from Japan and South America [Holmes, L. V. 2013]. It will be interesting to examine the genetic basis of meningococcal susceptibility in patients from Africa, where epidemic disease occurs every 5-15 years [Wall, E. C. 2014]. These studies provide a mechanistic basis for the GWAS that identified CFHR3 as being involved in susceptibility to meningococcal disease in the general population. GWAS studies, including the work on *N. meningitidis*, do not assay copy number variation. Therefore it will be important in the future to determine whether the  $\Delta CFHR3/CFHR1$  deletion and other polymorphisms are associated with changes in susceptibility to meningococcal disease.

## 6. CHARACTERISATION OF FHBP FROM NEISSERIA CINEREA AND POTENTIAL IMPLICATIONS OF VACCINATION AGAINST MENB

### 6.1 Introduction

The bacterial genus *Neisseria* includes two major human pathogens, *N. meningitidis* and *N. gonorrhoeae* [Knapp J. S. 1988. Smith, N. H. 1999]. *N. meningitidis* is an asymptomatic commensal of the human nasopharynx in approximately 10-40% of the general population but can transverse the epithelial layer and cause systemic disease [Stephens, D. S. 2007]. Non-pathogenic *Neisseria* species can also colonise the human oral cavity and/ or nasopharynx of healthy individuals [Gold, R. 1978. Knapp, J. S. 1988].

Capsular polysaccharide-conjugated vaccines have been developed for protection against some serogroups of *N. meningitidis* i.e. A, C, W and Y, while outer membrane vesicle (OMV) vaccines have been employed to combat epidemic disease outbreaks caused by a single clone [Vipond, C. 2012. Kelly, C. 2007]. Neither of these approaches have been successful against endemic serogroup B strains of *N. meningitidis*, which still cause significant numbers of meningococcal disease cases in Europe and the USA. This is due to concerns of inducing autoimmunity, as the  $\alpha$ 2-8 linked polysialic acid of the serogroup B capsule is identical to a modification of human N-CAM1 [Finne, J. 1983. Finne, J. 1987. Jennings, H. J. 1981], while OMV vaccines offer limited cross protection [Fredriksen, J. H. 1991. Holst, J. 2009. Tappero, J. W. 1999. Tondella, M. L. 2000]. Recently developed meningococcal vaccines against serogroup B *N. meningitidis* are composed of multiple protein antigens to enhance immunogenicity and cross protection [Giuliani, M. M. 2006]. Two, recently licensed vaccines, Bexsero® and Trumemba®, both contain fHbp [Maignani, V. 2003. Fletcher, L. D. 2004].

fHbp is a 27 kDa surface lipoprotein expressed by almost all disease isolates of *N. meningitidis* and elicits SBA, a known correlate of protection against the meningococcus [Maignani, V. 2003. Fletcher, L. D. 2004. Lucidarme, J. 2011. Gotschlich, E. C. 1969]. fHbp can be classified into one of three variant groups (V1, V2 or V3), based on its amino acid sequence [Fletcher, L. D. 2004. Brehony, C. 2009].



fHbps within the same variant groups generally can induce cross protection due to high amino acid sequence identity, whereas there is little or no cross protective immunity between variant groups [Massignani, V. 2003]. fHbp promotes survival of *N. meningitidis* in human blood by binding CFH at high affinity via CCP domains 6 and 7 [Madico, G. 2006. Schneider, M. C. 2006. Schneider, M. C. 2009]. In addition, CFHR3 competes for binding to fHbp and acts as a competitive antagonist of CFH, whereby surface bound CFHR3 decreases meningococcal survival (Chapter 5).

Recent genome analysis of 80 strains of pathogenic and commensal *Neisseria* found *fhhp* in all *N. meningitidis* (n=27) and *N. cinerea* isolates (n=5) examined [Muzzi, A. 2013]. *fhhp* was also present in all *N. gonorrhoeae* (n=17) and *N. polysaccharea* (n=7) included in the study, although most of the *fhhp* genes in these species contain a frame shift mutation; this frame shift has been shown to render the protein unable to bind CFH in *N. gonorrhoeae*, isolate F62 [Hadad, R. 2012. Jongerius, I. 2013. Muzzi, A. 2013]. *N. cinerea* *fhhps* have a significant nucleotide identity with V1 *fhhp*, whereas *N. gonorrhoeae* and *N. polysaccharea* harbour homologues of V3 *fhhp* [Muzzi, A. 2013]. The commensal *Neisseria* spp. have been speculated to serve as reservoirs for virulence-associated alleles as high rates of genetic exchange can potentially occur due to the diversity of commensal *Neisseria* spp. on human mucosal surfaces [Marri, P. R. 2010. Stabler, R. A. 2005. Sáez Nieto, J. A. 1998]. For example, *N. cinerea* contains two genes for the major Tfp subunit, *pile*, which is required for virulence, although in contrast to *N. meningitidis*, Pile is not involved in bacterial association with epithelial cells [Wörmann, M. E. 2016]. Although other virulence associated genes, including *fhhp*, *opa*, and *nadA* have been identified in the genome of *N. cinerea*, their expression and function have not been studied [Toleman, M. 2001. Muzzi, A. 2013].

Previous evidence indicates that commensal *Neisseria* spp. can induce immunity against the meningococcus. For example, colonisation with *N. lactamica* is thought to offer immunity against *N. meningitidis* [Gold, R. 1978. Cann, K. J. 1989. Snyder, L. A. 2006]. Moreover, human challenge with *N. lactamica*, have identified salivary IgA and serum IgG antibodies that cross react with *N. meningitidis* within four weeks of carriage; non-carriers and the control group did not develop antibodies against *N. lactamica* or *N. meningitidis* [Evans, C. M. 2011]. Furthermore, inoculated

individuals who were colonised by *N. lactamica* were protected against subsequent acquisition of *N. meningitidis* [Evans, C. M. 2011]. The presence and conservation of *fhbp* in commensal *Neisseria* shown by Muzzi *et al.* and the shared bacterial niche in the human nasopharynx between *N. meningitidis* and *N. cinerea* raises the issue of whether implementation of protein-based vaccines containing V1 fHbp could impact the nasopharyngeal flora.

Therefore, the aim of this study was to investigate the expression and function of *N. cinerea fhbp* and to evaluate the immunogenicity of Bexsero® against *N. cinerea*.

## 6.2 Results

### 6.2.1 *N. cinerea* fHbp is predicted to be structurally similar to meningococcal fHbp.

Previous studies have identified a homologue of *fhbp* in the *N. cinerea* genome with the *fhbp* locus conserved compared to *N. meningitidis* [Muzzi, A. 2013]. Genome sequences of five *N. cinerea* isolates, CCUG 346 T, CCUG 5746, CCUG 25879, CCUG 27178 A, and CCUG 53043, were obtained from the PubMLST *Neisseria* BIGSdb database (<http://pubmlst.org/neisseria/>). All isolates contain *fhbp* with a predicted signal peptide, conserved lipobox, and a variable linker sequence (Figure 6.1).

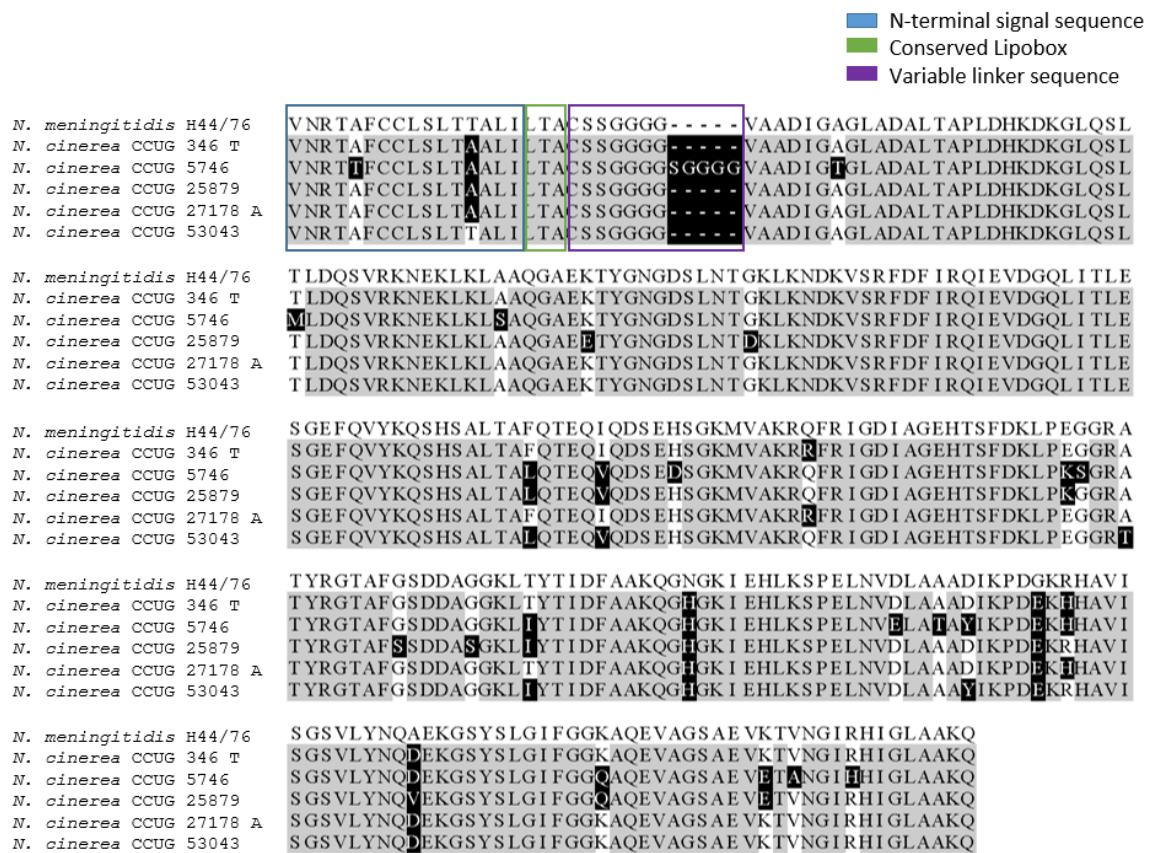


Figure 6.1: Sequence alignment of *N. meningitidis* fHbp V1.1 with *N. cinerea* fHbp from (<http://pubmlst.org/>)

Black boxed residues show sequence variation of fHbp and white boxed residues show identical residues compared with *N. meningitidis* V1.1 fHbp sequence. Grey boxed residues show residues conserved in all strains. Sequence analysis indicates a conserved signal motif (blue box), lipobox (green box) and variable linker sequence (purple box).

