Complement Abnormalities in Membranoproliferative Glomerulonephritis and C3 Glomerulopathy

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Abstract

Membranoproliferative glomerulonephritis (MPGN) and C3 glomerulopathy (C3G) are rare diseases that associate with dysregulation of the alternative pathway (AP). The earliest abnormalities associated with these diseases were C3 nephritic factor and also rare genetic variants in the gene *CFH* that caused factor H (FH) deficiency. Since then, other acquired and genetic abnormalities in AP have been reported in MPGN and C3G. The aim of this project was to screen cohorts of MPGN and C3G for such abnormalities.

Screening for rare sequence variants in genes encoding proteins involved in AP activity in two cohorts revealed a low prevalence of genetic abnormalities. Compared to the prevalence of C3 nephritic factor and autoantibodies to complement proteins, it was clear that the predominant abnormalities in these cohorts were acquired.

Though few, rare genetic variants identified in *CFH* were studied in functional studies. The first was identified in a case of familial MPGN in the N-terminal domain of *CFH*. Functional studies included surface plasmon resonance and haemolytic assays to study a mutant protein in the setting of a short fragment comprising the N-terminal domain of FH. This initial study confirmed loss of function in a familial variant and formed the basis for further studies.

In studies of eight other variants identified in MPGN and C3G and two other diseases that share complement risk factors, only two variants were likely to be functionally significant as demonstrated by complete loss of function. This highlights the need for such studies to correctly identify important variants. The significant functional effects initially identified in studies using short fragments were then confirmed in studies using full length protein.

The significance of rare genetic variants in *CFH* needs to be considered even though MPGN and C3G are largely an autoimmune phenomenon.

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Publications Arising from This Work

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Publications from work described in this thesis.

Primary data papers.

Wong EK, Anderson HE, Herbert AP, Challis RC, Brown P, Reis GS, et al. Characterization of a factor H mutation that perturbs the alternative pathway of complement in a family with membranoproliferative GN. Journal of the American Society of Nephrology : JASN. 2014;25(11):2425-33

R.C. Challis*, G.S.R. Araujo*, **E.K.S. Wong***, H.E. Anderson, A. Awan, A.M. Dorman, M. Waldron, V. Wilson, V. Brocklebank, L. Strain, B.P. Morgan, C.L. Harris, K.J. Marchbank, T.H.J. Goodship, D. Kavanagh. A de novo deletion in the RCA cluster resulting in a novel hybrid CFH/CFHR3 gene causing impaired cell surface complement regulation in atypical haemolytic uraemic syndrome. Journal of the American Society of Nephrology : JASN. 2015

Yu Y*, Triebwasser MP*, **Wong EK***, Schramm EC*, Thomas B, Reynolds R, et al. Whole-exome sequencing identifies rare, functional CFH variants in families with macular degeneration. Human molecular genetics. 2014;23(19):5283-93

***These authors contributed equally**

Publications from work related to but not described in this thesis.

Primary data papers.

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Abbreviations

Ab	Antibody
aHUS	Atypical haemolytic uraemic syndrome
AMD	Age-related macular degeneration
ANOVA	Analysis of variance
AP	Alternative pathway
APL	Acquired partial lipodystrophy
BDC	Blood donor control
BMG	Buffered minimal glycerol
BMM	Buffered minimal methanol
BSA	Bovine serum albumin
C2/3/4/5/6/7/8/9	Complement component 2/3/4/5/6/7/8/9
$C3b(H_2O)$	Initial proconvertase of alternative pathway
$C3b(H_2O)Bb$	Initial C3 convertase of alternative pathway
C3bB	Proconvertase of alternative pathway
C3bBb	C3 convertase of alternative pathway
C3bBbC3b	C5 convertase
C3G	C3 Glomerulopathy
C3GN	C3 Glomerulonephritis
C4bC2a	C3 convertase of the classical pathway
C4BP	C4 binding protein
CCP	Complement control protein
CD35	Complement receptor 1
CD46	Membrane co-factor protein
CD55	Decay accelerating factor
cDNA	Complementary Deoxyribonucleic acid
CFB	Complement factor B gene
CFD	Complement fixation diluent
CFH	Complement factor H gene
CFHR	Complement factor H-related gene
CFI	Complement factor I gene
CKD	Chronic kidney disease
CNV	Copy number variation
CO_2	Carbon Dioxide
CP	Classical pathway
CR1	Complement receptor 1
DAF	Decay accelerating factor
Db SNP	Single nucleotide polymorphism database
DDD	Dense deposit disease
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotides
DO	Dissolved oxygen

dsDNA	Double-stranded deoxyribonucleic acid
EA	Sensitised sheep erythrocyte
EAC3b	C3b-coated sheep erythrocyte
EAGLE	Eculizumab in Primary MPGN
EDC	N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESRD	End-stage renal disease
FB	Complement factor B
FD	Complement factor D
FH	Complement factor H
FHL-1	Complement factor H like protein 1
FHR	Complement factor H related protein
FI	Complement factor I
GAG	Glycosaminoglycan
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
HSQC	Heteronuclear single quantum coherence
HUS	Haemolytic uraemic syndrome
iC3b	Inactivated C3b
IC-GN	Immune-complex glomerulonephritis
IC-MPGN	Immune-complex membranoproliferative glomerulonephritis
Ig	Immunoglobulin
JC-1	Mouse monoclonal antibody to FB
K_D	Affinity constant
LB	Luria-Bertani
MAC	Membrane attack complex
MAF	Minor allele frequency
MASP	MBL-associated serine protease
MBI6	Mouse monoclonal antibody to FH 402Y
MBI7	Mouse monoclonal antibody to FH 402H
MBL	Mannose-binding lectin
MCP	Membrane co-factor protein
MLPA	Multiplex-ligation dependent probe amplification
MMF	Mycophenolate mofetil
MPGN	Membranoproliferative glomerulonephritis
NHS	N-hydroxysuccinimide
NMR	Nuclear magnetic resonance
OMCI	*Ornithodoros moubata* complement inhibitor
OR	Odds ratio
OX24	Mouse monoclonal antibody to FH SCR5
P:Cr	Protein:Creatinine ratio

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEX	Plasma exchange
pKa	Acid dissociation constant
RaDaR	National Registry of Rare Kidney Diseases
RBC	Red blood cells
RCA	Regulators of complement activation
rpm	Revolutions per minute
RPMI	Roswell park memorial institute
RU	Response units
SCR	Short consensus repeat
SDS	Sodium dodecyl sulphate
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SPR	Surface plasmon resonance
UK	United Kingdom
VUS	Variant of uncertain significance
VWA	Von Willebrand type A domain
WT	Wildtype
YPD	Yeast peptone dextrose
ΔBΔHNHS	Norman human serum depleted of factor B and factor H

Chapter 1 - Introduction

The complement system comprises an integrated network of proteins that forms part of the innate immune system. The ability of the complement system to discriminate between foreign and host cells is critical to maintain health. Dysregulation of the alternative pathway of complement leads to damage of host cells and is associated with several renal diseases. Greater understanding of this pathway and the abnormalities that lead to dysregulation of the alternative pathway can improve treatment and prognosis in these diseases. The focus of this thesis is the identification and study of abnormalities of the complement system in these complement-mediated renal diseases.

1.1 The Complement System

The complement system comprises three activation pathways that generate C3b via C3 convertases. These lead to amplification of complement and activation of the terminal pathway. Host cells are protected from complement by a number of regulatory proteins. A simplified overview highlighting the interplay between complement activation pathways, the amplification loop and terminal pathway and a number of regulatory proteins is summarised in Figure 1 (Walport, 2001a; Walport, 2001b; Ricklin *et al.*, 2010).

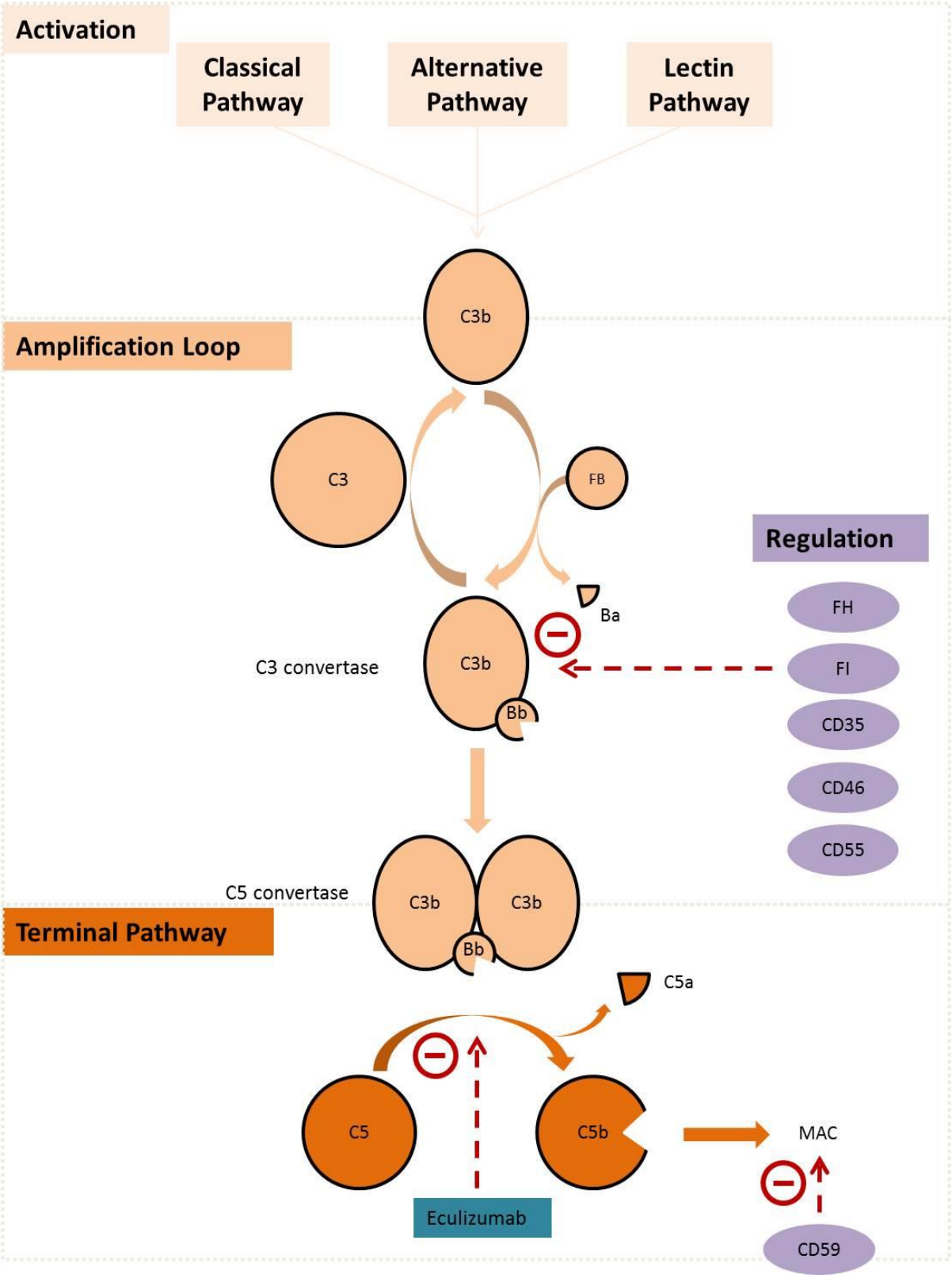

Figure 1 The complement system

Adapted from Oxford Textbook of Medicine, 6th Edition. The activation pathways generate C3b leading to amplification of complement via the C3 convertase, C3bBb. This leads to generation of a C5 convertase (C3bBbC3b), which cleaves C5 and activates the terminal pathway. Circulating and membrane-bound proteins can regulate complement at the level of the amplification loop and also inhibit MAC. Eculizumab is the only licensed complement inhibitor drug available. It inhibits the cleavage of C5. FB=complement factor B, FH=complement factor H, FI=complement factor I, MAC=membrane attack complex.

1.1.1 Classical Pathway

The classical pathway (CP) is activated by antibody-antigen immune complexes binding to C1q. In a calcium-dependent process, C1q binds C1r and C1s and cleaves C4 into its subunits C4a and C4b and cleaves C2 into C2a and C2b. Both C4a and C2b are released, leaving C2a to bind to C4b to form the C3 convertase of the classical pathway (C4bC2a). This convertase then cleaves C3 into C3a and C3b (Wallis *et al.*, 2010).

1.1.2 Lectin Pathway

The lectin pathway is activated by carbohydrate residues present on the cell surfaces of certain pathological organisms including bacterial and fungal organisms. These are recognised by proteins collectively known as pattern recognition molecules such as mannose-binding lectin (MBL). MBL then aggregates with MBL-associated serine protease (MASP) to form a complex that then cleaves C2 and C4 resulting in the formation of C4bC2a and the generation of C3b (Wallis *et al.*, 2010).

1.1.3 Alternative Pathway

The alternative pathway (AP) is the most ancient of the three complement pathways and is continually active due to 'tickover'. Tickover is a process in which C3 spontaneously hydrolyses leading to a structural change exposing the thio-ester domain of C3, in the form C3(H_2O) (Pangburn *et al.*, 1981). C3(H_2O), structurally similar to C3b, binds complement factor B (FB) to form an intermediate proconvertase (C3b(H_2O)B) that is cleaved by complement factor D (FD) to form an initial C3 convertase (C3b(H_2O)Bb) that cleaves C3 into C3a and C3b and deposits small amounts of C3b onto cell surfaces. On host cells, this initial surface bound C3b is inactivated by regulatory proteins.

Surface bound C3b on foreign cells binds FB to form a proconvertase (C3bB) which is cleaved by FD, to form the surface bound C3 convertase of the AP (C3bBb) (Forneris *et al.*, 2010). Surface bound C3bBb is a short-lived complex with a half-life of about 90s (Pangburn and Muller-Eberhard, 1986). C3bBb can be stabilised by properdin (Fearon and Austen, 1975). C3bBb cleaves C3 and releases C3b to complete an efficient amplification loop thus increasing the density of C3b deposition. Eventually, C3b binds C3bBb to form a C5 convertase (C3bBbC3b).

1.1.4 Regulators of the AP C3 convertase

Regulators of C3bBb are present on the surface of host cells and in the fluid phase. Complement factor H (FH) is a regulator of C3bBb in the fluid phase that can be recruited to host surfaces via recognition domains (Schmidt *et al.*, 2008a). The membrane-bound regulators include membrane co-factor protein (CD46, MCP) (Seya and Atkinson, 1989), decay accelerating factor (DAF, CD55) (Kim and Song, 2006) and complement receptor 1 (CR1, CD35) (Khera and Das, 2009).

C3bBb decay can be accelerated by FH, CR1 or DAF (Hourcade *et al.*, 2002). Inactivation of C3b into iC3b by complement factor I (FI) results in a degradation product that is unable to bind FB (Lambris *et al.*, 1996). The FI-mediated proteolysis of C3b requires a co-factor, such as FH, CD46 or CR1. C4-binding protein (C4BP) can also bind C3b and act as a co-factor for FI-mediated inactivation of C3b (Blom *et al.*, 1999).

1.1.5 Terminal complement pathway

The formation of C3bBbC3b results in the cleavage of the complement component C5 and activates the terminal pathway. This results in the release of the anaphylatoxin C5a into the circulation. Meanwhile, C5b binds C6, C7, C8 and C9 to form the membrane attack complex (MAC) and initiate cell lysis (Bubeck, 2014). Regulators of this pathway include CD59, a membrane-bound protein that prevents polymerisation of C9 in the formation of MAC (Farkas *et al.*, 2002). Eculizumab, the only licensed complement inhibitor approved for clinical use, binds C5 and prevents its cleavage by C3bBbC3b (Rother *et al.*, 2007).

1.2 Membranoproliferative Glomerulonephritis and C3 Glomerulopathy

Membranoproliferative glomerulonephritis (MPGN), dense deposit disease (DDD), C3 glomerulonephritis (C3GN) and *CFHR5* nephropathy are rare renal diseases that associate with dysregulation of the AP. These classifications are based upon pathological appearances on renal biopsy. The diseases DDD, C3GN and *CFHR5* nephropathy are collectively known as C3 glomerulopathy (C3G).

1.2.1 MPGN

Membranoproliferative glomerulonephritis (MPGN) is a pathological term describing a pattern of glomerular injury seen on light microscopy. It is characterised by mesangial expansion, cellular proliferation and double contouring of basement membrane (Sethi and Fervenza, 2012). It is associated with immunoglobulin (Ig) and complement deposition. The classification of MPGN was historically based upon the position of electron dense deposits relative to the glomerular basement membrane. Three types of MPGN were defined in this manner, type 1 (subendothelial), type 2 or DDD (intramembranous) and type 3 (subendothelial and subepithelial) (Anders and Thoenes, 1975). Type 1 and type 3 MPGN typically stained positive for Ig and complement on immunofluorescence whereas type 2 MPGN typically stained for complement only. Low C3 levels associate with all types of MPGN. Associations with low C4 and a C3 nephritic factor, suggestive of a different underlying causes of a low C3 via classical pathway and alternative pathway activation respectively, do not exclusively associate with individual types of MPGN, 1, 2 or 3 (Rennke, 1995) (Table 1).

A revised classification (Sethi and Fervenza, 2011) of MPGN based on the presence or absence of Ig on immunofluorescence was suggested. In MPGN, the presence of Ig (an immune-complex MPGN or IC-MPGN) would suggest complement deposition via the classical pathway and prompt the clinician to search for an underlying secondary cause (Table 2). The absence of Ig would be suggestive of a disorder of AP regulation leading to complement deposition such as the presence of a C3 nephritic factor (Masani *et al.*, 2014).

	MPGN Type 1	MPGN Type 2	MPGN Type 3
Position of Electron Dense Deposits	Sub-endothelial	Intramembranous	Sub-endothelial and sub-epithelial
C3 deposition	Present	Present	Present
Ig deposition	Typically present	Often absent	Typically present
Low complement levels	Usually C3 and C4 Sometimes low C3 only	Usually low C3 only	Usually C3 and C4 Sometimes low C3 only
C3 Nephritic Factor	Sometimes present	Usually Present	Sometimes present

Table 1 Historical classification of MPGN

Clinical and pathological characteristics of MPGN type 1, 2 and 3 are summarised.