There was a high nucleotide sequence identity between *N. cinerea* *fhbp* with the meningococcal *fhbp* V1.1 (approximately 95%). Phylogenetic analysis indicates that all *N. cinerea* *fhbps* belong to the variant 1 family (Muzzi, A. 2013). *fhbp* from *N. cinerea* isolate, CCUG 346 T was further characterised

as this strain is genetically transformable [Wörmann, M. E. 2016]. Comparison of the predicted amino acid sequence of *fhbp* from *N. cinerea* CCUG 346 T with *N. meningitidis* V1.1 fHbp revealed six nonsynonymous substitutions in *N. cinerea fhbp*, of which one residue is in the signal peptide (Figure 6.2A). To evaluate the impact of amino acid substitutions, a structural model of *N. cinerea* fHbp was generated using V1.1 fHbp (Schneider, PDB: 2W80) as the threading template (Figure 6.2B and Supplementary Information Figure SI.1). Three of the residues unique to *N. cinerea* fHbp, (H243, H269 and D282) are in the C terminal barrel and predicted to be distinct from the CFH:fHbp interface. However, residues R193 and E267 of *N. cinerea* fHbp reside at the CFH:fHbp interface and could potentially alter this key host pathogen interaction. The close proximity (less than 4 Å) of these residues with CFH residues, S366 and N338 respectively, has the potential to lead to additional salt bridges, so could increase the binding affinity of CFH to *N. cinerea* fHbp compared with V1.1 fHbp.

### 6.2.2 Functional characterisation of *N. cinerea* fHbp.

Initially binding of fHbp from *N. cinerea* and *N. meningitidis* to CFH was determined by far western blot analysis using CFH from NHS. Previous investigations have shown that an Ile to Ala substitution at position 311 of V1.1 fHbp (V1.1<sup>I311A</sup> fHbp) significantly decreases the binding affinity to CFH<sub>(6,7)</sub> (Johnson, S. 2012); therefore this non-functional protein was used as a control. *N. cinerea* fHbp, V1.1 fHbp and V1.1<sup>I311A</sup> fHbp were purified as recombinant proteins and detected by anti-V1.1 fHbp (Figure 6.3A). Both *N. cinerea* fHbp and V1.1 fHbp bind CFH by far western analysis, whereas far western analysis demonstrates that V1.1<sup>I311A</sup> fHbp had no detectable CFH binding (Figure 6.3A). To further characterise this interaction, the binding affinity of *N. cinerea* fHbp was determined for CFH<sub>(6,7)</sub> by SPR. Results show that *N. cinerea* fHbp binds CFH<sub>(6,7)</sub> with similar affinities as V1.1 fHbp, with  $K_D$ s of 3.5 nM  $\pm$  0.5 and 3.0 nM  $\pm$  0.5, respectively (Figure 6.3B-C), indicating the substitutions in *N. cinerea* fHbp do not affect its ability to bind CFH. Next, the level of CFH binding to *N. cinerea* fHbp and V1.1 fHbp was analysed by ELISA using full length recombinant CFH (Figure 6.3D). ELISA data was consistent with results from both far western and SPR analyses, with no significant difference in CFH binding to *N. cinerea* fHbp and *N. meningitidis* V1.1 fHbp ( $p > 0.05$  by two-tailed  $t$

test) over a range of concentrations (Figure 6.3E). Taken together, these results demonstrate that *N. cinerea* fHbp binds CFH and that this interaction is mediated by CCPs 6 and 7.

Initial functional characterisation of *N. cinerea* fHbp (Figure 6.3) and fHbp surface localisation experiments (Figure 6.5) were performed by Katy Poncin, University of Namur, under my direct supervision at the University of Oxford during an internship for her MSc.

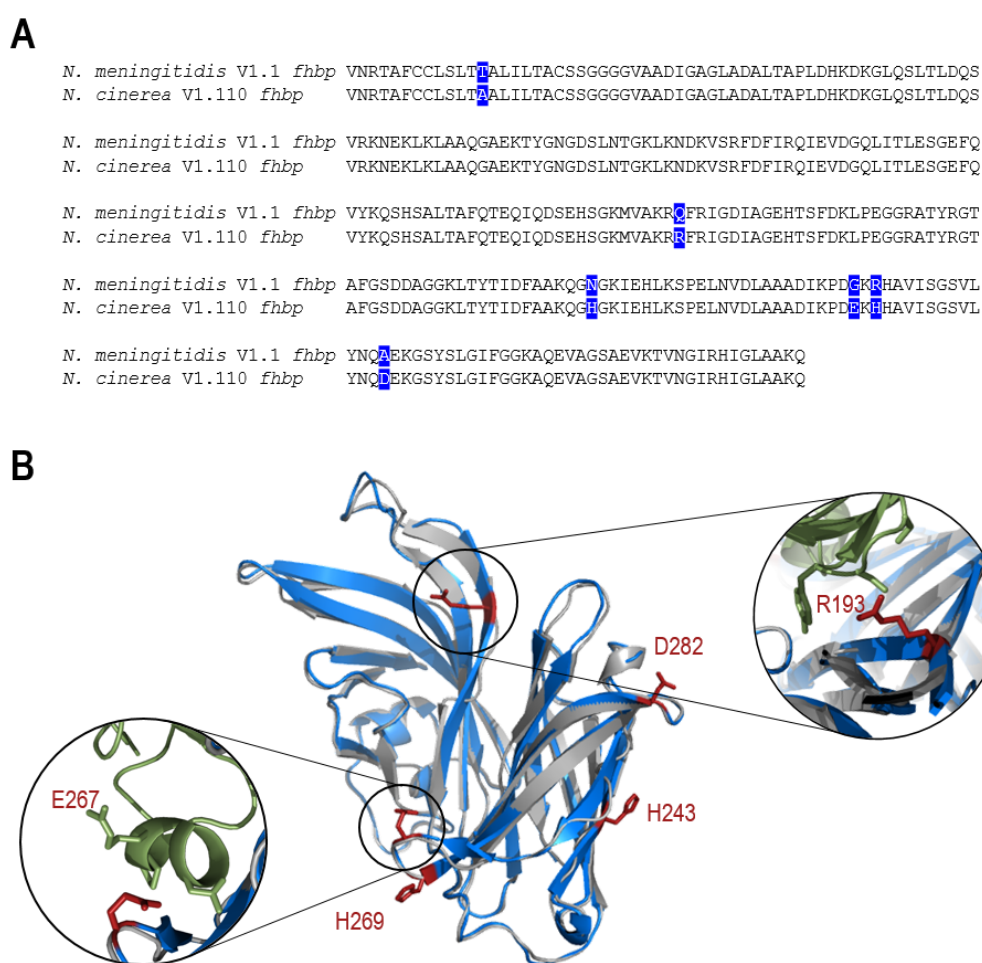
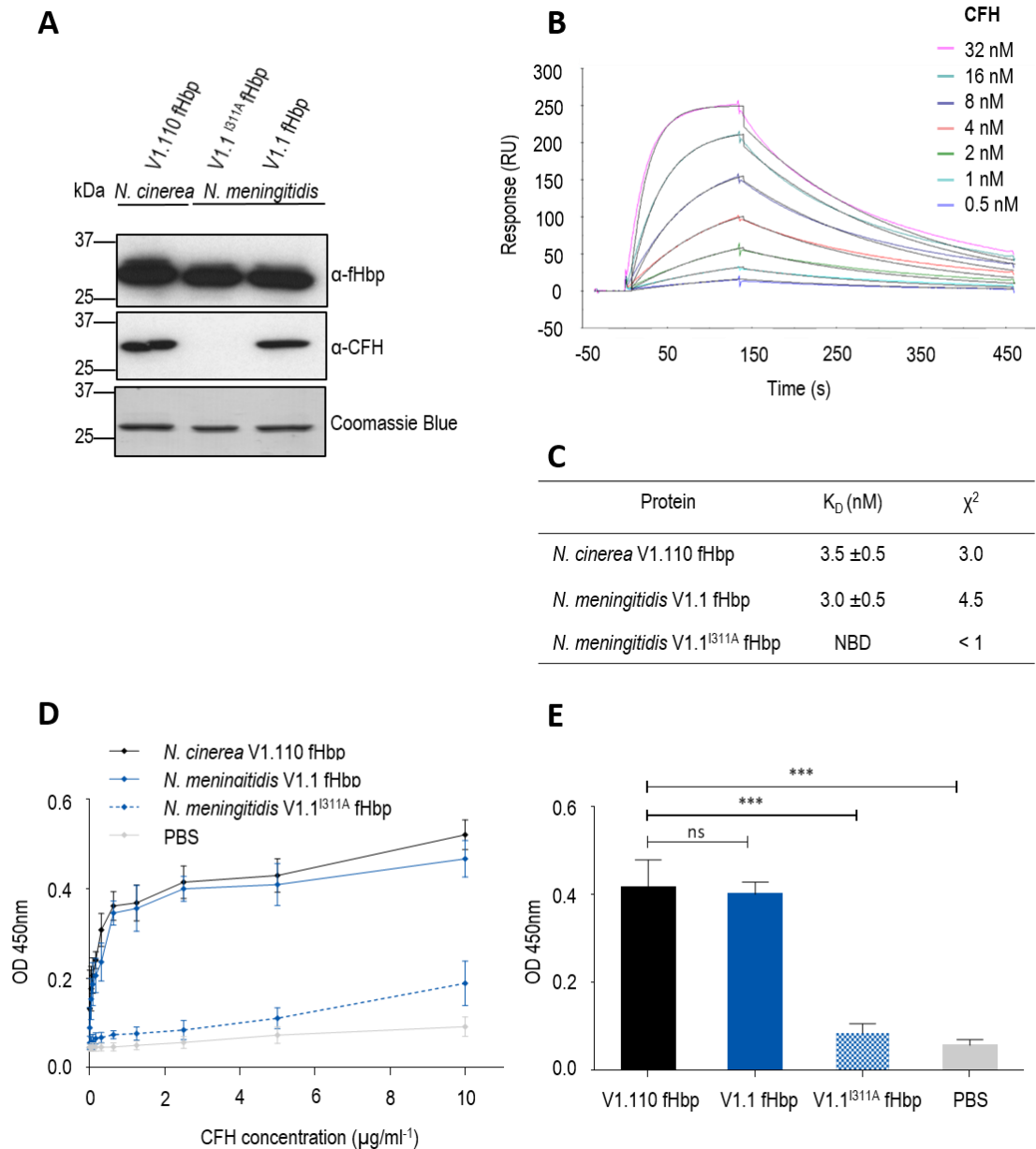


Figure 6.2: *N. cinerea* fHbp is predicted to be structurally similar to *N. meningitidis* fHbp

(A) Sequence alignment of *N. meningitidis* V1.1 fHbp and *N. cinerea* fHbp. Blue boxed residues show amino acid differences. (B) Predicted structure of *N. cinerea* fHbp (grey ribbon) generated with the I-TASSER server and V1.1 fHbp (blue ribbon) [Schneider, PDB: 2W80]. Figure drawn using Pymol. Residues in red (shown as ball-and-stick representations) are different in *N. cinerea* fHbp compared to V1.1 fHbp. Residues Arg193 and Glu267 (red) are near to the interface of fHbp with CFH (expanded view, green ribbon).