1.2.2 C3 Glomerulopathy

In 2007, a new pathological entity of primary glomerulonephritis with isolated C3 deposits was identified (Servais *et al.*, 2007). Abnormalities of AP were identified in this group of patients and not restricted to those who had an MPGN pattern of glomerular injury. This led to the introduction of the term C3 glomerulopathy. C3G was initially defined as 'glomerular pathology characterized by C3 accumulation with absent or scanty immunoglobulin deposition' (Fakhouri *et al.*, 2010a). This classification has evolved since its initial introduction and the most recent interpretation was described in the C3 glomerulopathy consensus report (Pickering *et al.*, 2013). The introduction of the term was to alert the clinician to a group of diseases in which dysregulation of the AP was likely. DDD, C3GN and *CFHR5* nephropathy are all forms of C3G.

Autoimmune diseases	Infections	Paraproteinemias
Systemic lupus	Hepatitis B	Monoclonal gammopathy of undetermined significance
Sjogren's syndrome	Hepatitis C	Waldenstrom macroglobulinaemia
Rheumatoid arthritis	Endocarditis	Chronic lymphocytic leukaemia
Mixed connective tissue disease	Shunt infections	Low-grade B-cell lymphoma
	Visceral abscesses	Cryoglobulinaemia type 1 and 2
	Leprosy	Immunotactoid glomerulopathy
	Malaria	Monoclonal Ig deposition disease
	Schistosomiasis	Fibrillary glomerulopathy
	Mycoplasma	

Table 2 Secondary causes of MPGN

Adapted from Masani et al (Masani et al., 2014). Chronic circulating antigen from numerous causes is thought to result in classical pathway activation and the appearance of immune-complexes in MPGN (type 1, 3 or immune-complex MPGN).

According to the C3G consensus report, a diagnosis of C3G should be considered if there is dominant C3 deposition, where dominant is defined as C3 intensity ≥2 orders of magnitude more than any other immune reactant on a scale of 0 to 3 (including 0, trace, 1+, 2+, 3+) (Pickering *et al.*, 2013). The presence of Ig staining on biopsy does not exclude C3G (Pickering *et al.*, 2013; Hou *et al.*, 2014). Light microscopy may show one of several patterns of glomerular injury, but usually membranoproliferative glomerulonephritis or mesangial proliferative glomerulonephritis (Hou *et al.*, 2014).

Dense deposit disease (DDD) is a term defined by the laminar electron dense deposits within the glomerular basement membrane and was and considered a form of MPGN (type 2) (Habib *et al.*, 1975). However, only 25% of cases with DDD have an MPGN pattern of glomerular injury (Fogo and Kashgarian, 2005; Walker *et al.*, 2007). The most common pattern of glomerular injury is mild mesangial cell hypercellularity in approximately 45% of cases of DDD. Other patterns of injury include a crescentic pattern (approximately 18%) or

an acute proliferative and exudative pattern (12%) (Walker *et al.*, 2007). DDD is currently considered a form of C3G and not a form of MPGN. Cases of C3G that do not fulfil the diagnostic criterion for DDD are termed C3GN.

CFHR5 nephropathy is a distinct form of C3GN (Gale *et al.*, 2010). Two large families of Cypriot patients with renal disease had distinct characteristics of persistent microscopic haematuria and synpharyngytic macroscopic haematuria. Biopsy appearances were consistent with C3GN and MPGN. Further genetic studies identified an internal duplication of exons 2 and 3 of *CFHR5* in affected family members. This led to the expression of a novel FHR5 protein. This inherited form of C3GN was subsequently reported in a total of 91 patients from 16 pedigrees of Cypriot descent (Athanasiou *et al.*, 2011). *CFHR5* nephropathy has since been identified in a family of non-Cypriot descent (Medjeral-Thomas *et al.*, 2014a).

1.2.3 Overlap of MPGN and C3G

The classification and aetiology of MPGN and C3G overlap (Sethi *et al.*, 2012b). Patients with an MPGN pattern of glomerular injury can also have C3G, defined by dominant C3 deposition. In cases of MPGN without dominant C3 deposition, an underlying secondary cause should first be considered. This is applicable to patients with IC-MPGN and those previously classified as MPGN type 1 or 3. The possibility of an underlying abnormality of AP can then be considered in cases of C3G and types of MPGN in which no secondary cause can be found (Figure 2). In this thesis, I shall refer to cohorts that include either of these pathological entities as MPGN/C3G, in which abnormalities of AP should be considered.

1.2.4 Epidemiology of MPGN/C3G

All forms of MPGN/C3G are rare. The exact incidence of MPGN is unknown but is in the order of 1- 4 cases per million (West, 1992; Briganti *et al.*, 2001). The incidence of C3G in a recent study was estimated at 1-2 cases per million (Medjeral-Thomas *et al.*, 2014b) and similar for DDD (Appel *et al.*, 2005). These diseases, especially DDD are a disease of childhood and young adulthood (Servais et al., 2012; Medjeral-Thomas et al., 2014b).

1.2.5 Clinical Features of MPGN/C3G

Patients with MPGN/C3G have similar clinical features (excepting *CFHR5* nephropathy as described above). Presenting features include proteinuria, haematuria and renal failure (Servais *et al.*, 2012; Medjeral-Thomas *et al.*, 2014b). Renal failure is progressive and 40% of patients develop end-stage renal disease (ESRD) at 10 years (Cansick *et al.*, 2004; Servais *et al.*, 2012; Medjeral-Thomas *et al.*, 2014b). Recurrence in transplantation is common in all types, ranging from 30-40% in MPGN type 1 to 80-90% in DDD (Braun *et al.*, 2005). Plasma C3 levels are often low (Varade *et al.*, 1990; Servais *et al.*, 2012; Medjeral-Thomas *et al.*, 2014b; Iatropoulos *et al.*, 2016). Several extra-renal manifestations also associate with patients in these cohorts and are described section 1.4.5.

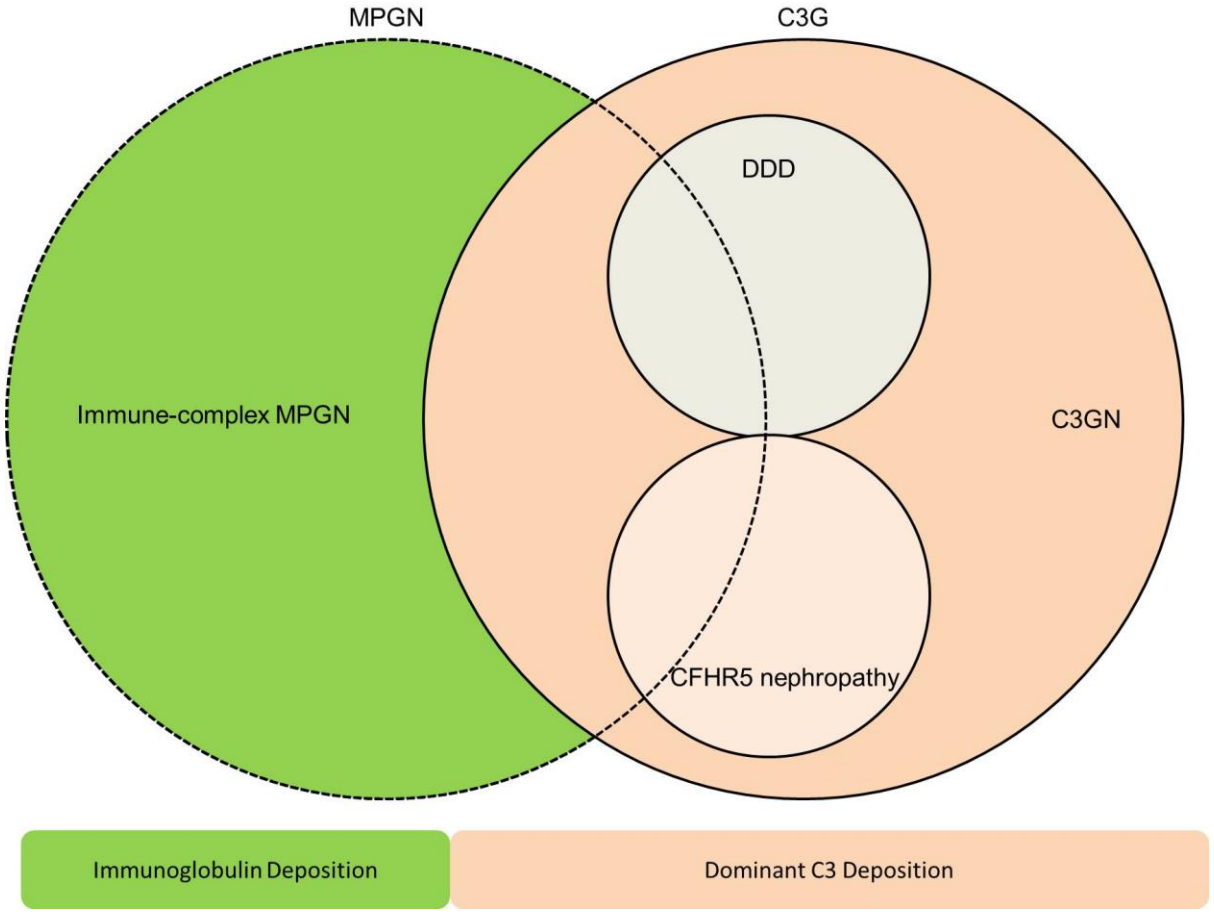

Figure 2 Current overview of MPGN and C3 Glomerulopathy

Once secondary causes (table 3) have been excluded, an abnormality of AP should be considered in MPGN and C3G. In these overlapping conditions, C3 deposition is usually present. MPGN is shown in dashed circle and includes IC-MPGN (Ig and C3 deposition - Green) and C3G (Dominant C3 deposition). C3G is shown in large circle (pink) any can associate with an MPGN pattern of glomerular injury. Specific forms of C3G include C3GN, DDD and CFHR5 nephropathy.

1.3 Complement Abnormalities in MPGN/C3G

A number of abnormalities of AP that have been described in MPGN/C3G are summarised in Figure 3. Uncontrolled activation of the AP in the fluid phase due to C3 nephritic factor (Spitzer *et al.*, 1969) and FH deficiency have been associated with MPGN for over 30 years (Levy *et al.*, 1986). In recent studies, the C3 nephritic factor remains a common finding in patients with MPGN/C3G, especially those with DDD (Servais *et al.*, 2012; Zhang *et al.*, 2012). Autoantibodies against other complement components (Strobel *et al.*, 2010; Goodship *et al.*, 2012) have also been described.

Studies describing rare genetic variants in genes encoding proteins of the AP have been reported in cohorts of MPGN/C3G. The prevalence of rare genetic variants in *CFH*, *CFI* or *CD46* was 17.9% in a French cohort of MPGN/C3G patients (Servais *et al.*, 2012). In cohorts screened for rare genetic variants in *CFH*, *CFI*, *CD46*, *C3* and *CFB*, the prevalence ranged from 18% in an Italian cohort of MPGN/C3G (Iatropoulos *et al.*, 2016) to 43.3% in an American cohort of C3G (Bu *et al.*, 2015). Individual complement abnormalities have not been associated with a greater risk of adverse outcomes (Servais *et al.*, 2012; Medjeral-Thomas *et al.*, 2014b) although the absence of a C3 nephritic factor or a rare genetic variant in an Italian cohort did have a higher risk of progression to ESRD (Iatropoulos *et al.*, 2016).

These abnormalities and others are described in more detail below.

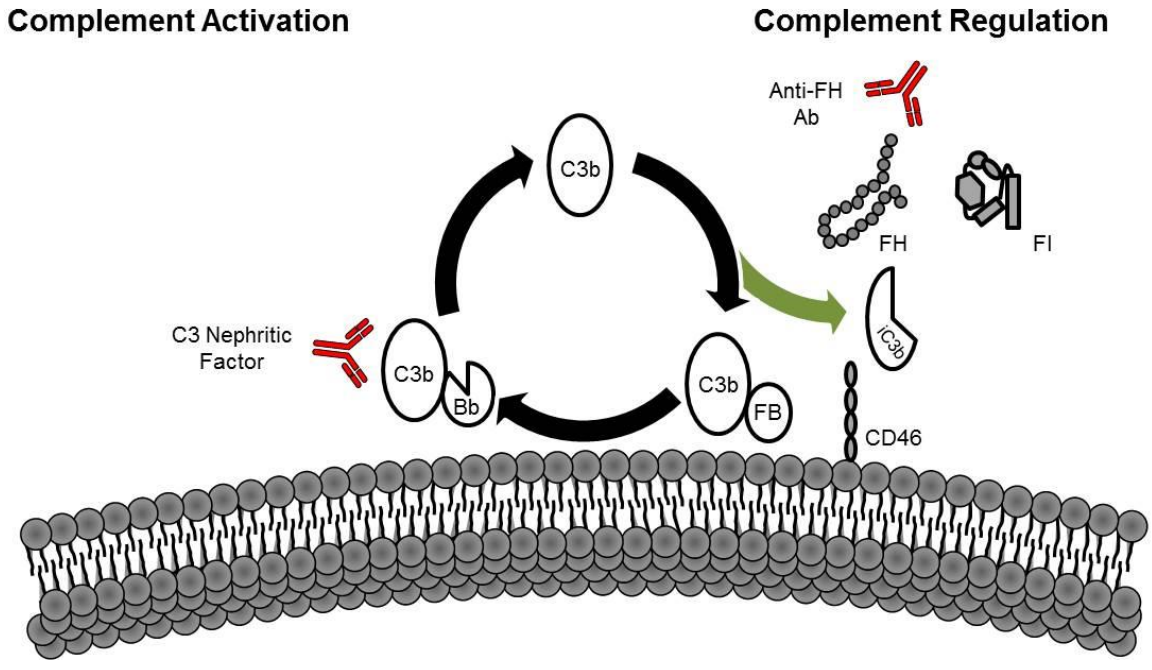

Figure 3 Abnormalities of alternative pathway in MPGN/C3G

1.3.1 Complement Factor H

Complement factor H (FH) is a single chain, 155kDa glycoprotein that is organised into 20 repetitive subunits called short consensus repeats (SCR) each of ~ 60 amino acids (Ripoche *et al.*, 1988). FH regulates the AP in the circulation and on cell surfaces via regulatory and recognition domains that are localised to specific SCRs shown in Figure 4 (Schmidt *et al.*, 2008b). In order to regulate AP, FH binds C3b and acts as a co-factor for the FI-mediated proteolytic cleavage of C3b into iC3b and accelerates the decay of the C3bBb. These regulatory effects of FH are mediated via its N-terminal domains (SCR1-4) (Wu *et al.*, 2009).

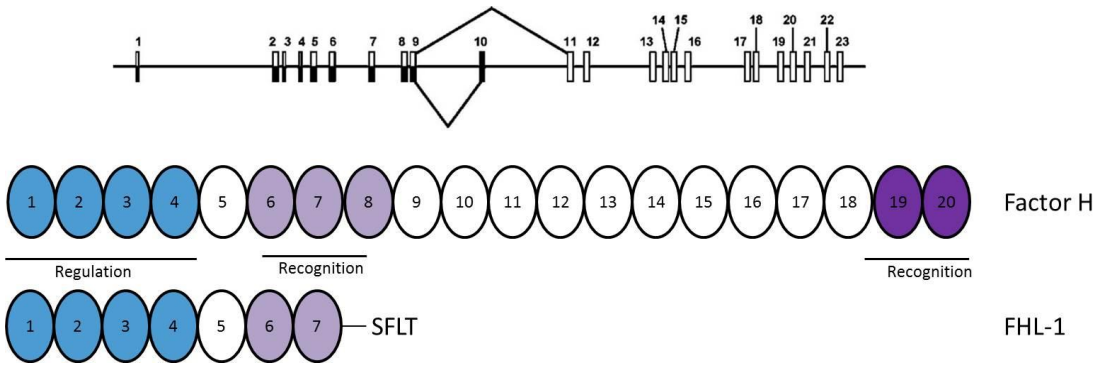

Figure 4 The CFH gene, FH and FHL-1

The schematic of the CFH gene is taken from (Rodriguez de Cordoba et al., 2004). FH is encoded by the gene, CFH, shown above comprising 23 exons. FHL-1 is an alternatively spliced protein, encoded by CFH. Exons 1-9 are common to both proteins. Exon 10 encodes a C-terminal 4 amino acid tail and a stop codon. Exons 11-23 encode FH only.

The regulatory functions of FH can be recruited to host surfaces via binding domains in SCR6-8 and the C-terminus of FH in SCR19-20. Differences in these two binding domains for glycosaminoglycans (GAGs), polyanions and C3b infer specificity of these regions to different host tissues (Clark *et al.*, 2013). The domain in SCR6-8 has specificity for Bruch's membrane within the eye (Clark *et al.*, 2013). The domain in SCR19-20 has specificity for tissues bearing GAGs and sialic acids, localising FH to the glomerular endothelium via a second main C3b binding site (Ferreira *et al.*, 2009; Morgan *et al.*, 2011). Furthermore, FH appears to be able to bind to C3b simultaneously via SCR1-4 and SCR19-20 (Morgan *et al.*, 2011).

Functional studies of disease-associated genetic variants in *CFH* have a predilection to these three domains (Sanchez-Corral *et al.*, 2002; Ferreira *et al.*, 2009; Wong *et al.*, 2014; Yu *et al.*,

2014; Recalde *et al.*, 2015). Two domains in particular SCR1-4 and SCR19-20 are the focus of functional studies that I will be describing later in this thesis.

Interactions of FH to other proteins have previously been identified and include the lipid peroxidation product malondialdehyde (Weismann *et al.*, 2011), C-reactive protein (Sjoberg *et al.*, 2007), pentraxin 3 (Kopp *et al.*, 2012), adrenomedullin (Pio *et al.*, 2001), as well as necrotic cells (Sjoberg *et al.*, 2007). Furthermore, proteins that bind FH have been synthesised by micro-organisms allowing them to hijack FH and evade the activity of the complement system. The number of organisms that have developed these strategies is vast and includes *Streptococcus* (Horstmann *et al.*, 1988) and *Meningococcus* (Malito *et al.*, 2013).

A lack of FH leads to uncontrolled activation of AP in the fluid phase (Thompson and Winterborn, 1981) and associates with a phenotype that includes MPGN or C3G in both human (Levy *et al.*, 1986; Dragon-Durey *et al.*, 2004; Servais *et al.*, 2007) and animal studies (Hegasy *et al.*, 2002; Pickering *et al.*, 2002). Screening of cohorts of MPGN/C3G identifies rare genetic variants in *CFH* in 4-16.2% of patients (Servais *et al.*, 2012; Bu *et al.*, 2015; Iatropoulos *et al.*, 2016). In some cases but not all, these associate with low FH levels. In other cases, where FH levels are normal, functional testing of a variant is required to confirm their significance in disease (Kavanagh and Anderson, 2012).

Functional studies of rare genetic variants identified in *CFH* in MPGN/C3G are rare and include the study of the significant disease-associated variants delK224 (Licht *et al.*, 2006) and R1210C (Recalde *et al.*, 2015). A detailed discussion of rare genetic variants in MPGN/C3G forms part of chapter 5 of this thesis.

Common single-nucleotide polymorphisms (SNPs) in *CFH* have been studied in cohorts of MPGN/C3G (Table 3). In DDD, the Y402H polymorphism (Abrera-Abeleda *et al.*, 2006; Abrera-Abeleda *et al.*, 2011; Servais *et al.*, 2012) associates with an increased risk of disease. Conversely, the V62I polymorphism was shown to be protective against DDD (Pickering *et al.*, 2007; Iatropoulos *et al.*, 2016).

Risk haplotypes in *CFH* (Pickering *et al.*, 2007) (Table 4) also associate with disease. The H1 haplotype that carries the at–risk Y402H SNP in DDD associates with an increased risk of DDD (Abrera-Abeleda *et al.*, 2006; Pickering *et al.*, 2007). The H2 haplotype that carries the protective SNP V62I was shown to be protective in DDD (Pickering *et al.*, 2007).

These two SNPs that associate with increased risk of disease have been functionally studied. The V62 variant is less effective in co-factor activity compared to I62 resulting in greater AP activity (Tortajada *et al.*, 2009; Pechtl *et al.*, 2011). The Y402H variant does not associate with any effect on AP activity (Francis *et al.*, 2012) but there is an effect on tissue recognition within Bruch's membrane (Clark *et al.*, 2010; Clark *et al.*, 2013).

SNP	Amino Acid Change	Db SNP identifier	MAF (%)
c.-331C>T	N/A	rs3753394	26.2[1]
c.184G>A	V62I	rs800292	22.1[2]
c.1204T>C	Y402H	rs1061170	38.2[2]
c. 2016A>G	Q672Q	rs3753396	17.3[2]
c.2808 G>T	E936D	rs1065489	17.5[2]

Table 3 Common SNPs defined in CFH haplotypes

Db SNP = Single Nucleotide Polymorphism Database, MAF = minor allele frequency, % = percentage values taken from [1] http://www.1000genomes.org/ for non-coding or [2]European-American population of evs.gs.washington.edu. for coding regions. N/A = not applicable.

	c.-331C>T	c.184G>A	c.1204C>T	c. 2016A>G	c.2808 G>T	Frequency (%)
HI	C	G	C	A	G	22.8
H2	C	A	T	A	G	26.6
H3	T	G	T	G	T	19.2
H4	C	G	T	A	G	16.7
H5	T	G	C	A	G	7.5
H6	C	G	T	G	T	3.9

Table 4 Common CFH haplotypes (H1 to H6)

Frequency of CFH haplotypes (%) percentage values taken from (Pickering et al., 2007)

1.3.2 RCA Gene Cluster

The *CFH* gene is within the regulators of complement activation (RCA) gene cluster on chromosome 1q32 (Rodriguez de Cordoba *et al.*, 2004) and encodes the 20 SCR FH protein and an alternatively spliced gene product FHL-1 (Figure 4). FHL-1 is identical to the first 7 SCRs of FH before the alternatively spliced transcript translates into a C-terminal 4 amino acid tail and the introduction of a stop codon, leading to the truncated protein (Figure 4).

A number of other genes within this cluster arose as a series of genetic duplications resulting in the formation of several genes that have a high sequence homology to *CFH*, known as *CFH*-related *(CFHR)* genes 1-*5* (Figure 5). They encode the FH-related (FHR) proteins 1-5. The characteristics of FHL-1 and the FHR proteins are summarised in Table 5. Estimations of serum concentration of the FHR proteins are consistently lower than those of FH (Timmann *et al.*, 1991; Heinen *et al.*, 2009; Skerka *et al.*, 2013).

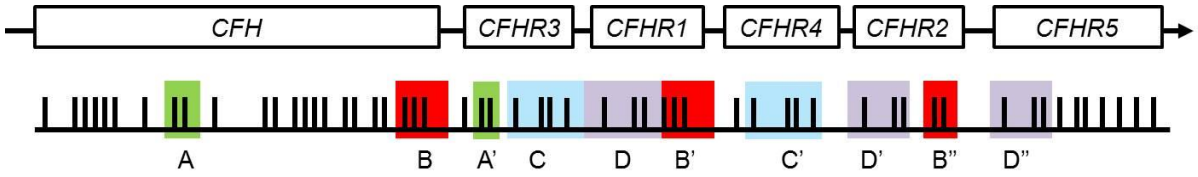

Figure 5 CFH and CFHR genes

Adapted from (Heinen et al., 2006). Coloured boxes represent regions of sequence duplication.

The N-terminal regulatory domains of FH are shared by FHL-1 but not the FHR proteins. The roles of FHL-1 and the FHR proteins are not completely known though a role for FHL-1 in maintaining health within Bruch membrane has been postulated (Clark *et al.*, 2014).

Although the FHR proteins do not contain the regulatory domains of FH, some studies report complement regulatory activity for FHR3 and FHR4 (Hellwage *et al.*, 1999) and FHR5 (McRae *et al.*, 2005). Other possible complement regulatory roles have been suggested via inhibition of C3 convertase by FHR2 (Eberhardt *et al.*, 2013) and FHR5 (McRae *et al.*, 2005) and C5 convertase by FHR1 (Heinen *et al.*, 2009).

	SCRs	Glycosylated forms	Size (kDa)	Suggested complement regulatory roles	Reference
FHR1	5	2	1β - 42 1α - 37	Regulates C5 convertase	(Heinen *et al.*, 2009)
FHR2	4	2	24 28	Regulates C3 convertase	(Eberhardt *et al.*, 2013)
FHR3	5	4	35-56	Weak co-factor activity	(Hellwage *et al.*, 1999)
FHR4	4A 9 4B 5	2	4A 86 4B 45	Weak co-factor activity Stabilises C3 convertase	(Hellwage *et al.*, 1999; Hebecker and Jozsi, 2012)
FHR5	9	1	62	Weak co-factor and decay activity Regulates C3 convertase	(McRae *et al.*, 2005)

Table 5 Characteristics of FHR proteins

Not shown in this table is that FHR 1,2 and 5 have been identified in serum in dimeric form. Studies of these proteins suggest a complement de-regulatory role (Goicoechea de Jorge et al., 2013).

More recent studies suggest that the FHR proteins may compete with FH for C3b binding and prevent the regulatory activities of FH on surface-bound C3b (Goicoechea de Jorge *et al.*, 2013). SCR1 and 2 of FHR1, 2 and 5 have a very high degree of sequence homology (Figure 6) and share a structure that allows dimerization. These studies show that these FHR proteins exist in dimeric form. The avidity of these dimeric forms of FHRs enables them to act as competitive antagonists, preventing the normal regulatory function of FH and therefore 'deregulating FH' (Goicoechea de Jorge *et al.*, 2013).

Due to the high degree of sequence homology, the *CFH-CFHR* gene region is prone to genomic abnormalities such as genetic rearrangements, insertions and deletions (Lupski and Stankiewicz, 2005). One frequently observed abnormality is the *CFHR3/1* gene deletion. The allele frequency of the *CFHR3/1* gene deletion ranges from 0% to 54.7% depending upon the geographical location of the cohort studied (Holmes *et al.*, 2013). The allele frequency in the United Kingdom of the *CFHR3/1* deletion has reported between 6.3% and 18.4% (Zipfel *et al.*, 2007; Holmes *et al.*, 2013). In the largest study of a UK cohort of 505 patients, this frequency was 17.3% (Moore *et al.*, 2010).

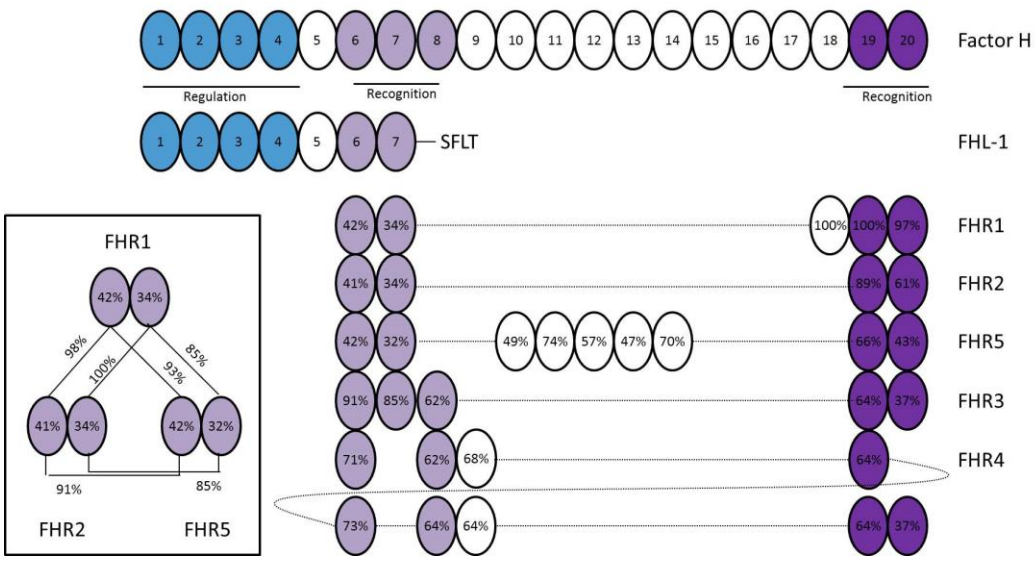

Figure 6 FH, FHL-1 and FHR proteins have a high degree of sequence homology

Coloured ovals denote regulatory and recognition domains of FH. Percentages shown within ovals indicate degree of homology shared with corresponding SCR of FH depicted directly above. FHR4 is a 9-SCR protein shown on two rows. In boxed inset, percentages along lines indicate degree of shared homology between SCR1 and 2 of FHR1, 2 and 5.

A number of genomic abnormalities in this region have been described in familial cases of C3G (Table 6). In all cases, the genomic abnormality resulted in the formation of a larger *CFHR* gene resulting in FHR proteins with additional SCRs. Functional study of dimeric

forms of abnormal FHR proteins demonstrated an enhanced ability to compete with FH resulting in enhanced deregulation of FH as a possible mechanism of disease. (Goicoechea de Jorge *et al.*, 2013; Tortajada *et al.*, 2013). In the case of the $FHR2_{12}FHR5$ hybrid protein, functional studies showed evidence of stabilisation of C3bBb in the presence of the hybrid $FHR2_{12}FHR5$ protein resulting in complement activation in the fluid phase (Chen *et al.*, 2014) and most recently a possible role in binding properdin (Chen *et al.*, 2015).

Rare genetic variants in *CFHR5* have also been reported in C3GN and include C269R (Besbas *et al.*, 2014) and N216P (Sethi *et al.*, 2012a). The functional significance of either variant has yet to be determined.

Abnormal FHR protein	Phenotype	Effect	Reference
$FHR2_{12}FHR5$	DDD	Stabilises C3bBb	(Chen *et al.*, 2014; Chen *et al.*, 2015)
$FHR5_{12}FHR5$	*CFHR5* nephropathy	De-regulates FH	(Gale *et al.*, 2010; Goicoechea de Jorge *et al.*, 2013)
$FHR5_{12}FHR12$	C3GN	Not known	(Zhang *et al.*, 2013b)
$FHR1_{1234}FHR1$	Low C3	De-regulates FH	(Tortajada *et al.*, 2013)
$FHR3_{12}FHR1$	C3GN	De-regulates FH	(Malik *et al.*, 2012; Goicoechea de Jorge *et al.*, 2013)

Table 6 Abnormal FHR proteins described in C3G

1.3.3 Complement Factor I

Complement Factor I (FI) is a glycoprotein, 88kDa in size. It is a serine protease that cleaves the α-chain of C3b and C4b. Cleavage of the α-chain of C3b irreversibly inactivates C3b and prevents its entry into the amplification loop of the AP, and requires co-factors that include FH (Pangburn *et al.*, 1977) and CD46 (Liszewski *et al.*, 1991). It is composed of a heavy

chain and a light chain – the active catalytic site is contained within the light chain (Roversi *et al.*, 2011).

In studies of MPGN/C3G, a total of 10 rare genetic variants in *CFI* have been reported in four cohorts of MPGN/C3G (Table 7) (Servais *et al.*, 2012; Sethi *et al.*, 2012a; Bu *et al.*, 2015; Iatropoulos *et al.*, 2016). Not all variants lead to impairment of FI levels or function. The significance of some variants is unknown.

Variant	Disease	Effect	Reference
c.1-4C>T	C3GN	Unknown	(Iatropoulos *et al.*, 2016)
G57D	DDD	Unknown	(Iatropoulos *et al.*, 2016)
G119R	MPGN1	Low FI levels	(Bienaime *et al.*, 2010; Servais *et al.*, 2012; Kavanagh *et al.*, 2015)
A240G	C3GN	Impaired Secretion	(Nilsson *et al.*, 2010; Servais *et al.*, 2012)
G261D	C3GN	Normal function	(Nilsson *et al.*, 2007; Servais *et al.*, 2012; Sethi *et al.*, 2012a)
I306S	C3GN	Normal function	(Servais *et al.*, 2012)
C309R	MPGN1	Low FI levels	(Servais *et al.*, 2012)
C327R	MPGN1	Low FI levels	(Servais *et al.*, 2012)
Not stated*	C3GN DDD	Unknown	(Bu *et al.*, 2015)

Table 7 Rare CFI variants in MPGN and C3G identified in literature

*MPGN1 = MPGN type 1, * two variants not stated in paper.*

1.3.4 Membrane Co-factor Protein - CD46

Membrane-cofactor protein (CD46) is a cell surface bound regulator of complement and comprises a transmembrane domain and 4 extracellular SCRs. Like FH, it acts as a co-factor for the FI-mediated proteolytic cleavage of C3b but it does not have any decay activity (Liszewski *et al.*, 1991).

Rare genetic variants in *CD46* have rarely been reported in MPGN/C3G and include the variants K66N (Iatropoulos *et al.*, 2016) and V181M (Servais *et al.*, 2012).

Common SNPs (Table 8) and haplotypes (Table 9) in *CD46* have been studied in cohorts of MPGN/C3G. The intronic SNP c.-652G was protective in MPGN and C3GN. This association was also observed in cases of MPGN type 1 and C3GN, in which the haplotype *CD46*$_{AAGGT}$ that was observed more frequently, whist the haplotype *CD46*$_{GAGGT}$ was observed less frequently (Servais *et al.*, 2012). These findings were not observed in a later study (Iatropoulos *et al.*, 2016). In this later study, the SNPs c.-366A>G and c. *4070T>C were observed more frequently in IC-MPGN than controls (Iatropoulos *et al.*, 2016). In the same later study, the SNP c.-366A was observed more frequently in DDD compared to controls (Iatropoulos *et al.*, 2016).

SNP	Db SNP identifier	MAF (%)
c.-652A>G	rs2796267	39.1
c.-366A>G	rs2796268	36.4
IVS9 -78G	rs1962149	29.3
IVS12 +638G	rs859705	31.7
c.*4070T>C	rs7144	34.7

Table 8 SNPs that define common CD46 haplotypes

Db SNP = Single Nucleotide Polymorphism Database, MAF – minor allele frequency % percentage values taken from http://www.1000genomes.org/.

	-652G>A	-366A>G	IVS9 -78G	IVS12 +638G	*4070T>C	Frequency
CD46 1	A	A	G	G	T	0.5
CD46 2	G	G	A	A	C	0.25
CD46 3	G	A	G	G	T	0.12
CD46 4	A	G	A	A	C	0.09

Table 9 Common CD46 haplotypes

Frequency of CD46 haplotypes taken from (Servais et al., 2012.)

1.3.5 Components of the AP C3 Convertase - C3 and FB

Complement component C3 is a 186kDa protein that comprises an α- and β- subunit. It is cleaved by C3 convertases, releasing a 9kDa anaphylatoxin, C3a and undergoes conformational change to C3b thus exposing the thioester domain and allowing binding to cell surfaces (Janssen *et al.*, 2006). Complement factor B (FB) is a 90kDa protein that comprises 3 SCR domains, a Von Willebrand type A domain (VWA) and a serine protease domain (Milder *et al.*, 2007). FB binds to C3b to form C3bB. A linker region between the SCR domains and the VWA domain is then cleaved by FD, releasing Ba and resulting in the formation of C3bBb, the key molecule in the amplification loop of AP (Forneris *et al.*, 2010). Key regulators of this interaction include FH, FI and CD46. FH accelerates the natural decay of C3bBb. FH and CD46 act as co-factors for the FI-mediated proteolytic cleavage of the α-chain of C3b.

Detailed functional studies of several familial rare genetic variants in *C3* in MPGN/C3G have been described. In a case of familial DDD, the variant Δ923-924 does not undergo conformational change to C3b but does form a C3 convertase that is resistant to decay by FH (Martinez-Barricarte *et al.*, 2010). In a report of familial C3GN, the variant I756T results in defective C3b inactivation by FI in setting of co-factors CR1 and FH (Chauvet *et al.*, 2015).

Rare genetic variants have also been described in cohorts of MPGN/C3G in *C3* (Table 10) and *CFB* (Table 11), and includes the S367R variant in *CFB* in 1 familial case of C3GN (Imamura *et al.*, 2015). Most of these variants have not been functionally studied.

Variant						Reference
R148Q	R161W[1]	A443S	L1100P	L1318R		(Bu *et al.*, 2015)
V86I	R505C	V619M	G637R	R1042Q	K1051M[2]	(Iatropoulos *et al.*, 2016)
S1063N	R1303H	R1320Q	D1362N	C1518R	D1625H	

Table 10 Rare C3 variants in MPGN and C3G identified in literature

Most rare variants in C3 in MPGN/C3G have not been functionally studied. [1] functionally significant, [2] not-functionally significant (Schramm et al., 2015).