**Figure 6.3: *N. cinerea* fHbp binds CFH at high affinity**

(A) Recombinant fHbp from *N. cinerea* CCUG 346 T, *N. meningitidis* V1.1 fHbp and V1.1<sup>I311A</sup> fHbp analysed by western blot using polyclonal anti-V1.1 fHbp sera. *N. cinerea* fHbp and V1.1 fHbp bind CFH by far western analysis with NHS as the source of CFH. Molecular masses shown in kDa and Coomassie blue staining of proteins as the loading control. (B) SPR analysis of *N. cinerea* fHbp binding to CFH<sub>(6,7)</sub> (concentrations indicated) with 1:1 Langmuir fit (black lines). (C) Calculated  $K_D$  values for CFH<sub>(6,7)</sub> binding to *N. cinerea* fHbp and V1.1 fHbp; NBD, no binding detected.  $\chi^2$ , chi-squared test of independence. (D) ELISA of full length CFH binding to *N. cinerea* fHbp, V1.1 fHbp and V1.1<sup>I311A</sup> fHbp. (E) Significance of CFH binding by ELISA analysed  $5 \mu\text{g}/\text{ml}^{-1}$  CFH. Error bars are  $\pm$  SEM of three independent experiments,  $p$  values (\*\*\*,  $p < 0.001$ ; ns,  $p > 0.05$ ) calculated using a two-tailed unpaired  $t$ -test. H.L. conceived and designed experimental plan, experiments were performed by K.P. ad data analysed by both H.L. and K.P.

### 6.2.3 *N. cinerea* isolates express fHbp on the bacterial surface.

Next, the expression of fHbp by *N. cinerea* isolates was analysed using polyclonal mouse sera raised against V1.1 fHbp which cross reacts with *N. cinerea* fHbp from CCUG 346 T (Figures 6.3A). fHbp was detected at approximately 27 kDa in all five *N. cinerea* isolates by western blot analysis of whole cell lysates from these strains (Figure 6.4A). Moreover, *N. meningitidis* H44/76, H44/76 $\Delta$ fHbp and five *N. cinerea* isolates were analysed for their ability to bind full length CFH using NHS. Far western analyses demonstrates that *N. meningitidis* H44/76 and *N. cinerea* isolates are able to bind CFH; no CFH binding was detected for *N. meningitidis* H44/76 $\Delta$ fHbp (Figure 6.4A). Next, an fHbp deletion strain of *N. cinerea* CCUG 346 T was constructed (*N. cinerea* CCUG 346 T $\Delta$ fHbp) by insertional inactivation with a kanamycin resistance cassette to determine if binding of CFH to *N. cinerea* is fHbp-dependent. Analysis of the wild-type *N. cinerea* and the  $\Delta$ fHbp strain demonstrated no expression of fHbp in the  $\Delta$ fHbp, while far western blot analysis demonstrates that CFH binding to *N. cinerea* is dependent on fHbp expression (Figure 6.4B). *N. meningitidis* H44/76 and H44/76 $\Delta$ fHbp were used as positive and negative controls, respectively.

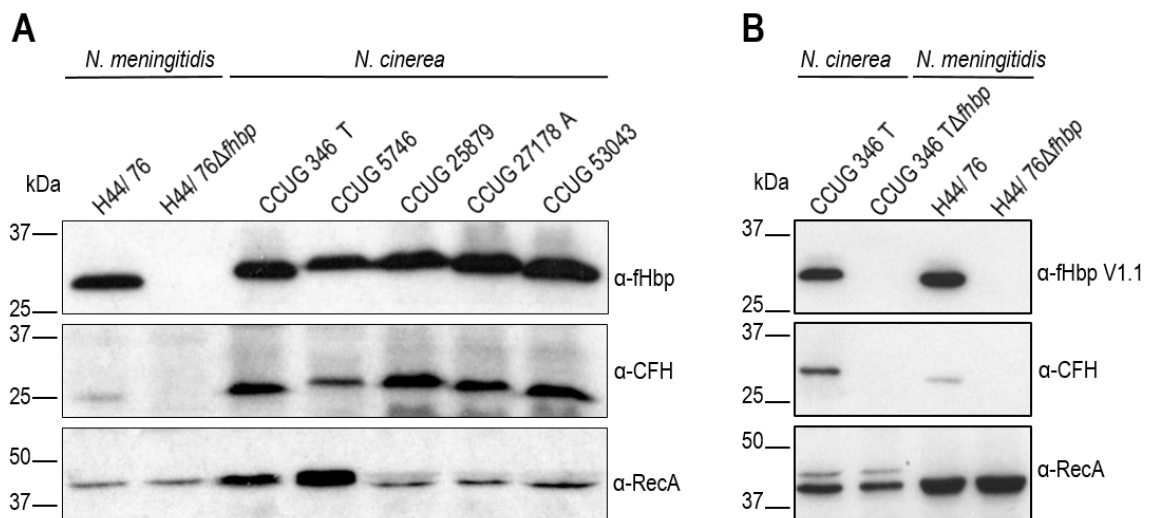
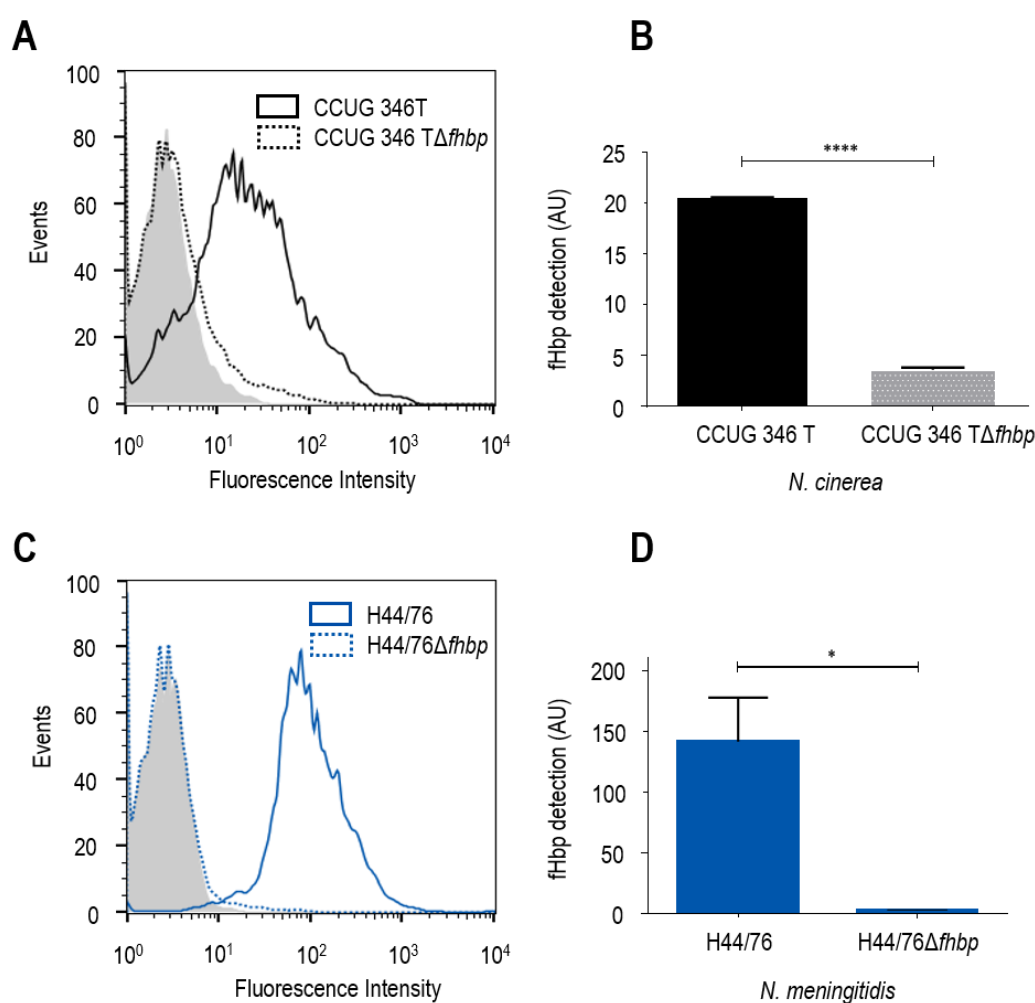


Figure 6.4: Functional fHbp is expressed by all *N. cinerea* strains examined

(A) Western blot analysis of fHbp expression using anti-V1.1 fHbp sera against whole cell lysates of *N. cinerea* strains (indicated), *N. meningitidis* H44/76 and H44/76 $\Delta$ fHbp. Far western analysis, using NHS as the source of CFH, demonstrates that fHbp from *N. cinerea* binds CFH; RecA used as a loading control. (B) Western and far western analyses demonstrate that binding of CFH to *N. cinerea* is dependent on fHbp expression.

To determine whether *N. cinerea* fHbp is localised on the surface of bacteria, flow cytometry analysis was performed using anti-V1.1 fHbp sera to detect fHbp on the surface of wild-type *N. cinerea*, CCUG 346 T and CCUG 346  $\Delta$ fHbp (Figure 6.5A-B); *N. meningitidis*, H44/76 and H44/76 $\Delta$ fHbp were examined as positive and negative controls (Figure 6.5C-D). The results demonstrate that fHbp is present on the surface of *N. cinerea* CCUG 346 T and *N. meningitidis* H44/76 but not CCUG 346  $\Delta$ fHbp or H44/76 $\Delta$ fHbp (two tailed *t* test  $p = < 0.0001$  and  $p = 0.0183$ ) (Figure 6.5B, D). Taken together these results show that in *N. cinerea* fHbp is surface localised and *N. cinerea* fHbp binds CFH at a similar affinity to meningococcal V1.1 fHbp.



**Figure 6.5:** *N. cinerea* fHbp is present on the bacterial surface

Flow cytometry analysis with anti-V1.1 fHbp sera demonstrates surface localisation of fHbp on (A-B) *N. cinerea* (black-solid line) and (C-D) *N. meningitidis* (blue-solid line). fHbp was not detected on the surface of (A-B) *N. cinerea*  $\Delta$ fHbp (black-dotted line) and (C-D) *N. meningitidis*  $\Delta$ fHbp (blue-dotted line). (A, C) Grey shaded area indicated bacteria incubated in the absence of sera. (B, D) Error bars are  $\pm$  SEM of three independent experiments; Significance was calculated using a two-tailed unpaired *t*-test; \*\*\*\*,  $p < 0.0001$ ; and \*,  $p < 0.05$ . H.L. conceived and designed experimental plan, experiments were performed by K.P. and data analysed by both H.L. and K.P.