Several common SNPs in *C3* and *CFB* have been studied in MPGN/C3G (Table 12). The SNPs, R102G (Finn and Mathieson, 1993; Abrera-Abeleda *et al.*, 2011) and P314L in *C3* are associated with DDD (Abrera-Abeleda *et al.*, 2011). Functional studies of the SNPs in *CFB*, R32W and R32Q have been described (Marinozzi *et al.*, 2014) but no significant association with MPGN/C3G was identified (Iatropoulos *et al.*, 2016).

1.3.6 Complotypes

Increased AP activity has been demonstrated in functional studies for the SNPs R102G in *C3* and R32Q in *CFB* and I62V in *CFH* (Heurich *et al.*, 2011). The effect of inheriting a combination of SNPs that conferred greater AP activity was studied. C3, FB and FH were depleted from serum and replaced with purified C3, FB and FH of specific polymorphic variants in a series of sheep erythrocyte haemolytic assays. It was found that using purified C3, FB and FH of the most active variants C3 102G, FB 32R and FH 62V, the haemolytic activity was 6x greater than if the less active variants C3 102R, FB 32Q and FH 62I were used (Heurich *et al.*, 2011). Therefore inheriting certain polymorphisms or a complotype can lead to a more active complement system and may explain the association of some SNPs in MPGN/C3GN (Harris *et al.*, 2012).

Variant	Reference
I242L[1]	(Bu *et al.*, 2015)
D279E[2]	(Bu *et al.*, 2015)
S367R[3]	(Imamura *et al.*, 2015)
G161R	(Iatropoulos *et al.*, 2016)
H451R	(Iatropoulos *et al.*, 2016)
R679W	(Iatropoulos *et al.*, 2016)

Table 11 Rare CFB variants in MPGN and C3G identified in literature

[1]had enhanced formation of C3 convertase but no evidence of enhanced haemolytic activity (Marinozzi et al., 2014), [2]the D279G mutation was gain of function (Hourcade et al., 1999) (Marinozzi et al., 2014), [3]familial.

Gene	SNP	Amino acid change	Db SNP ID	MAF (%)
C3	c.304C>G	R102G	rs2230199	20.9
C3	c.941C>T	P314L	rs1047286	20.5
CFB	c.94C>T	R32W	rs641153	9.8
CFB	c.95G>A	R32Q	rs12614	8.7

Table 12 Common polymorphisms in C3 and CFB associated with disease

MAF = minor allele frequency, % = percentage values taken from http://evs.gs.washington.edu/

1.3.7 C3 Nephritic Factor

The earliest evidence of a C3 nephritic factor resulting in dysregulation of complement in MPGN was described in 1969 (Spitzer *et al.*, 1969). A circulating factor in the serum was found to increase cell lysis in the fluid phase. This was later discovered to be IgG that stabilised C3bBb by 10-fold (Daha *et al.*, 1976; Daha *et al.*, 1977) now known as C3 nephritic factor. There is a strong association of C3 nephritic factor with DDD, MPGN and C3GN and is prevalent in up to 80% of DDD and 50% of MPGN and C3G (Servais *et al.*, 2012; Zhang *et al.*, 2012). There is also a genetic association of C3 nephritic factor with the *C3* polymorphism, R102G (Finn and Mathieson, 1993). C3 nephritic factors are not specific to MPGN and C3G and have been observed in APL (Sissons *et al.*, 1976) and in normal individuals (Gewurz *et al.*, 1983).

1.3.8 Autoantibodies to C3b and FB

Autoantibodies to FB have been described in DDD. In one patient, these were shown to stabilise C3bBb, causing C3 consumption and terminal pathway activation (Strobel *et al.*, 2010). No functional data are available in a further report in which three DDD patients with autoantibodies to FB are described (Zhang *et al.*, 2012). Autoantibodies to C3b and FB in the same patient were described in two cases of DDD. These patient lacked C3 nephritic factor but the antibodies enhanced C3bBb activity (Chen *et al.*, 2011).

1.3.9 Autoantibodies to FH

Autoantibodies to FH have also been described in patients with MPGN/C3G. These bind predominantly to the N-terminal domain of FH (Goodship *et al.*, 2012; Zhang *et al.*, 2012). The prevalence of autoantibodies to FH was 11% in a MPGN/C3G cohort (Blanc *et al.*, 2015). In this cohort, patients with autoantibodies to FH did not associate with homozygous deletion of *CFHR3/1*. These autoantibodies associated with C3 nephritic factor in children and monoclonal gammopathy in adults (Blanc *et al.*, 2015). The association of autoantibodies to FH and monoclonal gammopathy had been previously described in case reports (Meri *et al.*, 1992; Zand *et al.*, 2013).

1.4. Other Diseases Associated with MPGN/C3G

1.4.1 Atypical Haemolytic Uraemic Syndrome

Haemolytic uraemia syndrome (HUS) comprises the clinical trial of microangiopathic haemolytic anaemia, thrombocytopaenia and acute kidney failure (Kavanagh and Goodship, 2011). Most cases are due to Shiga-like toxin. The remainder of cases of HUS are termed atypical. The incidence of atypical HUS (aHUS) in the United Kingdom is estimated to be 0.2/million population (Sheerin *et al.*, 2015).

Over 50% of cases of aHUS are caused by abnormalities within the AP (Kavanagh *et al.*, 2013). Morbidity and mortality rates are high with 36% to 48% of children and 64% to 67% of adults dying or reaching ESRD within 3-5 years (Noris *et al.*, 2010; Fremeaux-Bacchi *et al.*, 2013; Kavanagh *et al.*, 2013). The adverse clinical course of patients with aHUS can be stratified from extensive cohort studies (Table 13). Prognosis is best in patients with rare genetic variants in *CD46*. Risk of recurrence in renal transplantation is also high except in those with a rare genetic variant in *CD46* and low anti-FH titres (Le Quintrec *et al.*, 2013). Penetrance of disease is only 50% for *CFH, CFI CD46* and *CFB* and slightly lower for *C3* (Bresin *et al.*, 2013). Other factors required for the development of aHUS include inheritance of a risk haplotype (Esparza-Gordillo *et al.*, 2005; Fremeaux-Bacchi *et al.*, 2005) and a trigger. The more common triggers include diarrhoea (Sellier-Leclerc *et al.*, 2007) and pregnancy (Fakhouri *et al.*, 2010b).

1.4.2 Genetic Abnormalities of the Complement System in aHUS

Rare genetic variants in *CFH* are found in a quarter of all cases of aHUS (Noris *et al.*, 2010; Fremeaux-Bacchi *et al.*, 2013). The majority of disease-associated variants are localised to the C-terminal domain of *CFH* (Noris and Remuzzi, 2009). The consequence of many of these variants is impaired cell surface recognition and has been confirmed for a large number of variants in functional studies (Sanchez-Corral *et al.*, 2002; Jokiranta *et al.*, 2005; Ferreira *et al.*, 2009) that includes the C-terminal R1210C variant mentioned in section 1.3.1 (Sanchez-Corral *et al.*, 2002; Recalde *et al.*, 2015). Variants that lead to FH deficiency also associate with aHUS (Dragon-Durey *et al.*, 2004).

Gene or subgroup	Frequency in aHUS	Risk of death or ESRD at 1st episode or within 1 year	Risk of recurrence after renal transplant
CFH	20-30%	50-70%	75-90%
CFI	4-10%	50%	45-80%
CD46	5-15%	0-6%	<20%
C3	2-10%	60%	40-70%
CFB	1-4%	50%	100%
Autoantibodies to FH	6%	30-40%	Yes, if high Ab titre

Table 13 Complement abnormalities in aHUS - Prevalence and prognosis

Adapted from (Loirat and Fremeaux-Bacchi, 2011). Ab = antibody.

Functional studies of aHUS-associated variants outside of the C-terminal domain of *CFH* are limited. Several functionally significant N-terminal variants in *CFH* have been described (Pechtl *et al.*, 2011). The importance of functional studies in *CFH* was confirmed in the study of the polymorphic variants I890/L1007. No effect on FH regulatory function could be demonstrated in co-factor assays in the fluid phase or in haemolytic assays on the cell surface (Tortajada *et al.*, 2012). The remaining aHUS-associated rare variants without functional studies should be termed variants of uncertain significance (VUS) (Kavanagh and Anderson, 2012).

A number of genetic deletions within the *CFH-CFHR* gene cluster associate with aHUS. The *CFHR3/1* deletion in homozygosity associates strongly with the presence of autoantibodies against FH in aHUS cohorts (Moore *et al.*, 2010).

Abnormal FH/FHR hybrid (Venables *et al.*, 2006; Maga *et al.*, 2011; Francis *et al.*, 2012; Challis *et al.*, 2015) or FHR1/FH 'reverse' hybrid proteins (Eyler *et al.*, 2013; Valoti *et al.*, 2015) have been described resulting from genetic deletions from within the *CFH-CFHR* gene cluster. These are shown in Figure 7. Functional studies have confirmed the significance of several of these hybrid proteins (Francis *et al.*, 2012; Challis *et al.*, 2015; Valoti *et al.*, 2015).

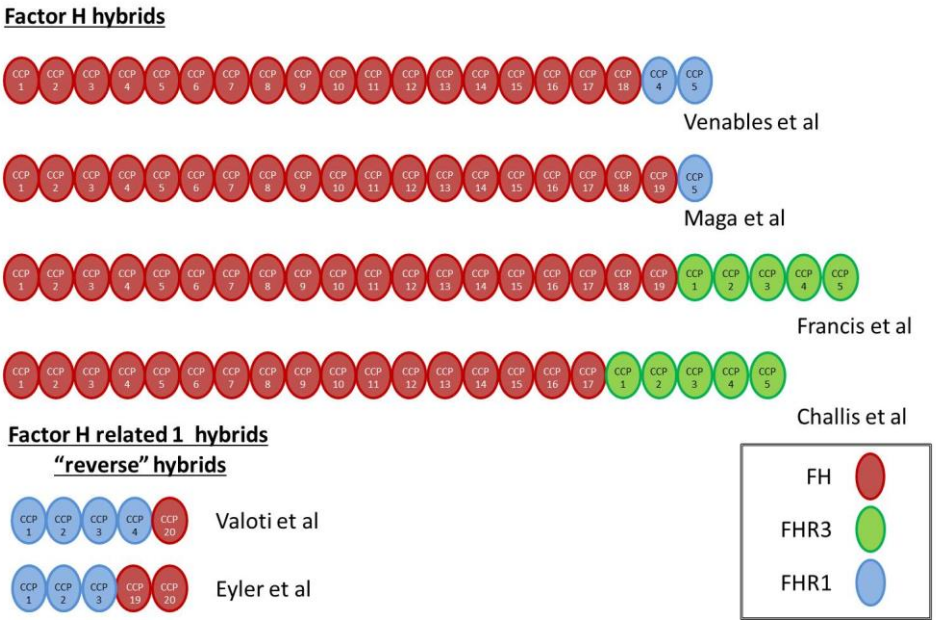

Figure 7 FH hybrid proteins and FHR1 'reverse' hybrids in aHUS

Adapted from Challis et al. Coloured ovals represent complement control protein (=CCP, synonymous with short consensus repeat and SCR) .from either FH, FHR3 or FHR1.

Rare genetic variants in *CFI* are found in 4-10% of cases of aHUS (Fremeaux-Bacchi *et al.*, 2004; Kavanagh *et al.*, 2005). These are all heterozygous and cluster in the light chain. Most result in a secreted protein. Functional studies of a number of these variants have been shown to result in loss of complement regulation of AP and CP (Kavanagh *et al.*, 2008).

Rare genetic variants in *CD46* are found in 5-15% of cases of aHUS. Variants mostly result in a failure to express CD46 on the surface of peripheral blood mononuclear cells (Richards *et al.*, 2007). Functional studies have confirmed loss of function of several secreted variants (Richards *et al.*, 2003; Caprioli *et al.*, 2006).

Rare variants in *C3* and *CFB* are uncommon in aHUS cohorts, and are found in only 2-10% and 1-4% of patients respectively. Nonetheless, detailed functional studies of rare genetic variants in *C3* and *CFB* have been described in several series. These studies have identified a number of different molecular mechanisms (Table 14) that ultimately result in over activation of AP.

Most rare genetic variants in *C3* lead to gain-of-function due to loss of binding to FH, CD46, or occasionally CR1 and subsequent loss of co-factor activity and impairment of inactivation of C3b by FI (Fremeaux-Bacchi *et al.*, 2008; Martinez-Barricarte *et al.*, 2015; Schramm *et al.*, 2015). In a familial case of aHUS, gain-of-function of a variant in *C3* was due to

enhanced FB binding and convertase formation has been previously described (Lhotta *et al.*, 2009).

Rare genetic variants in *CFB* lead to gain of function by enhanced convertase formation and resistance to convertase decay by complement regulators (Goicoechea de Jorge *et al.*, 2007; Marinozzi *et al.*, 2014).

Two variants (Y832X and C1136W) led to impaired secretion of C3 and haploinsufficiency (Fremeaux-Bacchi *et al.*, 2008). Their role in disease has not yet been elucidated.

C3 variant	*CFB* variant
Reduced co-factor binding by FH, CD46 or CR1 Reduced co-factor activity Enhanced FB binding and convertase formation	Enhanced C3bB formation C3bBb resistant to decay acceleration

Table 14 Mechanisms of pathogenicity of C3 and CFB variants in aHUS

1.4.3 Autoantibodies to Complement Proteins in aHUS

Autoantibodies to FH have been detected in approximately 10% of patients with aHUS (Dragon-Durey *et al.*, 2005; Loirat and Fremeaux-Bacchi, 2011). These autoantibodies bind predominantly to the C-terminal domain of FH, corresponding to the location of the majority of rare variants in *CFH* in aHUS (Jozsi *et al.*, 2007). However, autoantibodies to FH identified in a study of nineteen patients by Blanc et al did not bind exclusively to the C-terminal domain of FH suggesting that their pathological role may extend beyond the impairment of cell-surface recognition (Blanc *et al.*, 2012). The development of autoantibodies to FH in aHUS appears to be associated with homozygous deletion of *CFHR3/CFHR1* (Jozsi *et al.*, 2008; Moore *et al.*, 2010).

Autoantibodies to FI were described in one series of 3 patients out of 175 patients with aHUS (Kavanagh *et al.*, 2012). The autoantibodies were bound to circulating FI to form immune complexes but there was only a modest effect on complement regulation in functional studies. Autoantibodies to FI were found in a similar proportion of patients in a more recent study (Jozsi *et al.*, 2014). A pathogenic role for these autoantibodies also remains unclear. In this study, one patient with an autoantibody to C3b was also described (Jozsi *et al.*, 2014). Again,

a direct pathogenic role was uncertain. Recently, screening of a cohort of 89 patients for autoantibodies to CD46, CD35, CD55 and CD59 was performed (Watson *et al.*, 2015). No autoantibodies were identified to these surface-bound regulators.

1.4.4 Acquired Partial Lipodystrophy

Acquired partial lipodystrophy (APL) is the loss of subcutaneous tissue and is usually limited to the upper half of the body (Eisinger *et al.*, 1972; Misra *et al.*, 2004). APL has been described in association with DDD (Duvall-Young *et al.*, 1989; Finn and Mathieson, 1993). A shared risk factor between DDD and APL is C3 nephritic factor (Sissons *et al.*, 1976; Finn and Mathieson, 1993; Misra *et al.*, 2004). In one study, 22% of cases of APL developed MPGN after a median of 8 years (Misra *et al.*, 2004). A role of C3 nephritic factor in adipolysis and fat cell loss has been previously described (Mathieson *et al.*, 1993; Williams, 1997). The distribution of fat loss is thought to be dependent upon FD, expressed in adipocytes in a distribution that mimics the pattern of fat loss observed in APL (Mathieson *et al.*, 1994).

1.4.5 Drusen and Age-related Macular Degeneration

Drusen are a hallmark of early age-related macular degeneration (AMD), an important cause of blindness worldwide (Hageman *et al.*, 1999). Both drusen (Duvall-Young *et al.*, 1989) and AMD (Montes *et al.*, 2008; Savige *et al.*, 2016) have been described in patients with DDD. Drusen found in patients with glomerulonephritis are abundant in complement proteins and were of similar composition to those found in AMD (Mullins *et al.*, 2001). Furthermore, AMD and DDD shared the common polymorphism Y402H in *CFH* as a genetic risk factor, first described in AMD in 2005 (Edwards *et al.*, 2005; Haines *et al.*, 2005). Since then additional common polymorphisms and rare variants in several genes encoding proteins within the AP have now been described in AMD. These genes include *C3* (Yates *et al.*, 2007; Seddon *et al.*, 2013), *CFB* (Gold *et al.*, 2006), *CFH* (Boon *et al.*, 2008; Montes *et al.*, 2008; Raychaudhuri *et al.*, 2011; Duvvari *et al.*, 2015; Triebwasser *et al.*, 2015) and *CFI* (Kavanagh *et al.*, 2015). That complement genetic risk factors are common to AMD and MPGN/C3G is fascinating and may benefit our understanding of the complement system and these diseases. One notable example of such a risk factor is the rare genetic variants R1210C in *CFH* that is found in AMD, MPGN/C3G and aHUS (Recalde *et al.*, 2015).

1.5 Treatments of MPGN/C3G and aHUS

There are no universally effect treatments for MPGN/C3G. The only double blind randomised control trial in this group of patients was performed in 1992 (Tarshish *et al.*, 1992). Eighty children (with MPGN type 1, 2 and 3) were randomised to receive 40mg/m^2 of prednisolone on alternate days. In this study, long-term treatment with prednisolone did appear to improve the outcome of patients with MPGN. Other studies suggest some benefit from the use of cyclophosphamide, mycophenolate mofetil (MMF) and the combination of aspirin and dipyridamole (Table 15). Other treatments used in MPGN or C3G have been described in case reports and include the use of rituximab (Marques *et al.*, 2014; Farooqui *et al.*, 2015; Giaime *et al.*, 2015), soluble CR1 (Zhang *et al.*, 2013a) and plasma exchange (PEX) (Habbig *et al.*, 2009).

PEX had been considered the 'gold-standard' in the management of aHUS (Ariceta *et al.*, 2009; Taylor *et al.*, 2010). The rationale for use included the replacement of defective complement regulators and hyper-functional complement components. It remains an important treatment in the initial management of aHUS (Scully and Goodship, 2014) and also in the ongoing management of aHUS in countries where Eculizumab is not available.