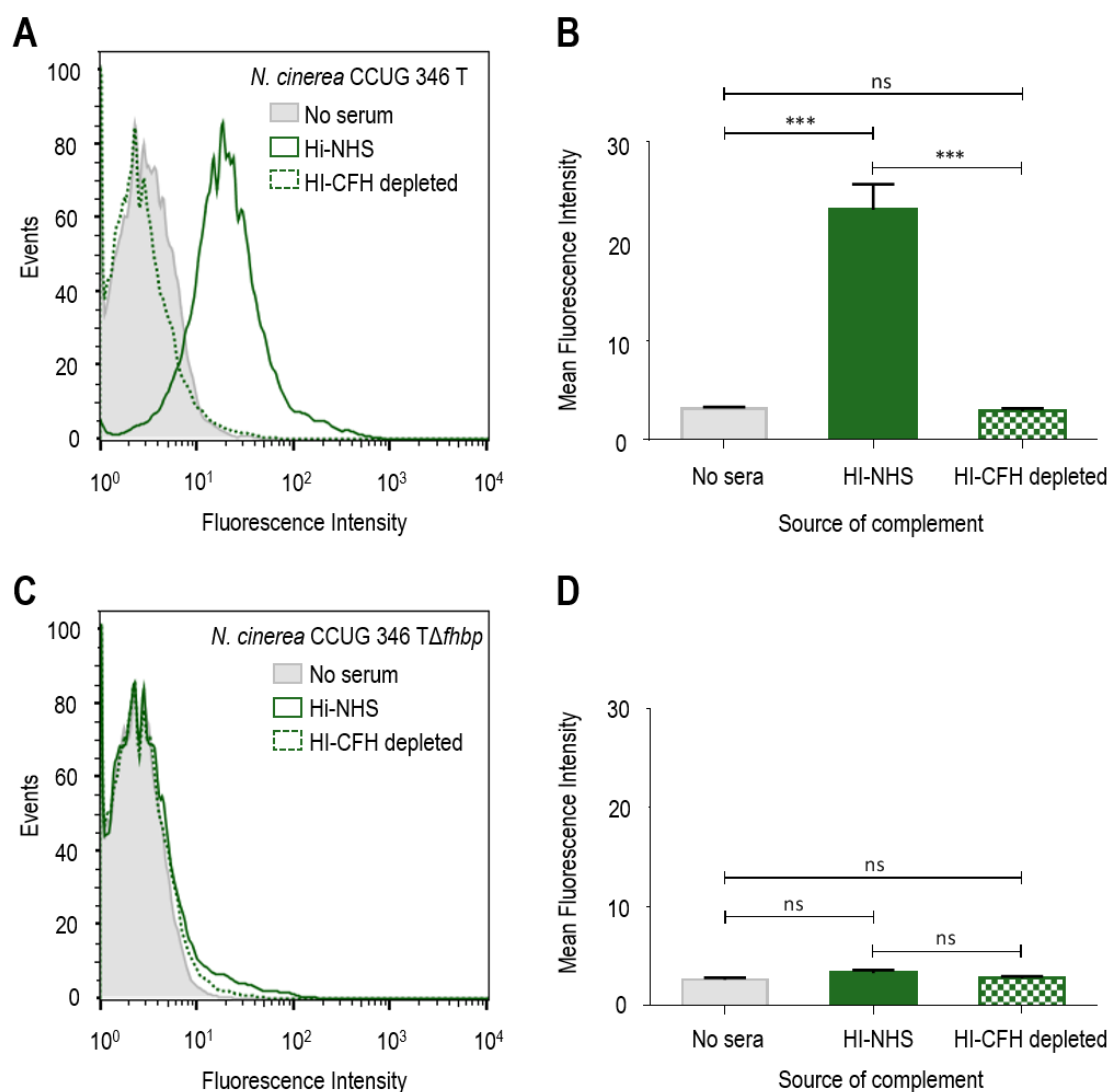


#### 6.2.4 Binding of CFH promotes complement resistance of *N. cinerea*.

Both NspA and PorB2 of *N. meningitidis* have previously been shown to bind CFH and promote serum survival of *N. meningitidis* [Lewis, L. A. 2010; Lewis, L. A. 2013]. Initially, the ability of *N. cinerea* to recruit CFH to its surface was analysed by flow cytometry. *N. cinerea* CCUG 346 T and CCUG 346  $\Delta fhb p$  were incubated in HI-NHS as a source of CFH, or in CFH depleted sera (HI-CFH depleted). CFH binding was detected with an anti-CFH mAb (OX24) which recognises CCP domain 5 of CFH (Figure 6.6) [Sim, R. 1983]; fluorescence was quantified as the geometric mean fluorescence intensity. Bacteria incubated with PBS, instead of HI-NHS, were used as a negative control. Following incubation with HI-NHS, CFH was detected on the surface of *N. cinerea*, CCUG 346 T (Figure 6.6A-B), but not the *fhbp* mutant, CCUG 346  $\Delta fhb p$  (two tailed *t*-test  $p=0.0002$ ) (Figure 6.6C-D). Furthermore, no CFH binding was detected to either strain following incubation with HI-CFH depleted sera (Figure 6.6). Taken together these results demonstrate that CFH binds to the surface of *N. cinerea* in an *fhbp*-dependent manner.

Next, the functional consequences of binding CFH on the bacterium was evaluated in serum survival assays. *N. cinerea* CCUG 346 T and CCUG 346  $\Delta fhb p$  were incubated in a range of NHS concentrations (0-20%) for 30 minutes to evaluate the effect of serum concentration on survival. The percentage survival of strains in NHS was calculated by comparing the number of CFU recovered from bacteria incubated in PBS. *N. cinerea* CCUG 346 T was more resistant against complement-mediated lysis than CCUG 346  $\Delta fhb p$  over a range of serum concentrations. For example, 54.3% and 31.24% survival was observed for CCUG 346 T and CCUG 346  $\Delta fhb p$ , respectively at 5% NHS (two tailed *t* test  $p = 0.0161$ ) (Figure 6.7A). Furthermore, we examined whether these differences in survival were observed in the absence of the CP and LP by adding 10 mM EGTA and 5 mM  $MgCl_2$  (Des Prez, R. M. 1975). The enhanced resistance of *N. cinerea* CCUG 346 T compared to CCUG 346  $\Delta fhb p$  to complement-mediated lysis was still observed when only the AP was active (67.1% and 58.55% survival was observed for CCUG 346 T and CCUG 346  $\Delta fhb p$ , respectively at 10% serum concentration, (two tailed *t* test  $p = 0.491$ ) (Figure 6.7B); although the enhanced resistance of *N.*

*N. cinerea* CCUG 346 T compared to CCUG 346  $\Delta fHbp$  to complement-mediated lysis was not significant in AP only complement assays.



**Figure 6.6:** *N. cinerea* recruits CFH to its surface in a fHbp-dependent manner

Flow cytometry analysis of CFH binding to (A-B) *N. cinerea* CCUG 346 T and (C-D) *N. cinerea* CCUG 346  $\Delta fHbp$ . Shaded area indicates results from bacteria incubated with PBS, solid green shows bacteria incubated in HI-NHS, and broken green shows bacteria incubated in HI-CFH-depleted sera. (B, D) Data presented as the mean fluorescence intensity. Error bars indicate mean  $\pm$  SEM of three independent experiments and p-values calculated using a two-tailed unpaired *t*-test; \*\*\*,  $p < 0.001$ ; and ns  $> 0.05$ .

Results in Chapter 5 demonstrate that CFHR3 can bind to fHbp with this interaction mediated by CCP domains 1-2, and that CFHR3 competes for binding to fHbp with CFH on the meningococcal surface. Competition of CFHR3 for fHbp renders *N. meningitidis* more susceptible to complement-mediated lysis. Therefore, the effect of CFHR3 on *N. cinerea* survival in serum was examined using CFHR3<sub>(1,2)</sub>

which binds fHbp. Furthermore, the effect of CFHR3<sub>(4,5)</sub> on bacterial survival was examined as CFHR3<sub>(4,5)</sub> is known to bind C3b but not fHbp [Caesar, J. J. 2014]. *N. cinerea* CCUG 346 T and CCUG 346 T $\Delta$ *fhbp* survival was measured following pre-incubation in a range of CFHR3<sub>(1,2)</sub> concentrations (0.01  $\mu$ M to 5  $\mu$ M) for ten minutes prior to incubating bacteria in 3% NHS. This serum concentration was selected as approximately 80% of *N. cinerea* CCUG 346 T survives after incubation in 3% NHS for 30 minutes (Figure 6.7A). Similar to *N. meningitidis*, CCUG 346 T was more susceptible to complement-mediated lysis after pre-incubation with CFHR3<sub>(1,2)</sub> (Figure 6.8A). Moreover, pre-incubation of CCUG 346 T  $\Delta$ *fhbp* with CFHR3<sub>(1,2)</sub> had no effect on bacterial survival indicating that the effect of CFHR3 on bacterial survival is fHbp dependent (Figure 6.8B). For example, 70.69% and 96.8% survival was observed for CCUG 346 T and CCUG 346 T  $\Delta$ *fhbp* respectively after pre-incubation with 1  $\mu$ M CFHR3<sub>(1,2)</sub>; percentage survival for these experiments was calculated relative to bacteria without pre-incubation with CFHR3<sub>(1,2)</sub> after 30 minutes incubation in NHS. Furthermore, CFHR3<sub>(1,2)</sub> did not affect survival of either CCUG 346 T or CCUG 346 T  $\Delta$ *fhbp* in HI-NHS (Figure 6.8A-B). Pre-incubation of CCUG 346 T or CCUG 346 T  $\Delta$ *fhbp* with CFHR3<sub>(4,5)</sub> had no effect on bacterial survival (Supplementary Information Figure SI.2) indicating that CFHR3 CCP domains 1 and 2 are essential for mediating the interaction between fHbp and CFHR3.

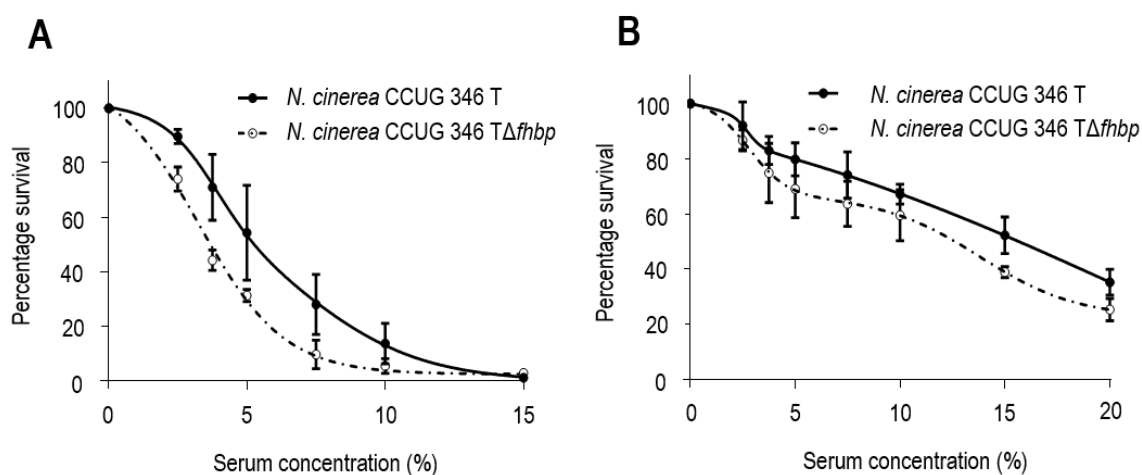


Figure 6.7: Serum survival of *N. cinerea*

Percent survival of *N. cinerea* (filled circles) and *N. cinerea* CCUG 346 T $\Delta$ *fhbp* (open circles) to (A) complement mediated lysis following 30 minute incubation in NHS (concentrations indicated) and to (B) lysis mediated by the AP (concentrations indicated). Survival was calculated relative to bacteria incubated in HI-NHS.

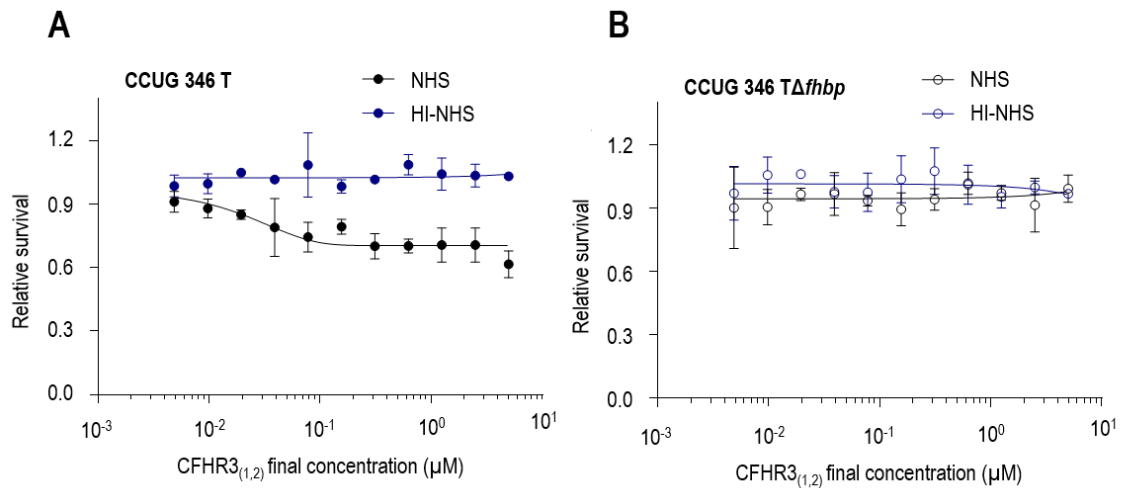


Figure 6.8: CFHR3 increases susceptibility of *N. cinerea* to complement mediated lysis

Survival of (A) *N. cinerea* CCUG 346 T (filled circles) and (B) CCUG 346  $\Delta$ fHbp (open circles) in the presence of 3% NHS (black) or HI-NHS (blue) following pre-incubation in different concentrations of CFHR3<sub>(1,2)</sub> (indicated). Data presented as survival relative to bacteria incubated in the absence of serum and CFHR3<sub>(1,2)</sub>. Error bars are  $\pm$  SEM of three independent experiments.

### 6.2.5 Mice immunised with Bexsero<sup>®</sup> elicit a serum bactericidal activity response which is comparable to mice immunised with fHbp alone.

The introduction of sub-unit vaccines against *N. meningitidis* containing fHbp has the potential to impact commensal species such as *N. cinerea* which expresses fHbp (Figure 6.4A) [Maignani, V. 2003; Fletcher, L. D. 2004; Muzzi, A. 2013]. To examine whether immune responses elicited by licensed meningococcal vaccines could mediate SBA against *N. cinerea*, mice were immunised with Bexsero<sup>®</sup>, V1.1 fHbp and *N. cinerea* V1.110 fHbp, and the resulting sera examined for SBA against *N. cinerea*. Non-immune serum, generated by immunising mice with adjuvant alone, was used as a negative control. Initially the antibody responses elicited by Bexsero<sup>®</sup> were analysed by assessing the ability of sera to recognise V1.1 fHbp, present in Bexsero<sup>®</sup>, and *N. cinerea* V1.110 fHbp by ELISA. Interestingly, there was no significant difference in the recognition of V1.1 and V1.110 fHbp by sera from mice immunised with V1.1 fHbp, *N. cinerea* fHbp, or Bexsero<sup>®</sup> ( $p = 0.7572, 0.2751$  and  $0.8965$ , respectively) (Figure 6.9A-C). Sera from mice immunised with V1.1 or V1.110 fHbp did not recognise BSA whereas mice immunised with Bexsero did react with BSA at lower serum dilutions which may

be due to more complex mixture of proteins in the vaccine (Figure 6.9A-C). Of note, non-immune sera did not recognise V1.1 fHbp, V1.110 fHbp, or BSA (Figure 6.9D).

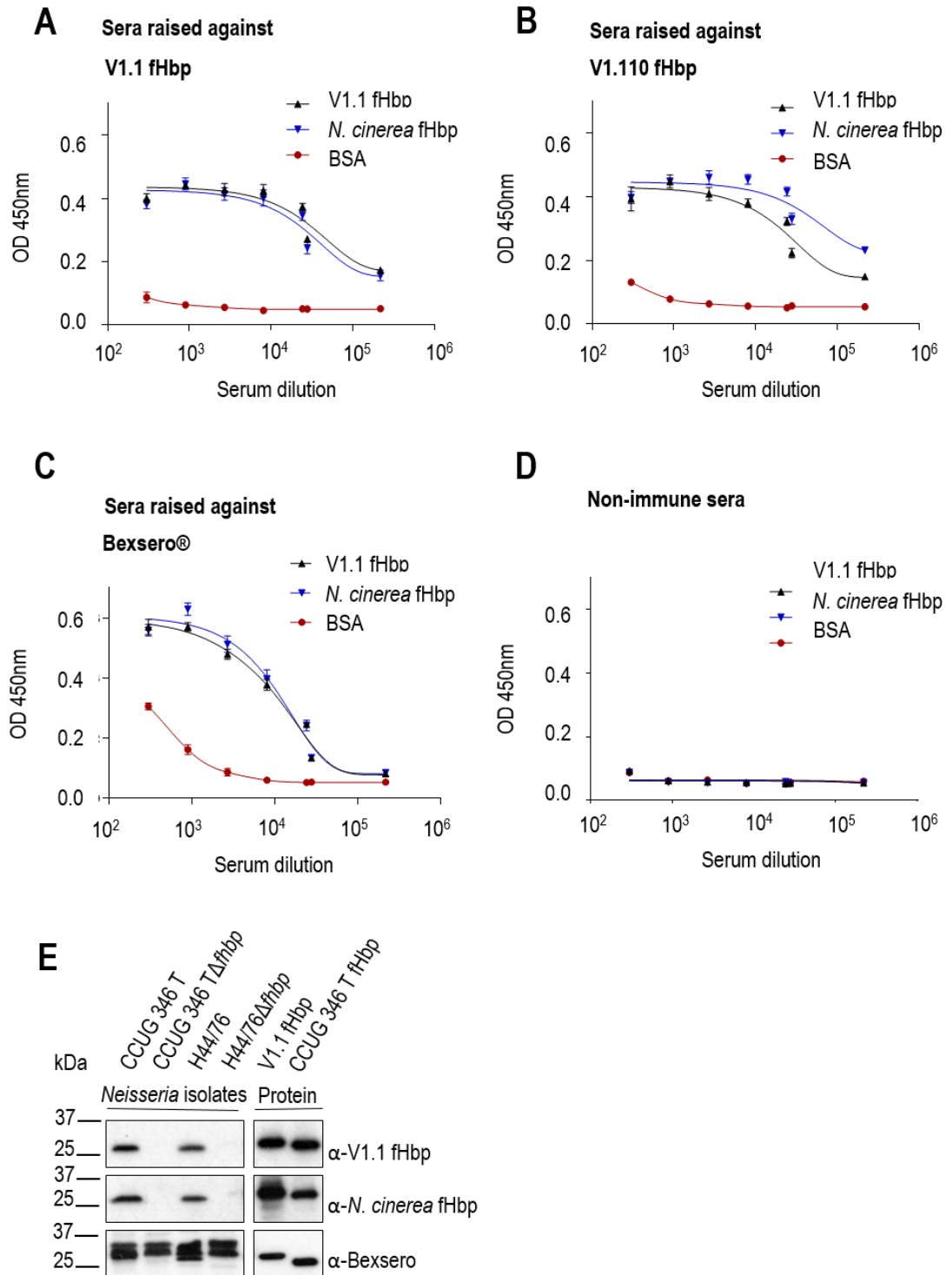


Figure 6.9: Immunisation with Bexsero® elicits immune responses against *N. cinerea*

ELISA analysis of sera raised against (A) V1.1 fHbp, (B) V1.110 fHbp, (C) Bexsero® and (D) non-immune sera (three-fold dilutions from 1/300) against recombinant V1.1 fHbp (black triangles) and V1.110 fHbp (blue triangles); BSA (red circles) was used as a control. (E) Western blot analysis of whole cell lysates (strains indicated) or recombinant V1.1 fHbp and V1.110 fHbp, fHbp is recognised by sera from mice immunised with recombinant fHbp or Bexsero®.

Next, the ability of pooled sera and individual mice sera to recognise fHbp was analysed by western blot analysis against whole cell lysates of *N. cinerea* CCUG 346 T and *N. meningitidis* H44/76 as well as recombinant V1.1 fHbp and V1.110 fHbp (Figure 6.9E; Supplementary Information Figure SI.3). Sera from groups immunised with fHbp V1.1 or *N. cinerea* fHbp detected a single band in wild-type strains corresponding to the expected size for fHbp (approximately 27 kDa), while no protein was detected in the  $\Delta fhb p$  knockout strains. However, sera from mice immunised with Bexsero<sup>®</sup> detected multiple protein bands which could be attributed to immune responses against other antigens in the OMVs in the vaccine. However, a band of approximately 27 kDa (corresponding to the approximate molecular weight of fHbp) was no longer detected in  $\Delta fhb p$  knockout strains. Furthermore, as expected antibodies elicited by V1.1 fHbp, V1.110 fHbp and Bexsero<sup>®</sup> also recognised V1.1 fHbp and V1.110 fHbp by western blot analysis (Figure 6.9E; Supplementary Information Figure SI.3)

Next the potential impact of immunisation with Bexsero<sup>®</sup> on *N. cinerea* survival was examined by measuring SBA against the bacterium (Table 6.1). In an SBA, the ability of antibodies to initiate bacterial lysis is measured by adding dilutions of antibodies (usually in the form of heat-inactivated immune serum) to bacteria in an exogenous source of complement (either baby rabbit complement or human serum). Currently, an SBA titer  $\geq 8$  in the presence of baby rabbit complement is used as the threshold for meningococcal vaccine efficacy, which has been correlated with protection from meningococcal disease [Borrow, R. 2006]. Of note, polysaccharide conjugate vaccines against serogroup C elicit SBA and lead to a significant reduction in carriage of serogroup C meningococci strains and a decrease in invasive serogroup A disease by 89-94% [Balmer, P. 2002. Maiden, M. 2008. Campbell, H. 2009]. Furthermore, introduction of a polysaccharide conjugate vaccine against serogroup A meningococci in Africa has almost eliminated serogroup A meningococcal disease through the reduction of carriage and induction of herd immunity [Sow, S. 2011. Sambo, L. 2015. Kristiansen, P. A. 2012]. A correlation between the SBA and reduction in serogroup B carriage has yet to be established and is under further investigation [Read, R. C. 2014].