1.5.1 Eculizumab

Eculizumab is a recombinant, humanised, monoclonal antibody directed against C5 (Rother *et al.*, 2007) (Figure 1). It binds C5 thus preventing its cleavage by C3bBbC3b into C5a and C5b, therefore inhibiting the terminal pathway.

Eculizumab has replaced PEX as the gold-standard in the management of aHUS in adults (Legendre *et al.*, 2013; Licht *et al.*, 2015) and children (Greenbaum *et al.*, 2016). Eculizumab is given in the treatment of aHUS in the native kidney and also to prevent recurrence of aHUS following renal transplantation (Wong *et al.*, 2013). Current controversies in the use of Eculizumab include the optimal duration of therapy (Ardissino *et al.*, 2015).

The benefit of Eculizumab in MPGN/C3G is less clear. There is a growing list of case reports describing the possible benefit of Eculizumab in MPGN/C3G but these reports are subject to publication bias. One small study by Bomback et al of 6 patients reported variable outcomes in a heterogeneous cohort of C3GN and DDD in both native and transplanted kidneys (Bomback *et al.*, 2012). Future studies of a well-characterised cohort of MPGN/C3G are required, such as the Eculizumab in Primary MPGN (EAGLE) trial currently underway (*Eculizumab in Primary MPGN - Full Text View - ClinicalTrials.gov*, 2016).

Treatment	Study Type	Outcome	Reference
Prednisolone	Double-blind randomised control trial in children with MPGN (42, type 1, 14, type 2, 17, type 3 and 7, non-typable disease)	The rate of treatment failure was lower in patients receiving steroids (40%) compared to controls (55%). After a median of 13 years, a higher proportion of patients had stable renal function in the group receiving steroids (61%) compared to controls (12%).	(Tarshish *et al.*, 1992)
Cyclophosphamide	Single arm study, 19 patients (concurrent prednisolone use)	Renal failure and progression in 1 patient only	(Faedda *et al.*, 1994)
MMF	5 patients receiving MMF and Prednisolone vs 6 controls (no treatment)	Improvement in proteinuria in treatment group	(Jones *et al.*, 2004)
	Retrospective cohort of C3GN 22 patients receiving MMF and Prednisolone	Renal function and renal survival better in patients receiving MMF (and prednisolone) compared to those receiving other regimens	(Rabasco *et al.*, 2015)
Aspirin and dipyridamole	18 patients with MPGN and nephrotic syndrome randomised to treatment (10 patients) vs control (8 patients)	Improvement in proteinuria in treatment group	(Zauner *et al.*, 1994)
	40 patients with MPGN type 1, double-blind randomised control trial (21 treatment, 19 control)	Less progression to ESRD in treatment group in initial study but re-analysis showed no benefit.	(Donadio *et al.*, 1984; Donadio and Offord, 1989)

Table 15 Studies of treatments in MPGN/C3G

1.6 Summary

Acquired and genetic abnormalities of the AP have been described in several renal phenotypes. The earliest associations were in MPGN. More recently, the term C3G has been introduced. These terms describe a cohort of patients in whom abnormalities of the AP may be found. Patients with MPGN/C3G often have low C3 levels. The most prevalent abnormality in these patients is the C3 nephritic factor; an autoantibody that stabilises C3bBb resulting in over activity of AP in the fluid phase. Additional abnormalities, including autoantibodies to FH and rare genetic variants in genes encoding proteins within the AP have also been identified. Functional studies of these abnormalities also demonstrate over activity of AP.

Previous detailed studies of AP abnormalities in aHUS led to clinical trials and the successful use of Eculizumab in aHUS. Further studies in cohorts of MPGN/C3G are required to clarify the role of these abnormalities, in terms of pathogenicity and their influence on the clinical course of disease.

1.7 Aims of Thesis

I have several main aims in this thesis.

1. I will identify and determine the prevalence of acquired and genetic abnormalities of AP in two cohorts of MPGN/C3G
2. I will study a rare genetic variant in the regulatory domain of FH to solve a long-standing conundrum and explain a case of familial MPGN
3. I will determine the range of functional effects of rare genetic variants in the regulatory domain of *CFH* associated with MPGN/C3G and other diseases
4. I will undertake functional studies using full length FH to confirm the effects of previous functional tests performed in the regulatory domain of FH.

Chapter 2 – Methods

2.1 Screening for Complement Abnormalities in MPGN and C3G Cohorts

The study was approved by Newcastle and North Tyneside 1 Research Ethics Committee, and informed consent was obtained in accordance with the Declaration of Helsinki.

2.1.1 Cohort Selection

Two cohorts for study of patients with MPGN/C3G were identified. Patients from paediatric renal units based in 10 UK hospitals were recruited for study in a prospective paediatric cohort (Table 16). Patients presented with haematuria, proteinuria and/or abnormal renal function and were investigated in their local paediatric renal unit. A diagnosis of MPGN, DDD or C3GN was made by the local pathology service following renal biopsy. All paediatric renal units were invited by the study investigators Professor Tim Goodship and Dr. Sally Johnson since 2011 to recruit patients with a diagnosis of MPGN, DDD or C3GN into the registry for rare renal disease (RaDaR) and the national study of MPGN. Patients consented to clinical data collection from the registry, and collection of pre-existing renal biopsy material and whole blood for DNA and serum storage (Figure 8). Following recruitment, I had access to the list of patients and oversaw the acquisition of data and subsequent analysis from clinical data registry, central pathology review, autoantibody screening and genetic screening. I will refer to this cohort as the RaDaR (prospective) cohort.

Alder Hey Children's Hospital	Leeds General Infirmary
Birmingham Children's Hospital	Queen's Medical Centre
Bristol Royal Hospital for Children	Royal Hospital for Sick Children
Evelina Children's Hospital	Royal Victoria Infirmary
Great Ormond Street Hospital	University Hospital of Wales

Table 16 Paediatric renal units recruiting to RaDaR

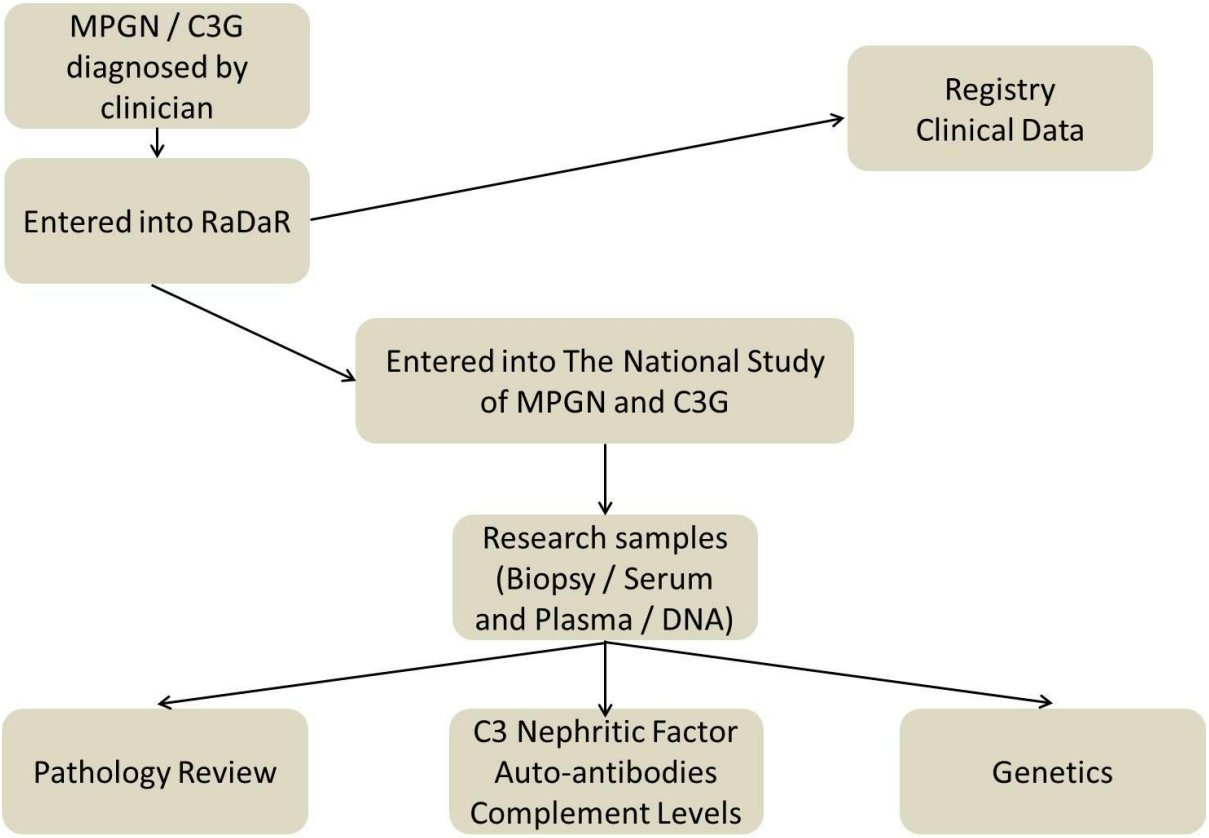

Figure 8 Flowchart of patient recruitment and sample acquisition for RaDaR

Patients were referred to Professor Tim Goodship or Dr. David Kavanagh at the Newcastle specialist centre for complement-mediated renal disease with a diagnosis of MPGN, DDD or C3GN by clinicians requesting specialist testing for complement abnormalities. Data was entered onto a central database of patients with complement–mediated renal diseases and was collected by me for analysis of complement abnormalities in a retrospective adult and paediatric cohort. I will refer to this as the Newcastle (retrospective) cohort.

2.1.2 Central Pathology Review

A central pathology review was conducted for patients in the prospective cohort study. Original slides or digital images were reviewed by Professor Terry Cook, an expert in the pathological diagnosis of MPGN/C3G. Immunofluorescence images and/or reports, and electron microscopy images and/or reports were also acquired for review. Following review a diagnosis given based upon the C3G consensus report (Pickering *et al.*, 2013).

2.1.3 Clinical Data

Clinical data from patients in the RaDaR cohort was entered by research nurses at local centres into the RaDaR database. The database was exported into an Excel spreadsheet by Dr. Fiona Braddon for analysis by me. Clinical data from patients in the Newcastle cohort was sent by the referring clinician at time of referral. Additional data was available if requested by Prof Tim Goodship or Dr. David Kavanagh. The data in the Newcastle MPGN database was analysed by me.

2.1.4 Complement Protein Analysis

Serum samples from patients in the RaDaR cohort study were tested for C3, C4, FH and FI in the laboratory at Newcastle Hospitals NHS Foundation trust. Serum samples from patients in the retrospective cohort study were also tested for C3, C4, FH and FI in the laboratory at Newcastle Hospitals NHS Foundation trust. Complement levels were analysed by me.

2.1.5 Autoantibodies to Complement Components

Serum samples from patients in the RaDaR cohort were screened for autoantibodies to complement proteins. Dr. Kevin Marchbank undertook screening for autoantibodies using an enzyme-linked immunosorbent assay (ELISA) to FH, FI, CD46, C3b, FB, CD35, CD55, CD59, FHR1, FHR2, FHR3, FHR4 and FHR5. Further analysis was performed for patients that were positive for autoantibodies to FH. Characterisation of epitope binding to fragments of FH was also performed in patients who had an autoantibody to FH. Descriptions of ELISA testing are in the references indicated in Table 17. Minor modifications were made to the protocol for screening for autoantibodies to FH for the study of proteins not referenced. C3b and FB were obtained from CompTech and were also coated onto 96-well plates at 5μg/ml. FHR2, 3 and 4 were expressed in mammalian cell lines and coated onto 96-well plates at 5μg/ml. FHR1 and FHR5 were expressed in mammalian cell lines and coated onto 96-well plates at 1μg/ml. FH SCR1-7 was expressed in mammalian cell lines and coated onto 96-well plates at molar equivalence to FH.

Serum samples from patients in the retrospective cohort were screened for autoantibodies to FH only. In a case of familial MPGN, detailed characterisation of autoantibodies against FH was also performed. Specificity of the autoantibodies to FH was tested using western blotting

(Goodship *et al.*, 2012; Wong *et al.*, 2014). Characterisation of epitope binding to fragments of FH was also performed (Wong *et al.*, 2014).

I undertook some of the screening for autoantibodies to FHR3 and FHR4. Flexible 96-well plates were filled with 50µL of coating buffer, pH 7.6 (AbD Serotec) containing 5µg/ml of purified FHR3 or FHR4 proteins (generated by Dr. Kevin Marchbank in mammalian cell culture) and incubated for 16 hours at 4°C. Plates were washed three times with 200µL of PBS containing 0.01% Tween 20 (Sigma) followed by blocking with 200µL ultrablock (AbD Serotec) for 45 minutes at 25°C. A duplicate plate was set up with block solution as a background binding control. After blocking, 50µL of 2% serum in PBS/0.01% Tween 20 was loaded in triplicate onto both plates and incubated for 1-2 hours. Plates were washed three times with 200µL of PBS containing 0.01% Tween 20 followed by blocking with 200µL ultrablock for 45 minutes at 25°C. 50µL of goat anti-human IgG HRP (Stratech Scientific UK) at 1:4000 dilution with PBS containing 0.01% Tween 20 was added to each well and incubated for 1 hour at 25°C. Plates were washed three times with 200µL of PBS/0.01%Tween 20. 50µL of tetramethyl benzidine standard kinetic solution (AbD serotec) was then added to each well for 7 minutes prior to the reaction being stopped using 10% sulphuric acid. Absorbance at 450nm (A_{450}) was measured using a SpectraMax 190 plate reader (MDS Analytical Technologies Limited). Triplicate data were analysed and mean ultrablock readings were subtracted from mean FHR readings to control for non-specific/false positive readings. Rabbit polysera (1:100000) for each FHR protein and goat anti-rabbit-HRP (1:10000) was used as a loading control. No positive control from a patient was available for use.

Protein	Reference
FH[1, 2, 3]	(Goodship *et al.*, 2012)
FH SCR1-4[1,, 3]	(Pechtl *et al.*, 2011)
FH SCR6-8[1, 3]	(Schmidt *et al.*, 2008b)
FH SCR8-15[1, 3]	(Herbert *et al.*, 2012)
FH SCR16-18[1, 3]	(Morgan *et al.*, 2011)
FH SCR19-20[1, 3]	(Ferreira *et al.*, 2009)
FI[1]	(Kavanagh *et al.*, 2012)
CD46[1]	(Watson *et al.*, 2015)
CD35[1]	(Watson *et al.*, 2015)
CD55[1]	(Watson *et al.*, 2015)
CD59[1]	(Watson *et al.*, 2015)

Table 17 Screening for autoantibodies to complement proteins

Screening for autoantibodies to different proteins was performed in the [1]RaDaR, [2]Newcastle cohort and [3]familial MPGN. Screening for autoantibodies to C3b, FB, FHR1-5 and FH SCR1-7 was performed for patients in the RaDaR cohort, and with small modifications to the FH protocol.

2.1.6 Genetic Studies in RaDaR and Newcastle Cohort

Screening for rare genetic variants in *C3*, *CFB*, *CFH*, *CFI* and *CD46* was performed for both the RaDaR and Newcastle cohorts using sequencing offered by the Northern Molecular Genetics Service in Newcastle upon Tyne during the course of the study. The techniques included Sanger sequencing and Next Generation Sequencing. In all cases, all coding exons of *C3*, *CFB*, *CFH*, *CFI* and *CD46* were sequenced. Exons that failed to sequence and all rare variants that were identified using Next Generation Sequencing were sequenced again using Sanger sequencing. Sanger sequencing of intronic sequences was also performed to identify common polymorphisms associated with risk haplotypes for patients in the RaDaR cohort.

Multiplex-ligation dependent probe amplification (MLPA) was used to screen for copy number variation (CNV). This was undertaken by the Northern Molecular Genetics Service. Screening for CNV in *CFH*, *CFHR3*, *CFHR1*, *CFHR2* and *CFHR5* was performed for patients in the prospective cohort using a proprietary kit from MRC Holland (www.mlpa.com; SALSA MLPA kit P236-A1 ARMD). GeneMarker software (Version 1.90) was used to calculate dosage quotients for all MLPA experiments. I undertook analysis of the data provided. Exons included in this analysis are summarised (Table 18)

CFH	*CFHR3*	*CFHR1*	*CFHR2*	*CFHR5*
CFH exon 1	*CFHR3* exon 1	*CFHR1* exon 3	*CFHR2* exon 1	*CFHR5* exon 1
CFH exon 2	*CFHR*3 exon 2	*CFHR1* exon 5	*CFHR2* exon 2	*CFHR5* exon 2
CFH exon 3	*CFHR*3 exon 3	*CFHR1* exon 6	*CFHR2* exon 3	*CFHR5* exon 3
CFH exon 4	*CFHR3* exon 4		*CFHR2* exon 4	
CFH exon 6	*CFHR3* exon 6			
CFH exon 11				
CFH exon 12				
CFH exon 13				
CFH exon 18				
CFH exon 23				

Table 18 Exons tested in MLPA for copy number variation

Screening was performed in Northern Molecular Genetics Service using SALSA MLPA kit P236-A1 ARMD.

2.1.7 Defining Rare Genetic Variants

The Exome Variant Server website (evs.gs.washington.edu/) is a population database, containing whole exome data generated from the NHLBI GO Exome Sequencing Project (ESP6500). The dataset contains exomes from 2203 African American and 4300 European American unrelated individuals. A rare genetic variant was defined as occurring at a frequency of <1% in a control cohort. The exome variant server was chosen for this purpose.

Rare genetic variants were modelled on the crystal structure of C3b in complex with FB (C3b:FB-2XWJ), C3b in complex with FH SCR 1-4 (C3b:FH1-4-2WII) and FH SCR19-20 in complex with C3d (C3d:FH19-20-3OXU) from .PDB files available from http://www.rcsb.org/pdb/home/home.do using Pymol (Schrödinger, Inc).

The minor allele frequency of exonic SNPs in *CFH, C3* and *CFB* was determined from the NHLBI GO Exome Sequencing Project (ESP6500). The minor allele frequency of intronic SNPs in *CFH* and *CD46* was determined from a previously screened cohort of UK and French controls (Fremeaux-Bacchi *et al.*, 2005).