The polysaccharide capsule is a key virulence factor of *N. meningitidis* and isolates which do not express polysaccharide capsule are more susceptible to complement-mediated lysis [Drogari-

Apiranthitou, M. 2002; Ram, S. 1999]. Genome analysis of *N. cinerea* indicate that *N. cinerea* lack expression of polysaccharide capsule as the key capsule biosynthesis genes are not present [Kim, J. 1989; Marri, P. R. 2010]. Therefore, *N. cinerea* were expected to be more susceptible to complement-mediated lysis. Consistent with this, approximately 50% bacterial survival was observed after incubation with 12% NHS for *N. meningitidis* (Figure 5.2C) whereas 50% survival was observed for *N. cinerea* after incubation in 5% NHS (Figure 6.7A). Prior to analysing the SBA from immunised mice, the concentration of rabbit complement was optimised (Supplementary Information Figure SI.4). *N. cinerea* was incubated in a range of complement dilutions from 0 to 1:64. At a 1:10 dilution of rabbit complement, in SBA buffer, and after a 60-minute incubation, 91.8% *N. cinerea* survival was observed (Supplementary Information Figure SI.4). This concentration of complement was used in subsequent SBA assays.

Immunisation with V1.1 fHbp and Bexsero<sup>®</sup> elicited SBA titres of 128 against *N. cinerea* CCUG 346 T but no SBA was detected against CCUG 346  $\Delta$ fHbp, indicating that the main vaccine target against *N. cinerea* on the bacterial surface is fHbp. Of note, *N. cinerea* CCUG 346 T complemented with *N. cinerea* V1.110 fHbp under an IPTG inducible promoter, had a higher SBA titre (256) using sera from mice immunised with Bexsero<sup>®</sup> than wild-type *N. cinerea*. This is correlated with the higher expression levels of fHbp in the complemented compared to the wild-type *N. cinerea* strain (Supplementary Information Figure SI.5). Furthermore, no SBA was detected against *N. cinerea* CCUG 346  $\Delta$ fHbp with V1.1 fHbp, V1.110 or Bexsero<sup>®</sup>. In contrast, mice immunised with V1.110 fHbp did not elicit SBA against either the wild-type *N. cinerea* CCUG 346 T or the  $\Delta$ fHbp mutant. However, mice immunised with V1.110 fHbp elicited an SBA titre of 512 against *N. cinerea* CCUG 346 T complemented with the homologous fHbp suggesting that *N. cinerea* fHbp either does not elicit a strong immune response or that *N. cinerea* fHbp is expressed at low levels on the bacterial surface. Moreover, no SBA was observed against *N. cinerea* CCUG 346 T, CCUG 346  $\Delta$ fHbp or CCUG 346  $\Delta$ fHbp::V1.110 fHbp using non-immune mouse sera. In conclusion, immune responses elicited by Bexsero<sup>®</sup> recognise *N. cinerea* fHbp and have SBA against *N. cinerea*.

Strain	fHbp variant	SBA elicited by		
		V1.1 fHbp	V1.110 fHbp	Bexsero®
CCUG 346 T	V1.110	128	NBT	128
CCUG 346 $\Delta$ <i>fhbp</i>	-	NBT	NBT	NBT
CCUG 346 $\Delta$ <i>fhbp::fhbp</i>	V1.110	128	512	256

Table 6.1: Serum bactericidal titres of pooled sera from mice immunised with recombinant fHbp, Bexsero® or control mice against *N. cinerea* strains

No bactericidal titre (NBT).



### 6.3 Discussion

fHbp is a key virulence factor for the meningococcus and binds the negative complement regulator, CFH, promoting bacterial survival in human blood [Madico, G. 2006. Schneider, M. C. 2006. Schneider, M. C. 2009]. Its importance as a vaccine candidate is evident as fHbp is a major component of two recently licensed meningococcal B vaccines, Bexsero® and Trumenba® [Masignani, V. 2003. Fletcher, L. D. 2004]. Data in this chapter shows that fHbp expression is not restricted to pathogenic *N. meningitidis* isolates but this antigen is also expressed by the commensal, *N. cinerea*. *N. cinerea* fHbp binds CFH at high affinity, and promotes resistance of *N. cinerea* to complement-mediated lysis. Furthermore, sera raised against Bexsero® had SBA against *N. cinerea* expressing fHbp. Therefore immunisation with Bexsero® has the potential to affect the survival of *N. cinerea*, a component of the nasopharyngeal flora.

Previous work has analysed the expression and function of fHbp in pathogenic *Neisseria* [Madico, G. 2006; Schneider, M. C. 2006]. fHbp is a highly variable protein which can be grouped into three distinct variants based on its amino acid sequence [Brehony, C. 2009]. Recent analysis of the genome sequence of commensal *Neisseria* spp. indicated that *fhbp* was present in *N. polysaccharea* and *N. cinerea*, with the latter showing high amino acid identity with V1 meningococcal *fhbp* (>95%). Using SPR, *N. cinerea* fHbp bound CFH<sub>(6,7)</sub> with  $K_D$ s in the nM range, that was similar to the affinity of CFH with V1.1 fHbp from *N. meningitidis* indicating that high affinity binding of CFH to the surface of the bacterium is not only involved during survival in the bloodstream but could aid colonisation and persistence in the human nasopharynx. In serum survival assays wild-type *N. cinerea*, which express fHbp, had enhanced survival compared to the fHbp knockout although the differences in survival were less pronounced than in *N. meningitidis* H44/76 [Madico, G. 2006]. Furthermore, the difference in survival of *N. cinerea* was not significant at most serum concentrations. In contrast to *N. meningitidis*, *N. cinerea* do not express a polysaccharide capsule, a major meningococcal virulence factor, as they lack the capsule biosynthesis genes and are consequently more susceptible to complement-mediated lysis [Kim, J. J. 1989. Marri, P. R. 2010]; this is evidenced by the serum concentration resulting in 50% bacterial lysis (LD<sub>50</sub>) being approximately 5% whereas it has been

previously been demonstrated that *N. meningitidis* H44/76 requires 40% NHS for LD<sub>50</sub> [Madico, G. 2006]. Therefore, to further examine the functional consequences of *N. cinerea* CCUG 346 T binding CFH, bacteria should be pre-incubated in purified recombinant CFH prior to the addition of CFH-depleted sera to evaluate the difference in serum survival as demonstrated previously [Schneider, M. C. 2006]. Pre-incubation with recombinant CFH would reduce competition with the known CFH antagonist, CFHR3, potentially making the assay more sensitive to differences in survival between the wild-type *N. cinerea* and the  $\Delta fhbp$  mutant [Caesar, J.J. 2014].

High affinity interactions between fHbp and CFH have evolved for *N. meningitidis* and *N. cinerea* which inhabit the nasopharynx, but not for *N. gonorrhoeae*. Indeed, a frame shift mutation in the nucleotide sequence at position 40 of *ghfp* disrupts the lipobox resulting in the loss of surface localisation of GHfp [Jongerijs, I. 2013]. Furthermore, sequence changes in the ORF of *ghfp* abolish CFH binding [Jongerijs, I. 2013]. GHfp has been shown to bind siderophores *in vitro*, although the precise function of this protein in the gonococcus has yet to be established even though GHfp is highly conserved unlike meningococcal fHbp [Veggi, D. 2012; Hadad, R. 2012; Jongerijs, I. 2013]. Furthermore, *N. gonorrhoeae* has been shown to bind CFH *via* surface expressed PorB.1 which is abundantly expressed on the gonococcal surface [Ram, S. 1998. Ngampasutadol, J. 2008].

For *N. meningitidis*, most work investigated the role of CFH on serum survival [Madico, G. 2006; Schneider, M. C. 2006]. However, disease is an evolutionary dead-end for the meningococcus. Therefore bacteria must be adapted for colonisation of the nasopharynx, which is its natural habitat. The mechanisms by which *N. meningitidis* and *N. cinerea* recruit CFH compared with *N. gonorrhoeae* might reflect differences in concentrations of CFH at these sites and the level of other complement proteins. Low levels of CFH have been detected in urothelial cells with higher levels detected in kidney tubules, while CFH could not be detected on respiratory epithelial cells of the nasopharynx by immunohistochemistry [Uhlén, M. 2015]. However, *N. meningitidis* is exposed to high levels of CFH in serum, 116–562  $\mu\text{g}/\text{mL}$  (0.7–3.6  $\mu\text{M}$ ) in a healthy Caucasian population, while the amount of CFH in the respiratory tract is significantly lower, with levels in saliva ranging from 0.007-0.059  $\mu\text{g}/\text{mL}$

(0.04-0.38 nM) [Esparza-Gordillo, J. 2004. Sofat, R. 2013]. Furthermore, complement proteins, including C3b, may be present in plasma exudates of the nasopharyngeal mucosa from the abundant sub-epithelial microvessels [Persson, C. G. 1991. Kaul, T. N. 1982]. Levels of C3 in nasopharyngeal secretions are estimated to be approximately 6.6% (4.2-20.2%) of serum C3 levels, (range from 0.1-0.3 mg/mL compared to serum levels of 1.5-2.1 mg/mL), indicating that there is a significant level of complement in the nasopharynx [Prellner, K. 1985]. Therefore high affinity interactions with CFH might be required to protect against complement activation on mucosal surfaces due to low availability of this important complement regulator [Esparza-Gordillo, J. 2004. Sofat, R. 2013]. CFH binding to meningococcal fHbp was previously estimated to have the highest affinity of any known CFH ligand, with  $K_D$ s in the low nanomolar range; *N. cinerea* also binds CFH at a similar affinity, which suggests that fHbp is required for colonisation [Schneider, M. C. 2009. Johnson, S. 2012].

Binding of CFH is important for colonisation of other bacteria which inhabit the nasopharynx, including *S. pneumoniae*. Binding of CFH by CCP domains 8-11 and CCP domains 19-20 to PspC of *S. pneumoniae* significantly increases bacterial attachment to host epithelial and endothelial cells by acting as a molecular bridge between the bacterium and host cell [Hammerschmidt, S. 2007]. There are other examples of host molecules including vitronectin, fibronectin, and plasminogen acting as molecular bridges to enable bacteria to engage host cells [Virji, M. 1994. Singh, B. 2010. Dziejwanowska, K. 2000. Sumitomo, T. 2016]. Moreover, the Arg-Gly-Asp at the C-terminus of CCP domain 4 of CFH was shown to mediate attachment of *S. pneumoniae* to epithelial cells via interactions with integrins to induce uptake of pneumococci into epithelial cells [Hellwage, J. 1997. Hammerschmidt, S. 2007]. A similar role for CFH acting as a molecular bridge has also been described for *Streptococcus pyogenes* [Pandiripally, V. 2003]. Similar mechanisms might influence colonisation of *N. meningitidis* and *N. cinerea*.

Asymptomatic colonisation of the oropharynx and nasopharynx by *N. lactamica* has been well documented in children, and has been suggested that colonisation by commensal *Neisseria* protects children from developing meningococcal disease through induction of natural immunity [Gold, R.

1978. Cann, K. J. 1989. Cartwright, K. A. 1987]. A live *N. lactamica* human challenge study indicated that colonisation by *N. lactamica* may reduce colonisation by *N. meningitidis* by eliciting opsonophagocytic antibodies or competing with *N. meningitidis* on mucosal surfaces [Evans, C. M. 2011]. A subsequent study, in which 149 individuals were inoculated with *N. lactamica*, demonstrated a significant reduction in *N. meningitidis* carriage in the challenge group after 8 weeks in participants colonised by *N. lactamica* (24.2 and 14.7% nasopharyngeal carriage of *N. meningitidis*) vs the control group (22.4 to 23.6% nasopharyngeal carriage of *N. meningitidis*) suggesting that bacterial competition on mucosal surfaces was likely to be the mechanism for the reduction on carriage and acquisition of *N. meningitidis* [Deasy, A. M. 2015]. Therefore, *N. lactamica* could compete with *N. meningitidis* for the same binding sites on mucosal surfaces, including CEACAMs and PAFr which are proposed receptors for Opa and Tfp, respectively [Toleman, M. 2001. Virji, M. 1996a Virji, M. 1996b. Jen, F. E. 2013]. However, *N. cinerea* lacks *opa* gene homologues and has been shown to bind epithelial cells independently of Tfp expression [Marri, P. R. 2010. Wörmann ME. 2016]. Future investigations should aim to identify molecules involved in initial adhesion which are independent of Tfp. *N. cinerea* lacks the capsule biosynthesis locus and therefore adhesins which mediate intimate attachment of bacteria to host cells may have a larger impact than in *N. meningitidis* [Kim, J. J. 1989. Marri, P. R. 2010]. MspA and NadA of *N. meningitidis* have been shown to mediate intimate attachment to epithelial cells and gene homologues have been identified in *N. cinerea* but the expression and function of *mspA* and *nadA* have yet to be confirmed [Wörmann ME. 2016. Muzzi, A. 2013]. Furthermore, the potential role of *N. cinerea* fHbp in mediating binding to epithelial cells by utilising CFH as a molecular bridge, as shown for *S. pneumoniae* and *S. pyogenes*, has yet to be analysed.

Genome analysis indicated that *fHbp* was present in all *N. cinerea* isolates. Western and Far western analysis showed that fHbp is expressed by all five *N. cinerea* isolates examined and bound CFH. Of note, *fHbp* is not present in other commensal *Neisseria* spp. with the exception of *N. polysaccharea*, although the majority of *N. polysaccharea* isolates have a frameshift in the ORF potentially producing a truncated protein [Muzzi, A. 2013]. This was in contrast to a recent genome analysis of the

commensal Streptococcal species, *Streptococcus mitis*, which showed that the CFH binding protein of *S. pneumoniae*, PspC, was absent despite the close relatedness of the two species [Denapaite, D. 2010]. Further comparative analysis of the genome sequences of *Staphylococcus epidermidis* with *Staphylococcus aureus* has also identified that the gene encoding a CFH-binding protein of *S. aureus*, SdrE, is absent from the genome of *S. epidermidis* [Gill, S. R. 2005. Sharp, J. A. 2012]. The absence of SdrE from *S. epidermidis* may be due to differences in its biological niche compared to *S. aureus*. *S. aureus* commonly colonises mucosal surfaces of the respiratory tract, whereas *S. epidermidis* is part of the skin flora and might not be exposed to complement at this site [Gill, S. R. 2005. Smith, E. J. 2011]. There are limited data investigating the role of CFH binding to commensal bacteria even though there is increasing evidence to indicate that CFH and CFH binding may be important for colonisation. Therefore future work to elucidate these mechanisms of CFH binding in commensal species should provide further insights into host-pathogen interactions and colonisation.

Bexsero<sup>®</sup>, a multi component vaccine consisting of fHbp fused to GNA2091, NadA, NHBA fused to GNA1030, and OMVs from NZ98/24, was recently licensed against serogroup B meningococci and implemented in the UK infant vaccination schedule [Oster, P. 2005]. Conjugate vaccines against *N. meningitidis* have been highly successful at reducing carriage of this pathogen but will not affect commensal *Neisseria* spp. as they target the capsular polysaccharide which is not present in the commensal species [Trotter, C. L. 2004. Balmer, P. 2002]. Limited data is currently available which assess the impact of Bexsero<sup>®</sup> on carriage, although a study of 2,954 18-24 year olds suggested that Bexsero<sup>®</sup> could reduce carriage [Read, R. C. 2014]. In the study, 987 participants were assigned to a control group and 979 participants received two doses of Bexsero<sup>®</sup>. Prior to immunisation 31% of the control group and 33% of the participants who received the Bexsero<sup>®</sup> immunisation were positive for meningococcal carriage. Three months after the second dose of Bexsero<sup>®</sup>, participants showed a significant reduction in carriage of *N. meningitidis* (18.2% reduction in carriage) compared to the control group which was independent of meningococcal serogroup [Read, R. C. 2014].

The antigens included in the Bexsero<sup>®</sup> vaccine have been shown to induce SBA against a panel of *N. meningitidis* isolates [Pizza, M. 2000. Masignani, V. 2003]. SBA is an accepted immunological correlate

of protection against meningococcal disease and rabbit SBA titres of  $\geq 8$  are considered to be protective [Gold, R. 1978. Borrow, R. 2005]. Mice immunised with Bexsero<sup>®</sup> elicited SBA against *N. cinerea*. Moreover, the SBA titres obtained with Bexsero<sup>®</sup> were similar to mice immunised with fHbp alone. Sequence analysis of *N. cinerea* indicates that *fhbp*, GNA2091 and GNA1030 are present in all *N. cinerea* isolates whereas only fragments of *nadA*, with low sequence identity to meningococcal *nadA4*, are present in some *N. cinerea* isolates [Muzzi, A. 2013]. NHBA was not found in any *N. cinerea* isolate analysed [Muzzi, A. 2013]. GNA2091 and GNA1030 do not induce SBA against *N. meningitidis* but are included in the vaccine as they act as adjuvants and increase immune responses. The lack of SBA responses against *N. cinerea* without *fhbp* indicates that immunisation with fHbp is enough to induce effective immune responses against *N. cinerea*.

Although SBA is the 'gold standard' correlate of protection against *N. meningitidis*, induction of SBA against *N. cinerea* does not necessarily mean that immunisation with Bexsero<sup>®</sup> will affect carriage of this species [Gold, R. 1978]. As IgG and complement are present on mucosal surfaces and in plasma exudates on epithelial surfaces [Robert-Guroff, M. 2000. Persson, C. G. 1991. Reichhardt, M. P. 2016], it is possible that induction of SBA against fHbp could affect the nasopharyngeal flora, especially bacteria expressing fHbp such as *N. meningitidis* and *N. cinerea*. SBA were performed using lower dilutions of complement for *N. cinerea* (1:10) compared with *N. meningitidis* (1:6). Furthermore, as *N. cinerea* lack capsule and expresses fHbp, *N. cinerea* may be more susceptible than *N. meningitidis* to immune responses against surface proteins elicited by Bexsero<sup>®</sup> *i.e.* bactericidal antibodies. Therefore, it will be interesting to evaluate the impact of Bexsero<sup>®</sup> on carriage of commensal *Neisseria* as well as the meningococcus. Furthermore, this study has not eliminated the possibility of *N. cinerea* inducing a bactericidal immune response in individuals colonised by the bacterium or assessed the impact of colonisation by *N. cinerea* on meningococcal carriage which could be the focus of further investigation.

Data in this chapter shows that not only does *N. cinerea* possess a *fhbp* gene but that fHbp is localised on the surface of the bacterium and accessible to mucosal surfaces. Moreover, Bexsero<sup>®</sup> elicits bactericidal antibodies against *N. cinerea*, with SBA responses similar those following immunisation

with fHbp alone. Given that meningococcal fHbp can elicit bactericidal antibodies against *N. cinerea*, *N. cinerea* could potentially elicit specific mucosal and serum antibody responses as previously demonstrated by cross-reactive antibodies against *N. meningitidis* following carriage by *N. lactamica* and therefore could be a source of natural immunity against the meningococcus [Evans, C. M. 2011]. Furthermore, carriage of commensal *Neisseria* species, including *N. cinerea*, may contribute to protection against meningococcal carriage and disease *via* direct competition in the nasopharynx as evidenced by reduced carriage of *N. meningitidis* in individuals colonised by *N. lactamica* although the impact of *N. cinerea* carriage on carriage of *N. meningitidis* has yet to be elucidated. [Evans, C. M. 2011. Deasy, A. M.]. Implementation of vaccines containing fHbp have the potential to affect the commensal nasopharyngeal flora which could alter the species which are naturally acquired and carried on the nasopharyngeal mucosa. If commensal *Neisseria* carriage is terminated through the introduction of fHbp containing vaccines, *i.e.* Bexsero®, other bacterial species could occupy a vacant niche and could potentially include other pathogens *e.g.* *N. meningitidis* which are not covered by available vaccines. Therefore the impact of multi-component vaccines on commensal *Neisseria* species should be assessed in future carriage studies.

## 7. REFERENCES

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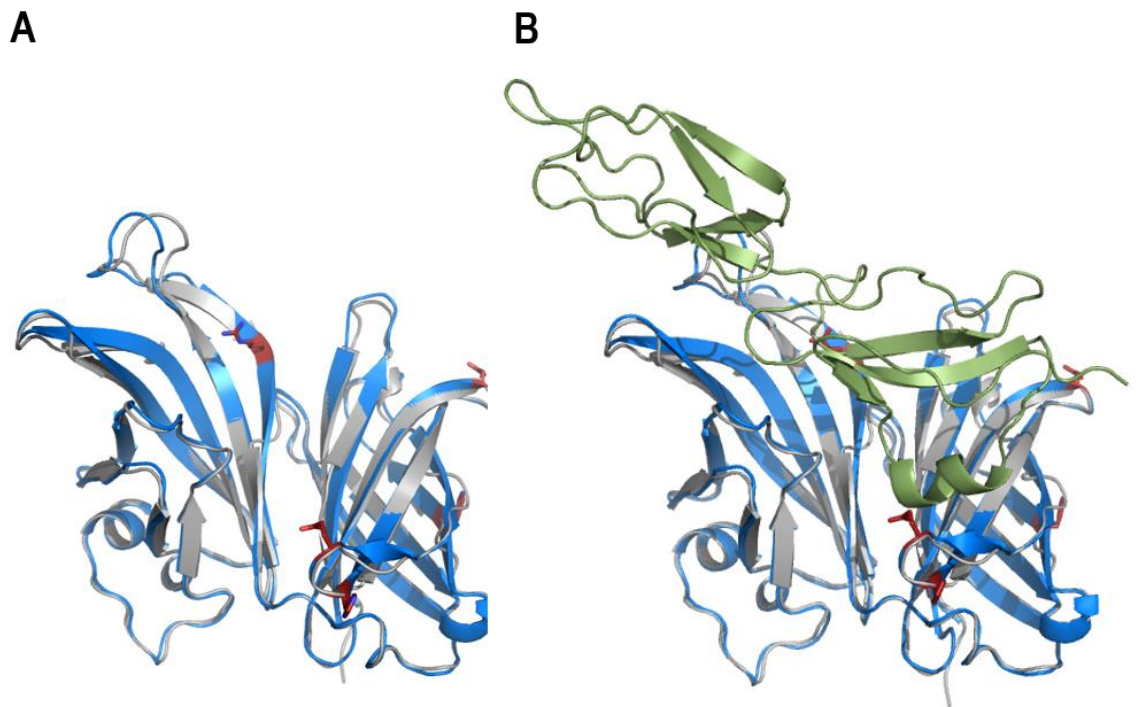