The effect of rare genetic variants on function was modelled *in-silico* using two online prediction software programs. Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/) and Provean (http://provean.jcvi.org/index.php) are both online prediction software programs that predict the effects a variant would have on a protein. Polyphen-2 reports the likely effect as benign, possibly damaging or probably damaging (Figure 9). Provean reports the likely effect as deleterious or neutral (Figure 10). These were discussed in the setting of the functional studies that were performed for some of the rare genetic variants identified.

2.1.8 Statistics

Clinical data was summarised in box and whisker plots, and demonstrate median values, quartiles, 10^{th} and 90^{th} percentiles and outliers. Comparison between multiple groups was performed using ANOVA or Kruskal-Wallis tests. Comparison between two groups was performed using t tests or Mann-Whitney test. Odds Ratio and Fisher's Exact test was used for proportional data. Statistical analysis and graphs were prepared using SigmaPlot®. Additional graphs were prepared using GraphPad Prism 6 (GraphPad Software) or Microsoft Excel®.

Figure 9 In-silico prediction of functional effects of variants using PolyPhen-2

Screenshot of prediction of effect of R83S variant using PolyPhen – (Polymorphism Phenotyping). Numbering entered into prediction software does not include the leader sequence. This is an in-silico tool used to predict the possible effects of a non-synonymous SNP. It reports a prediction based upon data held in two datasets, HumDiv and HumVar. The first data set HumDiv defines known disease-causing alleles as deleterious and variants that are different between human and other mammals, classed as benign. The second dataset HumVar defines known disease-causing alleles classed as deleterious and common variants (MAF>1%) as benign. The HumDiv score was preferred due to improved accuracy and higher true positive prediction rates (Adzhubei et al., 2010).

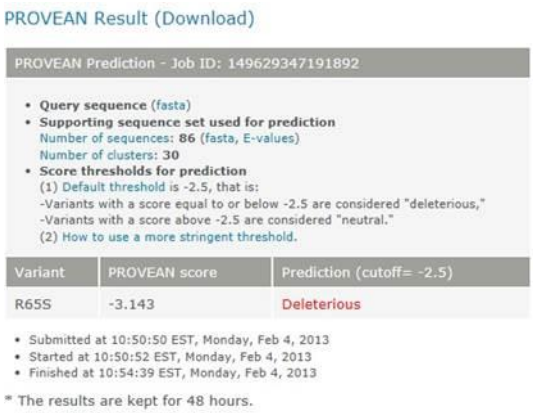

Figure 10 In-silico prediction of functional effects of variants using Provean

Screenshot of prediction of effect of R83S variant using Provean – (Protein Variation Effect Analyser). Numbering entered into prediction software does not include the leader sequence. This is an in-silico tool used to predict the possible effects of a non-synonymous SNP and also short sequences of genetic insertions or deletions. The algorithm used in Provean to predict the effect of amino acid substitutions has a prediction accuracy that is similar to Polyphen-2 (Choi et al., 2012).

2.2 Molecular Biological Techniques

Rare genetic variants in the N-terminal domain of FH were selected for study in structural and functional studies. A wildtype and mutant protein in the setting of FH SCR1-2 was expressed for the structural studies of the R83S variant in familial MPGN in chapter 4. Wildtype and mutant proteins in the setting of FH SCR1-4 were expressed for the functional studies of N-terminal variants identified for study in chapter 4 and chapter 5.

The pPICZαB vector, in *E. coli* and *Pichia pastoris* clones containing cDNA encoding SCR1-2 of FH (residues 19-142) previously used in the structural study of FH in the setting of SCR1-2 of FH (Hocking *et al.*, 2008) were obtained from Dr Kavanagh. The pPICZαB vector, in *E coli* and *Pichia pastoris* clones containing cDNA encoding SCR1-4 of FH (residues 19-263) with a C-terminal 6xHIS tag and N-terminal myc-tag previously used in the functional study of FH in the setting of SCR1-4 of FH (Pechtl *et al.*, 2011) were obtained from Dr Kavanagh. Plasmid DNA of the pPICZαB vector containing cDNA encoding both the 2 SCR and 4 SCR FH fragments were also obtained from Dr. Kavanagh and used as a DNA template for site-directed mutagenesis. The pPICZαB vector in *E.coli* containing cDNA encoding the mutant S159N SCR1-4 was a gift from Professor Alan Wright, University of Edinburgh.

2.2.1 pPICZαB Vector and KM71H

The pPICZαB vector for use in *Pichia pastoris* (both ThermoFisher Scientific) contains a 5'AOX promotor region that allows for methanol induced expression of protein and an α-factor signal sequence that allows secretion of protein. It also has a *Sac* I restriction site to linearise the plasmid and a Zeocin™ resistance gene that allows for selection of clones in *E.coli* and the KM71H strain of *Pichia pastoris* (Figure 11). Further advantages in using *Pichia pastoris* as an expression system include its ease to make high-yields of protein in an easily scalable manner. The importance of expressed protein that is correctly folded is important and functional wildtype protein was confirmed in previous studies using this technology (Pechtl *et al.*, 2011).

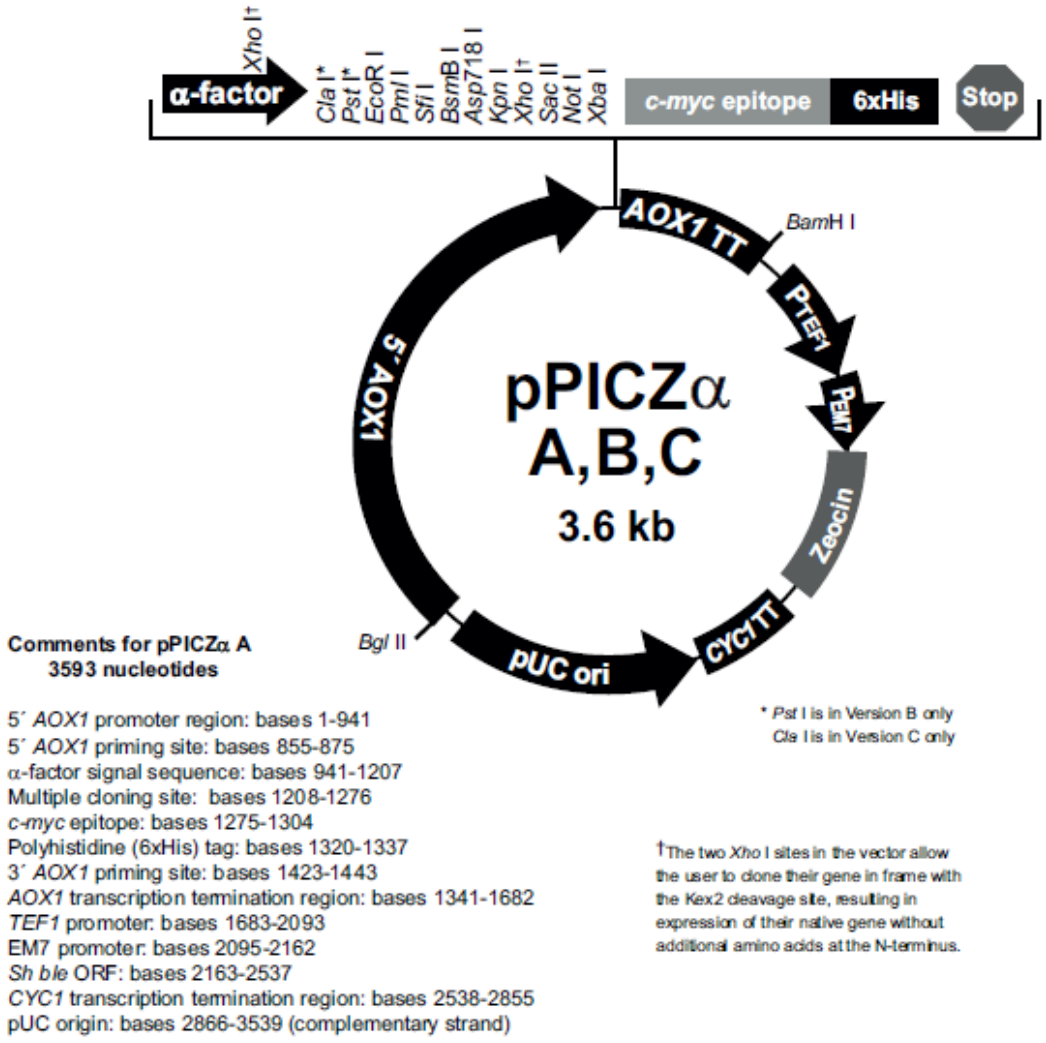

Figure 11 pPICZαB vector used for expression of FH fragments in Pichia pastoris

Taken from pPICZα Manual, Invitrogen.

2.2.2 Site-directed Mutagenesis

High performance liquid chromatography purified specific primers (Appendix 1 - Table 54) were designed and ordered from Integrated DNA Technologies. Primer pairs were used for the introduction of each rare genetic variant as a point mutation into plasmid DNA of the pPICZαB vector containing cDNA encoding either the 2 SCR or 4 SCR FH fragments. Site-directed mutagenesis was performed using QuikChange II XK Site-Directed Mutagenesis Kit (Stratagene).

Sample reactions were prepared by the addition of 5µL of a proprietary 10x reaction buffer, 1µL of DNA template (~50ng/µL), 1.25µL forward mutagenesis primer (~100ng/µL), 1.25µL reverse mutagenesis primer (~100ng/µL) and 1µL of dNTP mix in a final volume of

50µL dH_2O. 1µL of PfuUltra HF DNA polymerase (2.5U/µL) was then added to each sample reaction before cycling for 1 minute at 95°C, then 18 cycles for 50 seconds at 95°C, for 50 seconds at 60°C and for 7.5 minutes at 68°C before a final 7 minutes at 68°C.

Following mutagenesis, residual template DNA was digested by incubating the products of mutagenesis with 1µL of *Dpn* I (10U/µL) for 1 hour at 37°C. The mutagenesis product was then stored at 4°C until ready for transformation into *E. coli* competent cells.

2.2.3 Transformation of Mutated DNA into *E.coli* Competent Cells

2µL of the *Dpn* I-treated mutated DNA template was added to 45µL of thawed XL-1 Blue competent cells (Stratagene) and incubated for 10 minutes on ice. The mixture was then 'heat-shocked' for 45 seconds at 42°C before incubation for 2 minutes on ice. 500µL of low salt LB broth (Appendix 2) (prewarmed to 42°C) was then added to the competent cells. The mixture was incubated at 37°C for 1 hour. 450µL and 50µL of transformed cells were then inoculated onto low salt LB agar (Appendix 2) plates containing 25µg/ml Zeocin™ (Invivogen). Successfully transformed clones containing pPICZαB and a Zeocin™ resistance gene were identified following incubation for 16 hours at 37°C.

2.2.4 Extraction of Plasmid DNA – Small Scale

Clones containing mutated cDNA for FH SCR1-2 or SCR1-4 in the vector pPICZαB were picked and cultured in 5ml of LB broth (Appendix 2) containing 25µg/ml Zeocin™. Cultures were incubated at 37°C for 16 hours with shaking at 225rpm. Cells were then pelleted by centrifugation at 6800xg for 3 minutes at 25°C and the supernatant was removed. Plasmid DNA was then extracted from the cell pellet using QIAprep Spin Miniprep Kit (QIAGEN). The bacterial cell pellet was resuspended in 250µL of Buffer P1 containing 100µg/ml RNase. To this, 250µL of Buffer P2 was added and the mixture inverted 10 times. The mixture was incubated for 5 minutes during which time lysis of cell membrane occurred. The lytic reaction was stopped by the addition of 350µL of chilled Buffer N3 and the mixture inverted 10 times before centrifugation for 10 minutes at 13200xg. The supernatant was then applied to the QIAprep spin column before centrifugation for 60 seconds. The spin column was washed with 500µL Buffer PB and 750µL Buffer PE with a 60 second centrifugation step after each wash. Finally, the DNA was eluted by the addition of 60µL Buffer EB, incubation for 1 minute, followed by centrifugation for 1 minute to collect the eluted DNA.

2.2.5 Determining Concentration of Plasmid DNA

DNA was quantified using a NanoDrop 8000 (Thermo Scientific) at 260nm.

2.2.6 Sequencing of Plasmid DNA in pPICZαB Vector

20µL of plasmid DNA (~50ng/µL) and 20µL of sequencing primer (10pmol) (forward and reverse) (Appendix 1 - Table 55) were sent for Sanger sequencing at GATC Biotech. The sequencing data was checked using Sequencher (Genecodes).

2.2.7 Extraction of Plasmid DNA – Large Scale

Clones containing the correctly mutated cDNA for FH SCR1-2 or SCR1-4 in the vector pPICZαB were picked and initially cultured in 5ml of LB broth containing 25µg/ml Zeocin™ for 8 hours. The initial culture was then added to 500ml of LB broth containing 25µg/ml Zeocin™ for 16 hours. Cultures were incubated at 37°C with shaking at 225rpm. Cells were then pelleted by centrifugation at 6000xg for 15 minutes at 4°C. The supernatant was discarded and plasmid DNA was then extracted from the cell pellet using QIAprep Spin Maxiprep Kit (QIAGEN) The cells were resuspended in 10ml of Buffer P1 containing 100µg/ml RNase and mixed. To this, 10ml of Buffer P2 was then added and inverted to lyse the cells, then incubated for 5 minutes at 25°C. 10ml of chilled Buffer P3 was then added and the lysate was transferred to the QIAfilter Cartridge and incubated for 10 minutes. The lysate was then filtered through the QIAfilter Cartridge and collected in a QIAGEN-tip equilibrated with 10ml of Buffer QBT. The QIAGEN-tip was then washed with 60 ml of Buffer QC. The DNA was then eluted with 15ml Buffer QF and collected. To precipitate DNA, 10.5ml of isopropanol was then added to the eluted DNA, mixed and centrifuged at 15000xg for 30 minutes at 4°C. The supernatant was carefully decanted. The DNA pellet was then washed in 5ml of ethanol and centrifuged at 15000xg for 10 minutes. The ethanol was removed and the DNA pellet was allowed to air dry. The DNA pellet was resuspended in 500µL of dH_2O and quantified. Bidirectional Sanger sequencing was performed to confirm fidelity. DNA was stored at 4°C prior to linearization and transformation into KM71H cells.

2.2.8 Linearisation of Plasmid DNA and Phenol-chloroform Extraction

100µg of correctly mutated cDNA for FH SCR1-2 or SCR1-4 in the vector pPICZαB was linearised by adding to 1.4µL 100x BSA (Promega) and 14µL 10x reaction buffer (Promega) and 2µL *Sac* I (Promega) in a final volume of 140µL with dH_2O and incubated for 16 hours at 37°C.

Protein was then removed by phenol:chloroform extraction. An equal volume of phenol:chloroform, isoamyl alcohol 25:24:1 saturated with 10mM Tris pH 8.0, 1mM EDTA (Sigma) was added to the linearised DNA and vortexed. The mixture was centrifuged at 16800xg for 2 minutes. The aqueous phase was removed and added to an equal volume of phenol:chloroform, isoamyl alcohol 25:24:1 saturated with 10mM Tris pH 8, 0 1mM EDTA and vortexed again. Following centrifugation at 16800xg for 2 minutes, the aqueous phase was removed and added to an equal volume of chloroform (Sigma) in order to remove the phenol. The mixture was vortexed and centrifuged at 16800xg for 2 minutes. The aqueous phase was removed and to it, 0.1 volume of 3M sodium acetate pH 5.2, 2.5 volumes of 100% ethanol was added, vortexed and placed for 1 hour at -80°C. The mixture was then centrifuged at 16800xg for 20 minutes at 4°C and the supernatant was removed. 1ml of cold 70% ethanol was then added, centrifuged at 16800xg for 10 minutes and the supernatant removed. The DNA pellet was allowed to dry and then resuspended in 10µL dH_2O.

2.2.9 Transformation of KM71H

Wildtype KM71H cells (Invitrogen) were revived from -80°C stocks on YPD agar (Appendix 2) plates and incubated for 72 hours at 30°C. A single clone was picked and cultured in 5ml of YPD media (Appendix 2) for 24 hours at 30°C. 100µL of the initial culture was sub-cultured in 250ml of YPD media for ~18 hours at 30°C until it had an optical density at 600nm of 1.3-1.5.

The cells were centrifuged at 1500xg for 5 minutes at 4°C, and washed twice, each time by resuspending in 250ml ice-cold sterile water and centrifuged at 1500xg for 5 minutes at 4°C. After the second wash, cells were resuspended in 10ml of ice-cold 1M sorbitol and centrifuged at 1500xg for 5 minutes at 4°C. After this final wash, the cells were resuspended in 400µL of ice-cold 1M sorbitol.

For the electroporesis, 80µl of the KM71H cells were mixed with 10µg of linearised plasmid DNA in 10µl sterile water and transferred to an ice cold 0.2cm electroporation cuvette

(Biorad). The cuvette was incubated for 5 minutes on ice. Electroporesis was performed at 1500V, 200 Ω, and 25uF using a gene pulser (Biorad).

Following electroporesis, the cells were incubated for 1 hour at 30°C before 50µL, 200µL and 250µL aliquots of transformed cells were streaked onto 2 sets of YPD agar plates containing 100µg/ml and 300 µg/ml of Zeocin™ respectively. The plates were then incubated for ~72 hours at 30°C until the appearance of clones.

2.3 Protein Expression

2.3.1 Small Scale Test Expression of Transformed *Pichia* Clone

Successful clones of transformed KM71H with pPICZαB following electroporesis were picked and cultured in 10ml of BMG (Appendix 2) for 48 hours at 30°C with agitation at 225rpm. After 48 hours, the cells were pelleted at 1500xg for 5 minutes and resuspended in 3.3ml of BMM (Appendix 2) and incubated at 30°C with agitation at 225rpm. Further methanol was added at 24 and 48 hours to a final concentration of 0.5%. After a total 72 hours, some of the cell culture was inoculated onto YPD agar containing 100µg/ml Zeocin™ to enable stocks of successful clones to be kept. The remaining cells were pelleted at 1500xg for 5 minutes at 4°Cand the supernatant was collected for analysis. Samples were taken and detection of protein expression was determined by SDS-PAGE followed by staining with Coomassie blue (Figure 12) and/or Western blotting. Samples were then concentrated prior to SDS-PAGE and Western blotting if protein could not initially be detected.