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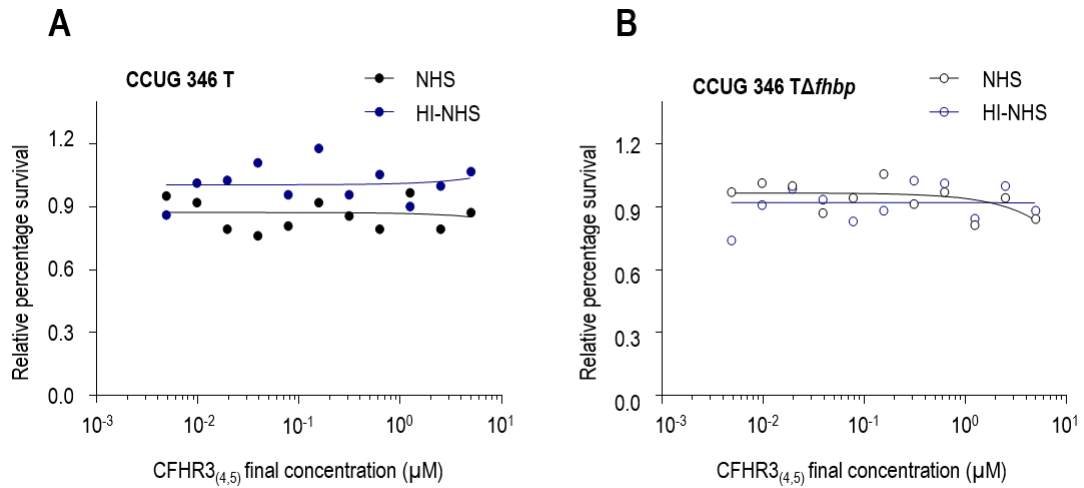
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## 8. SUPPLEMENTARY INFORMATION



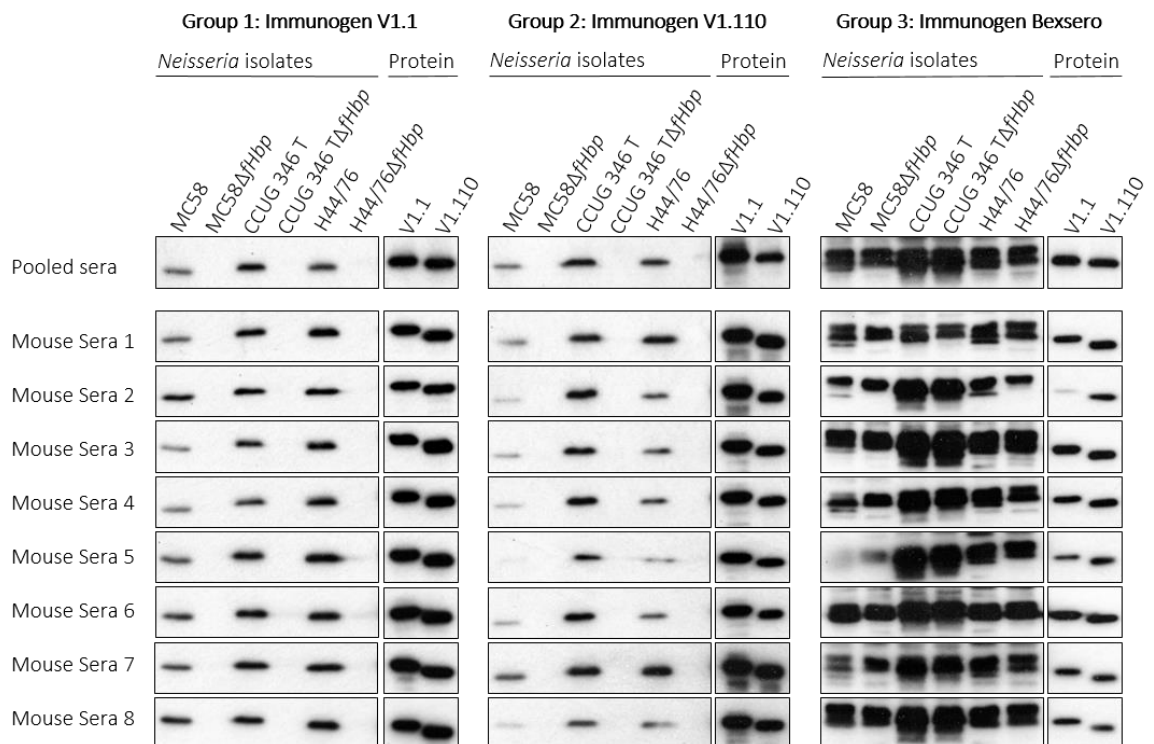
SI 1: *N. cinerea* fHbp is predicted to be structurally similar to *N. meningitidis* fHbp.

(A) Predicted structure of *N. cinerea* fHbp (grey ribbon) generated with the I-TASSER server and V1.1 fHbp (blue ribbon) [Schneider, PBD: 2W80] and (B) predicted structure of *N. cinerea* fHbp bound to CFH (green ribbon). Figure drawn using Pymol. Residues in red (shown as ball-and-stick representations) are different in *N. cinerea* fHbp compared to V1.1 fHbp.



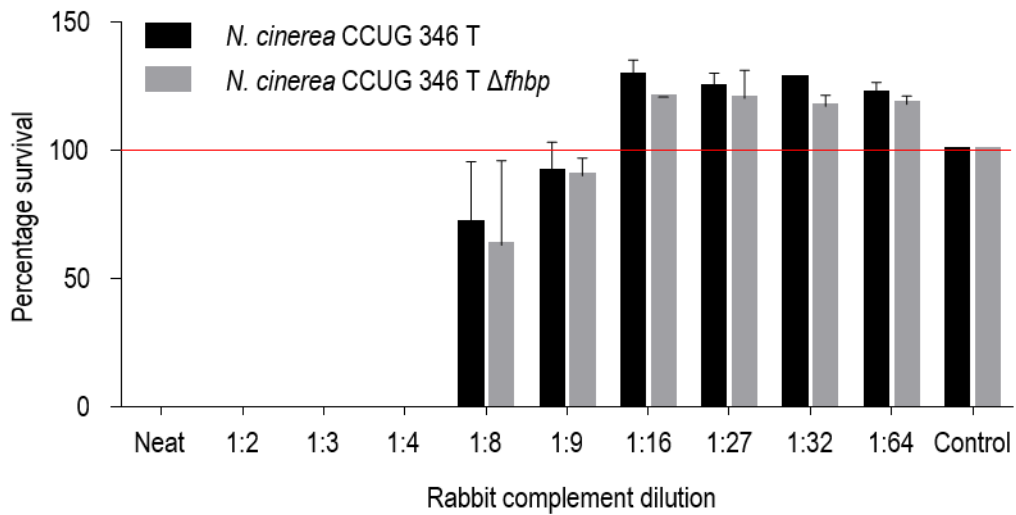
SI 2: CFHR3<sub>(4,5)</sub> does not influence susceptibility of *N. cinerea* to complement mediated lysis.

Survival of (A) *N. cinerea* CCUG 346 T (filled circles) and (B) CCUG 346 TΔfHbp (open circles) in the presence of 3% NHS (black) or HI-NHS (blue) following pre-incubation in different concentrations of CFHR3<sub>(4,5)</sub> (indicated). Data presented as percent survival relative to bacteria incubated in the absence of serum and CFHR3<sub>(4,5)</sub>.



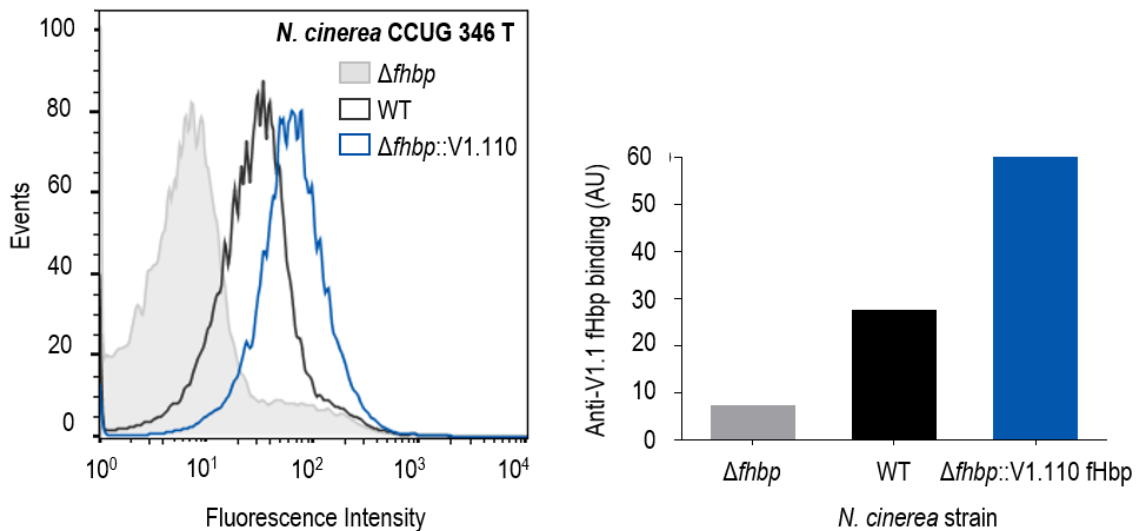
SI 3: Immunogenicity of individual mouse sera

Sera of individual mice from groups immunised with V1.1 fHbp, *N. cinerea* fHbp and Bexsero® recognises *N. cinerea* CCUG 346 T, *N. meningitidis* MC58 and H44/76 fHbp and detects recombinant V1.1 and V1.110 fHbp as determined by western blot analysis.



**SI 4: Optimisation of rabbit complement concentration for *N. cinerea* SBA assays.**

(A) Survival of *N. cinerea* CCUG 346 T (black bars) and CCUG 346 T  $\Delta fHbp$  (grey bars) after incubation in a range of rabbit complement dilutions (neat to no complement, as indicated). Red line indicates 100% bacterial survival. Approximately 90% bacterial survival is seen following incubation in 1:9 dilution of rabbit complement after 60 minutes incubation. Data presented as percent survival relative to bacteria incubated in no rabbit complement for 60 minutes. Error bars are  $\pm$  SEM of three independent experiments.



**SI 5: Characterisation of isogenic *N. cinerea* expressing the homologous fHbp.**

(A) Flow cytometry analysis of *N. cinerea* CCUG 346 T (black), and isogenic strain expressing V1.110 fHbp (blue) using anti V1.1 fHbp sera. Grey shaded area shows the plot for CCUG 346 T  $\Delta fHbp$ . (B) Data presented as arbitrary units from the mean fluorescence of FACS calibur FL-4 channel.

9. Publication: Competition between antagonistic complement factors for a single protein on *N. meningitidis* rules disease susceptibility. Caesar JJ. *et al.* (2014) *Elife*