2.3.2 Separation of Protein Samples by SDS-PAGE

Protein samples (20µL) were prepared for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) by the addition of 5µL of Pierce™ Lane Maker Non-Reducing or Reducing sample buffer (both ThermoFisher Scientific), and incubated for 5 minutes at 95°C. TGX Mini-Protean Pre-Cast gels (Biorad) and Mini-Protean Tetra Vertical Electrophoresis Cell (Biorad) were set up using 1x running buffer (100mM Tris, 100mM Glycine, 0.1% SDS, pH 8.3) after dilution from 10x Tris/glycine/SDS running buffer (Biorad). 4-15% gradient gels were used for the analysis of recombinant and purified protein samples. 25µL of sample was loaded per well and 5µL of a molecular weight ladder

(Appendix 3 Table 56) was loaded into one lane of each gel. Samples were then separated by SDS-PAGE at 190V.

Products of fluid-phase co-factor assay (section 2.5.5) were separated on a 10% gel. Detection of proteins in serum is described separately (section 2.4.9).

Following electrophoresis, the protein bands were visualised by Coomassie staining or Western blotting.

2.3.3 Coomassie Staining

Following separation of protein samples by SDS-PAGE, the gel was washed 3 times in dH_2O for 5 minutes. The gel was then incubated in 25ml of Bio-Safe™ Coomassie Stain (Biorad) for 30 minutes before destaining with dH_2O. The destained gel was then scanned using HP Scanjet G4050 (Hewlett Packard).

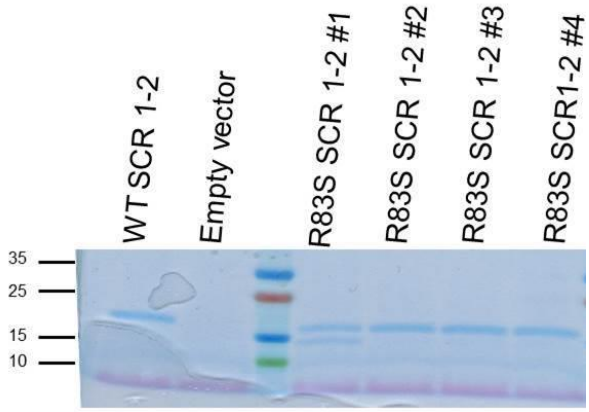

Figure 12 Representative small scale test expression of protein

Following transformation of KM71H and initial culture of successful clones, protein expression was induced using methanol. In this figure, protein expression is detected following separation of supernatant from small scale test expressions on 4-15% gradient SDS-PAGE under non-reducing conditions. The clone of FH SCR1-2 containing the R83S mutation was successfully transformed into the KM71H strain of Pichia pastoris. Clones #1-#4 all show a band at 15kDa consistent with a 2 SCR protein and comparable to the previously transformed WT FH SCR1-2. A pPICZαB empty vector (containing no cDNA for FH) was also transformed into KM71H. No band could be seen at a size of 15kDa for the empty vector.

2.3.4 Western Blotting

Mini-Protean Tetra Vertical Electrophoresis Cell (Biorad) was set-up for transfer of protein from TGX Mini-Protean Pre-Cast gels to nitrocellulose membrane (Invitrogen) by Western

blotting. Gels were transferred at 100V for 60 minutes in a transfer buffer (12mM Tris base, 96mM Glycine, pH 8.3, 20% methanol), pre-chilled to 4°C. The membrane was washed 3 times with dH_2O and then blocked for 1 hour at 25°C or 16 hours at 4°C in 5% milk powder (Marvel) in TBST (50mM Tris-HCl, pH 7.4, 150mM NaCl and 0.05% Tween 20). The membrane was then incubated in 5% milk power in TBST containing a primary antibody for 1 hour at 25°C. This was followed by a further incubation step in 5% milk powder in TBST containing a secondary antibody for 1 hour at 25°C. The membrane was washed 3 times in TBST for 5 minutes following each incubation step. The selection of respective primary and secondary antibodies and their dilutions are summarised (Appendix 3, Table 57).

The membrane was then incubated in SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific) for 1 minute prior to exposure onto Carestream® Kodak® X-Omat LS film (Sigma) in a hypercassette (Amersham Biosciences) for sufficient time for bands to develop. The film was then developed in a Konica SRX-101A Tabletop Processor (Konica Minolta Medical).

Alternatively, following incubation with chemoluminescence, the image was captured using BioSpectrum 500 Imaging System (UVP) and analysed using VisionWorks®LS Analysis Software (UVP).

2.3.5 Cryopreservation of successful clones

Single clones were cultured in 5ml YPD for 16 hours at 30°C with agitation of 225rpm. Cells were frozen at -80°C in YPD media containing 15% glycerol.

2.3.6 Expression of FH SCR1-2 Clone using ^{15}N Media

Expression of FH SCR1-2 using ^{15}N-media was performed for structural studies using NMR. WT and R83S FH SCR1-2 clones of KM71H were picked and cultured in a starter culture of 10ml BMG for 24 hours and transferred into a larger culture of 300ml BMG containing ^{15}N-ammonium sulphate (Appendix 2) as the sole nitrogen source. After a period of 24 hours of growth, cells were pelleted by centrifugation at 1500xg for 5 minutes and resuspended in a total of 3L of BMG containing ^{15}N-ammonium sulphate for a period of 48 hours. The cells were then pelleted and resuspended in a total of 1L of BMM containing ^{15}N-ammonium sulphate (Appendix 2) as the sole nitrogen source to induce protein expression. Methanol was

added to a final concentration of 1% after 24 and 48 hours. After a total of 72 hours of protein expression, the cells were pelleted by centrifugation at 1500xg for 15 minutes and the supernatant collected for protein purification. Culture of *Pichia pastoris* in shaker flasks was at 30°C and agitation of 225rpm throughout.

2.3.7 Expression of FH SCR1-4

Expression of FH SCR1-4 was performed for functional studies. WT and mutant FH SCR1-4 clones of KM71H were picked and cultured in an initial starter culture of 20ml BMG for 48 hours before transfer into an intermediate culture of 200ml for a further 48 hours at 30°C with agitation at 225rpm in shaker flasks. The intermediate culture was then added to 1.2L of fermentation basal salts medium (Appendix 2) supplemented with 4.35ml/L PTM1 trace salts (Appendix 2) for culture in a BioFlo®/CelliGen®115 Fermentor/Bioreactor (New Brunswick). 0.1% Antifoam A (Sigma) was also added to prevent foaming prior to inoculation and during fermentation when foaming was observed in the fermentor vessel.

Biocommand software® (New Brunswick) was used to optimise cell growth and protein expression during the fermentation process described below. The dissolved oxygen (DO) level measured by Mettler Ingold dO2 Probe, InPro 6800 (New Brunswick Scientific) prior to inoculation was set to 100% and monitored. During cell culture DO levels falling as a result of cell growth and oxygen consumption. In order to maintain DO levels at a set-point of 30%, the agitation was programmed to increase from a minimum of 200rpm to a maximum of 800rpm in response to DO levels falling below 30%. Monitoring of DO levels during the initial cell growth phase and subsequent induction of protein expression allowed careful evaluation of glycerol and methanol use as a source of carbon respectively so that the carbon source was limiting.

Metabolism from cell growth also resulted in a fall in pH. The pH was monitored by Mettler Ingold pH Probe (New Brunswick Scientific) and maintained at 5.0 by the automated addition of 50% ammonium hydroxide that also provided a constant nitrogen source.

Temperature was monitored and target temperature was maintained by the Fermentor/Bioreactor by a heating blanket and the flow of cold water within cooling elements within the fermentor vessel.

Following inoculation of the 200ml intermediate culture to the fermentation basal salts medium, the culture in the Fermentor/Bioreactor was allowed to grow at 30°C with an initial

agitation of 200rpm. No glycerol was added to the culture at this time. The glycerol in the initial media was metabolised by *Pichia* during cell growth resulting in an initial fall in DO from 100%. As growth and oxygen consumption continued, DO levels would fall to a set-point of 30% resulting in the increase in agitation to maintain DO levels of 30%. This initial phase is summarised in Figure 13.

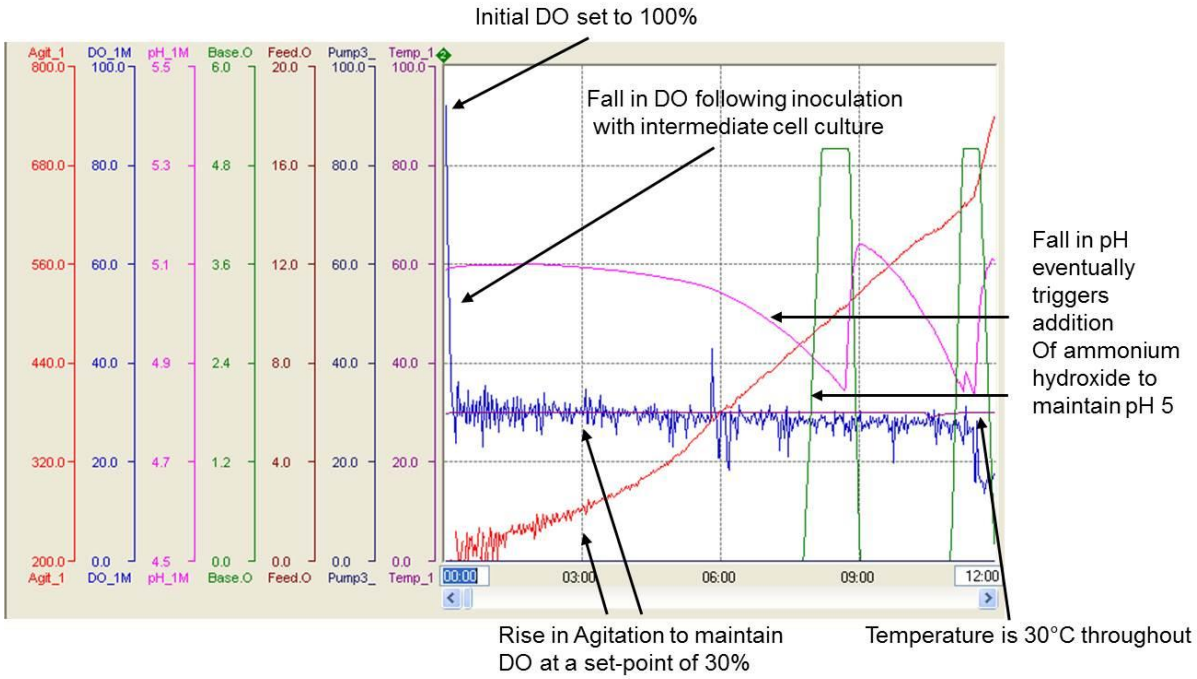

Figure 13 Monitoring of oxygen consumption during fermentation using Biocommand software

Screenshot from Biocommand® software representative of first 12 hours of Pichia culture in the fermentor. Y-axis indicates agitation (red), dissolved oxygen (blue), pH (pink), base feed (green), glycerol feed (brown) and temperature (purple). Pump 3 shown above was not used. X-axis represents time in hours. Calibration of dissolved oxygen (DO) was set to 100% immediately prior to inoculation of intermediate cell culture at time 0. Setpoint of DO was set to 30%. As the cell culture metabolises the available glycerol in the initial fermentation basal medium, oxygen is consumed and DO falls to the setpoint of 30%, at which point agitation increases to maintain DO at 30%. No glycerol is added until evidence of exhaustion of glycerol (indicated by sudden rise in DO – not shown in this example) to ensure it is the limiting factor during subsequent growth. Metabolism of Pichia also leads to a fall in pH. Base (in this case 50% ammonium hydroxide) is automatically added). Temperature during the initial glycerol growth phase is maintained at 30°C throughout.

Once the initial glycerol was exhausted, oxygen consumption would fall resulting in a sudden 'spike' in DO. At this point, the addition of glycerol was commenced at an initial rate of 0.5ml/min to allow continued cell growth. The DO set-point of 30% was maintained with increasing agitation as the metabolic rate of the culture increased with increasing cell mass. As the cell mass increased, the rate of glycerol was doubled if there was evidence of a fall in metabolic rate as determined by a sudden rise in DO or fall in agitation until a total of 36 hours of growth in the Fermentor/Bioreactor.

After 36 hours, no further glycerol was added. Given that the carbon source was limiting, there was a resultant 'spike' in DO. The cell culture was allowed to starve for a minimum of 4 hours ensuring that glycerol had completely exhausted as indicated by a rise in DO to 100% before the addition of methanol. Using methanol only as a limiting carbon source maximised the induction of the AOX1 promoter and protein expression during this induction phase. Maintaining the concentration of methanol at a basal amount also avoided the risk of toxicity due to elevated concentrations of methanol in the order of 1-2%.

After this time, the culture was cooled to 15°C to minimise degradation of subsequent protein expression and an initial volume of 100% methanol (supplemented with 12ml of PTM1 trace salts per litre) was added to a final concentration of 0.75% of the culture media to induce protein expression. Again, the DO was allowed to fall and the agitation was set to increase to a maximum of 800rpm to maintain a basal DO of 30%. After ~12 hours, and when there was evidence that the carbon source was limiting as observed by a DO 'spike', further methanol was added at 0.5ml/min. As the cell culture adapted to the utilisation of methanol, as seen by a further 'spike' in DO, the rate of methanol was doubled.

After 72 hours of protein expression, the cells were pelleted at 6000xg for 15 minutes and the supernatant collected for protein purification. During initial purification, it was noted that upon cooling supernatant to 4°C, the formation of crystals prevented sample loading during nickel affinity purification. The addition of potassium hydroxide to the cell culture to a pH of 7.4 prior to pelleting was an early modification to the protocol that allowed purification of protein.

2.3.8 Tissue Culture of OX24 Hybridomas

The OX24 antibody, a monoclonal to FH SCR5 was produced by a B-cell hybridoma cell line (Sigma). Hybridomas were cultured at 37°C and 5% CO_2 using Roswell Park Memorial Institute (RPMI) medium. RPMI media was supplemented with 10% low immunoglobulin foetal calf serum, 2mM L-glutamine, 0.1units/mL penicillin, 0.1mg/mL streptomycin, 1mM non-essential amino acids, 1mM sodium pyruvate (all PAA Laboratories) and 50µM β-mercaptoethanol (Sigma). A supernatant containing secreted OX24 was obtained following centrifugation of the cell suspension at 300xg for 2 minutes. The supernatant was stored at -20°C until purification.

2.4 Protein Purification

2.4.1 Cation Exchange

Cation exchange was used for the purification of WT and R83S FH SCR1-2 expressed using ^{15}N-ammonium sulphate as the sole nitrogen source. Supernatant containing FH SCR1-2 was diluted 1:5 in a binding buffer (250mM formic acid pH 4) and loaded onto a crude column filled with 5ml of SP Sepharose Fast Flow (GE Healthcare) equilibrated with binding buffer at 4°C. Supernatant containing unbound protein was collected in a flow-through. Once all of the supernatant had passed over the column, a further 5 column volumes of binding buffer was used to remove non-specific binding in a wash step. Bound FH SCR1-2 was eluted by the addition of 5 column volumes of an elution buffer (250mM formic acid pH4 and 1M NaCl).

Following cation exchange, the expressed protein (both WT and R83S) could be seen in elution fractions 1-3. The expressed protein was visualised following 4-15% gradient SDS-PAGE under non-reducing conditions as a 15kDa band as predicted and a smaller band suggestive of degradation (Figure 14) that would require further purification.

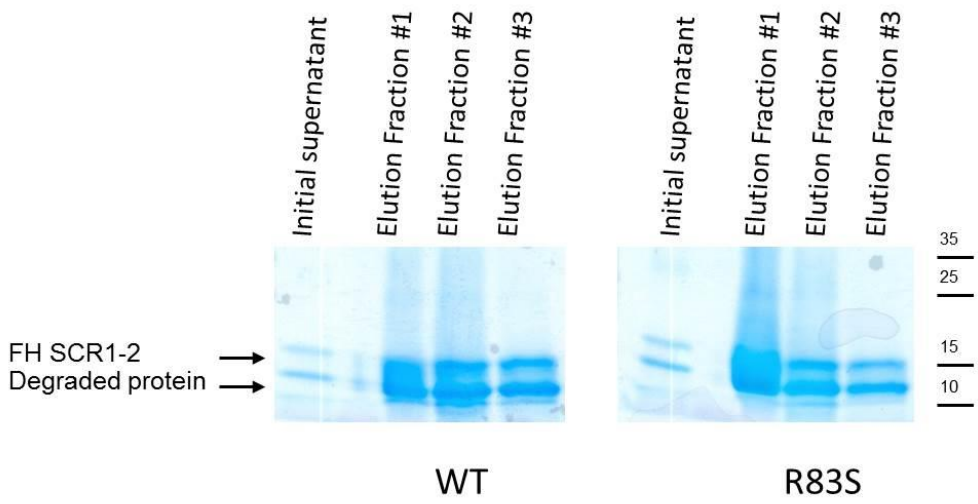

Figure 14 Initial purification step of ^{15}N FH SCR1-2

FH SCR1-2 was purified using cation exchange. WT and R83S FH SCR 1-2 were expressed using ^{15}N- ammonium sulphate as a sole nitrogen source. The expressed protein can be seen following separation on 4-15% gradient SDS-PAGE at 15kDa under non-reducing conditions with evidence of a degradation product in the initial supernatant. The expressed protein and degradation product are seen in the elution fractions following batched cation exchange of the available supernatant.

2.4.2 Nickel Affinity Purification

Nickel affinity chromatography was used to purify FH SCR1-4 (WT and all mutant proteins) expressed in KM71H. Proteins were histidine-tagged therefore allowing rapid purification on a HISTrap FF Crude pre-packed column (GE Healthcare). A 5ml HIS-Trap crude column was equilibrated with a binding buffer (40mM sodium phosphate pH 7.4, 500mM NaCl and 20mM imidazole). Following protein expression, the culture media was centrifuged at 6500xg at 4°C. The supernatant was collected and prepared for purification by the addition of an equal volume of 2x binding buffer (80mM sodium phosphate, pH7.4, 1000mM NaCl and 40mM imidazole). The sample was loaded onto the equilibrated column at a rate of 2ml/min using an ÄKTA purifier (GE Healthcare). After the sample had loaded, weakly bound protein was removed in a wash step using 25ml of binding buffer. Non-specific binding was removed in an additional wash step using 25ml of a binding buffer containing 40mM imidazole instead of 20mM.

Protein was eluted by applying an elution buffer (40mM sodium phosphate pH 7.4, 500mM NaCl and 500mM imidazole) at a rate of 2ml/min. The eluted protein was collected in 5ml elution fractions. Fractions containing protein were analysed by SDS-PAGE to confirm the presence of protein (Figure 15).

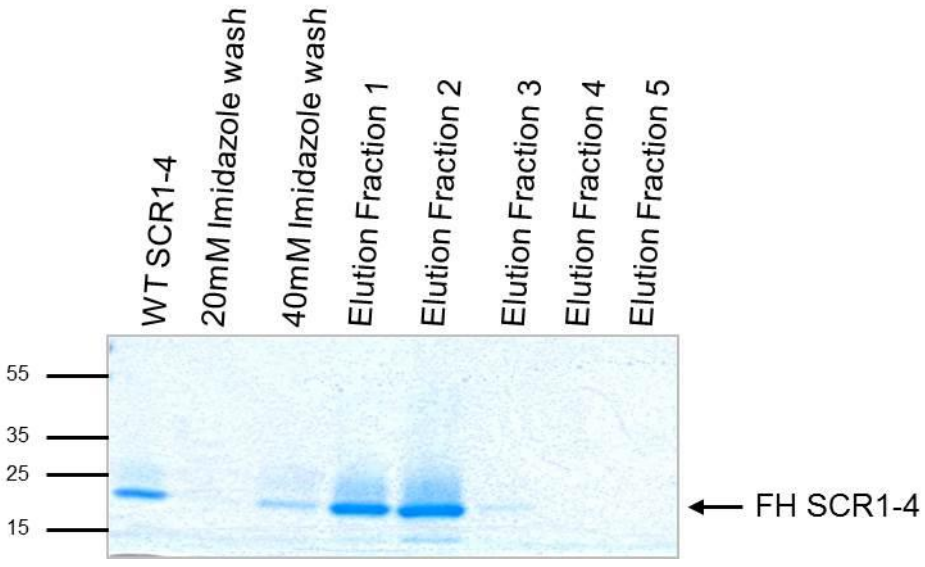

Figure 15 Purified R83S FH SCR1-4 following elution from nickel-affinity column

Representative gel following 4-15% gradient SDS-PAGE under non-reducing conditions showing the presence of a protein ~25kDa at the expected size for a FH SCR1-4 protein. In this example, R83S FH SCR1-4 has been eluted in fractions 1 and 2. Previously purified WT FH SCR1-4 is shown for reference.

2.4.3 Size Exclusion Chromatography

Size exclusion chromatography was used to remove any aggregates or degradation products following initial purifications steps. A 16/200 or 26/200 Superdex column (GE Healthcare) was equilibrated in 4 column volumes of buffer using an ÄKTA purifier (GE Healthcare). The buffer was chosen depending upon the functional assay for which the protein was required. A maximum volume of 5ml or 10ml of purified protein depending on whether the 16/200 or 26/200 Superdex column was used, was prepared using a 0.22µm filter and was loaded onto each column respectively using a superloop (GE Healthcare). Buffer was applied to the column for 1 column volume at a rate of 0.5ml/min. Elution fractions were collected and those containing protein as visualised on the chromatogram were analysed by SDS-PAGE to confirm the presence of protein. A representative chromatogram and corresponding bands on SDS-PAGE is shown (Figure 16).

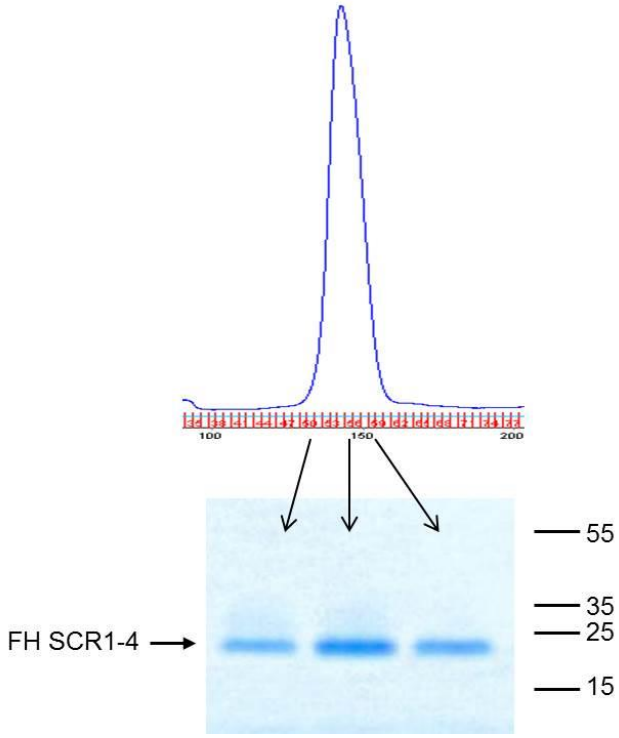

Figure 16 Representative trace of size-exclusion

R83S FH SCR1-4 was eluted from HIS-Trap FF column and loaded onto a 26/200 Superdex column. Protein was eluted using PBS. Chromatogram (blue trace) is a measure of absorbance at 280nm and the peak shown is evidence of protein elution. In this representative example, the peak was visible at a retention volume just under 150ml. Elution fractions corresponding to the peak were separated on a 4-15% gradient SDS-PAGE that confirmed the presence of a protein free of aggregates and degradation at ~25kDa under non-reducing conditions.

2.4.4 Reverse Phase Chromatography

WT and R83S FH SCR1-2 expressed in ^{15}N-media was purified using cation exchange followed by size exclusion. Unfortunately, due to the small difference in size between the intact protein and degradation product shown previously in Figure 14, size exclusion did not separate the intact protein to homogeneity. In order to separate the intact protein from the degradation product, reverse-phase chromatography was used.

WT and R83S FH SCR1-2 in 50mM potassium phosphate pH 7 was applied to a Discovery BIO Wide Pore C5 column (Sigma) equilibrated with 2.5 column volumes of a binding buffer (dH_2O with 0.05% tri-fluoroacetic acid). Protein was eluted using an elution buffer (acetonitrile with 0.05% tri-fluoroacetic acid) over a gradient of 0-100% over 20 minutes. The elution fractions containing protein were analysed by 4-15% SDS-PAGE confirming separation of intact protein from the degraded protein as shown in Figure 17.

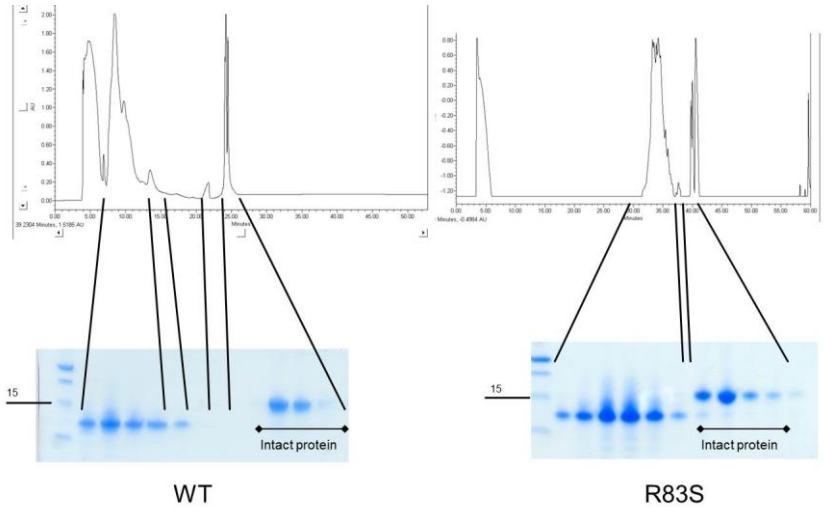

Figure 17 Final purification step of FH SCR 1-2 (WT and R83S) using reverse phase chromatography

WT and R83S FH SCR1-2 were loaded onto Discovery BIO Wide Pore C5 column and eluted using acetonitrile with 0.05% tri-fluoroacetic acid. Chromatogram (black trace) is a measure of absorbance at 280nm. Peaks are evidence of protein elution. Elution fractions corresponding to peaks were separated on 4-15% gradient SDS-PAGE and confirmed the separation of two similarly sized proteins, consistent with a larger intact protein at ~15kDa and a smaller degraded fragment, under non-reducing conditions.

2.4.5 Purification of OX24 using a Protein G Column

Protein G is a cell surface protein of Group G *Streptococci* that can bind mouse immunoglobulin of all sub-classes. Prepacked HiTrap Protein G HP column (Sigma) contains protein G and was used to purify OX24, a mouse monoclonal immunoglobulin. Supernatant containing OX24 antibody was prepared using a 0.22µm filter immediately prior to loading onto a HiTrap Protein G HP column equilibrated with 5 column volumes of a binding buffer (20mM sodium phosphate pH 7.0) using an ÄKTA purifier at 4°C. The column was washed with 5 column volumes of binding buffer. Bound protein was eluted using an elution buffer (0.1M glycine-HCl pH 2.7) and collected in 500µL fractions and immediately neutralised with 80µL 1M Tris pH 8.0. The elution fractions containing protein were analysed on 4-15% gradient SDS-PAGE under reducing conditions (Figure 18).

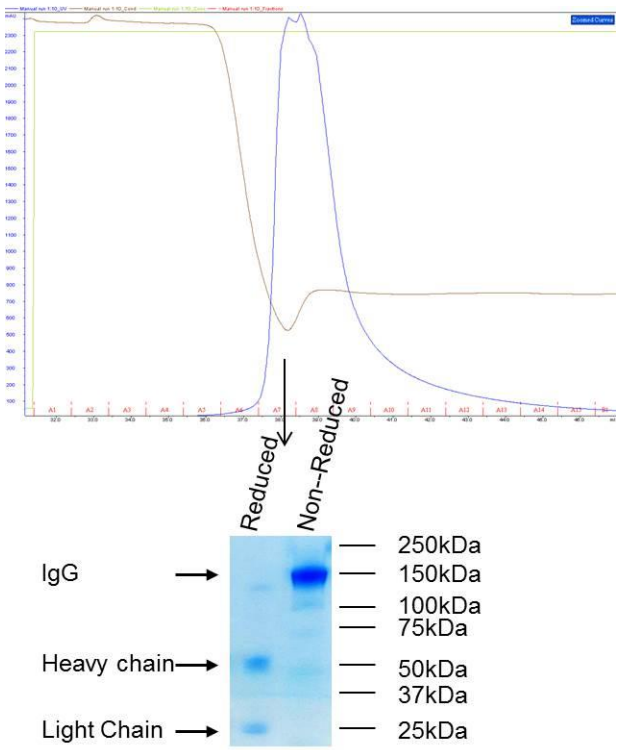

Figure 18 Purification of OX24 antibody

OX24 antibody binds to Protein G and was eluted using 0.1M glycine-HCl, pH 2.7 (green trace). The conductivity (brown trace) falls as the elution buffer is loaded onto the column and reflects the lower conductivity of the elution buffer. Chromatogram (blue trace) is a measure of absorbance at 280nm and the peak shown is evidence of protein elution. Elution fractions corresponding to the peak were separated on 4-15% gradient SDS-PAGE confirmed the separation of a ~160kDa band under reducing conditions into 2 smaller bands at ~ 50kDa and 25kDa, consistent with the elution of an immunoglobulin visible as a single protein and its heavy and light chains.

2.4.6 Generation of an Immunoaffinity Column

HiTrap NHS-activated HP (Sigma) is a prepacked column containing NHS-activated Sepharose™ High Performance and is designed for the covalent coupling of ligands containing primary amino groups. An affinity column can be generated by covalently coupling purified monoclonal antibody onto agarose beads activated by *N*-hydroxysuccinimide (NHS). Thereafter, the column with bound immunoglobulin can be used to capture a target antigen by immunoaffinity chromatography.

Purified antibody was buffer exchanged into a coupling solution (0.2M sodium carbonate and 0.5M NaCl, pH 8.3) and concentrated to 10mg/ml. 6ml of HCl was used to wash out isopropanol, used to maintain activity of the1 ml HiTrap NHS-activated HP column prior to first use. 1ml of purified antibody was then loaded onto the column and incubated for 30 minutes at 25°C. 6ml of Buffer A (0.5M ethanolamine and 0.5M NaCl, pH 8.3) and 6ml of buffer B (0.1M sodium acetate and 0.5M NaCl, pH 4) were alternately loaded onto the column to deactivate excess active groups for a total of 3 times each. The column was incubated for 30 min at 25°C following the second application of buffer A.

Three immunoaffinity columns were generated. One was using a monoclonal antibody to FB (JC-1), a kind gift from Professor Claire Harris, Cardiff University. One was a monoclonal antibody specific to SCR5 of FH (OX24), generated by tissue culture of OX24 hybridomas. One was a monoclonal antibody specific to the common polymorphic variant, 402Y on SCR7 of FH (MBI6), a kind gift from Professor Claire Harris, Cardiff University.

2.4.7 Preparation of Serum Samples

Freshly drawn whole blood was allowed to clot for 30 minutes at 25°C and centrifuged at 3000rpm for 15 minutes. Serum was removed and prepared using a 0.22µm filter and pooled.

2.4.8 Depletion of Serum of FB and FH

A 1ml HiTrap NHS HP column with bound monoclonal antibody to FB (JC-1) and a 1ml HiTrap NHS HP column with bound monoclonal antibody to FH (OX24) were equilibrated with PBS in series. Freshly prepared and filtered serum (up to 5ml at a time) was applied to the columns via a superloop using ÄKTA purifier to sequentially deplete FB and FH from serum at 0.5ml/min. Fractions of serum depleted of FB and FH (ΔBΔHNHS) containing peak

absorbance were analysed to confirm the removal of FB and FH. Fractions were then pooled and stored in 500µL aliquots before use in sheep erythrocyte haemolysis assays.

2.4.9 Detection of Proteins in Serum.

For the detection of proteins in serum, serum samples were diluted 1:100 in dH_2O and prepared for SDS-PAGE by the addition of 5x non-reducing buffer (Thermoscientific). Tris-glycine gels (Novex) and the XCell SureLock Mini-Cell (Novex) were set up using 1x running buffer (25mM Tris base, 192mM Glycine and 0.1% SDS pH 8.3) diluted from 10x Tris/Glycine SDS (Invitrogen). Diluted serum samples and 5µL of Precision Plus Protein™ Dual Color Standard (Biorad) were loaded into individual wells. Samples were separated by SDS-PAGE at 125V for 90 minutes.

XCell SureLock Mini-Cell was set up for gel transfer of Novex Tris-glycine gels onto a nitrocellulose membrane. Following SDS-PAGE, the protein was transferred to nitrocellulose membrane at 25V for 90 minutes in transfer buffer, pre-chilled to 4°C. The membrane was then blocked for 1 hour at 25°C in 5% milk powder in TBST. The membrane was then incubated in 5% milk power in TBST containing a primary antibody for 1 hour at 25°C followed by a 5% milk powder in TBST containing a secondary antibody for 1 hour at 25°C. The membrane was washed 3 times in TBST for 5 minutes following each incubation step. The selection of respective primary and secondary antibodies are summarised (Appendix 3, Table 57). The membrane was then incubated in SuperSignal™ West Pico Chemiluminescent Substrate and exposure onto Carestream® Kodak® X-Omat LS film as previously described.

2.4.10 Confirmation of depletion of serum

Fractions of serum depleted of FB and FH were tested using Western blot to confirm that FB and FH had been removed. Western blot analysis confirmed that serum was depleted of FB and FH (Figure 19). A sample of serum prior to FB and FH depletion and purified FB and FH were used as positive controls.

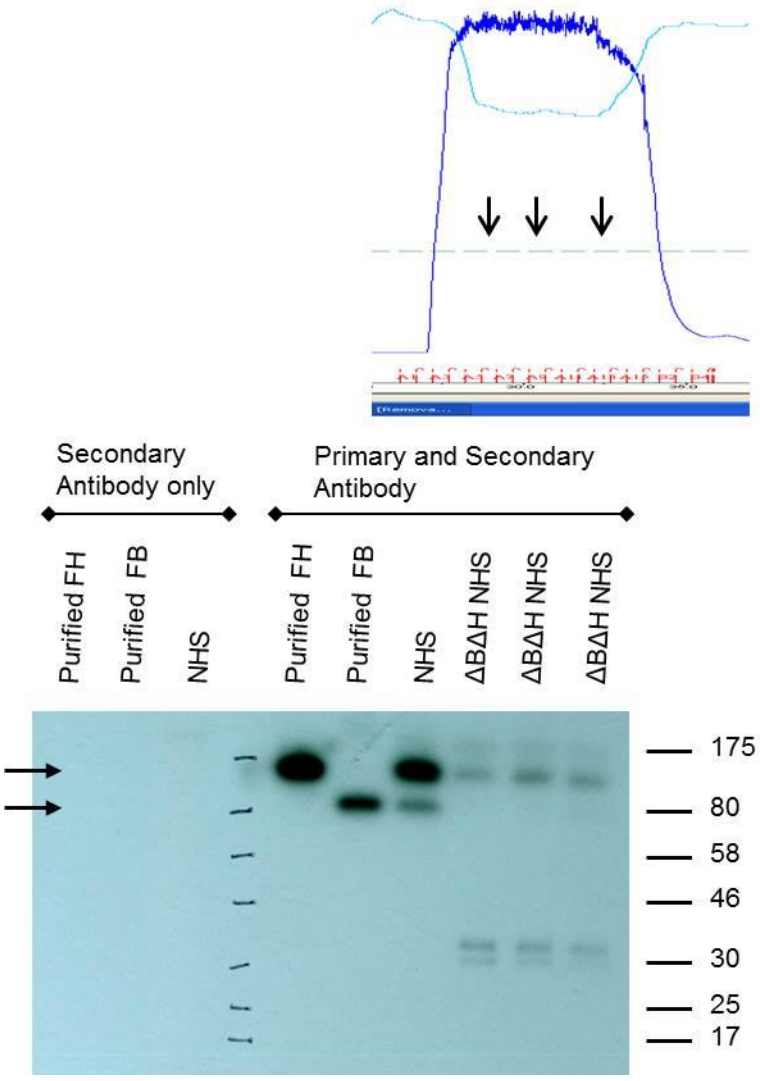

Figure 19 Confirmation of serum depletion of FB and FH

A 1ml HiTrap NHS HP column with bound antibody to FB (JC-1 column) and a 1ml HiTrap NHS HP column with bound antibody to FH (OX24 column) were equilibrated with PBS in series. Serum is loaded onto the columns in series. Conductivity (green trace) falls indicating differences between PBS and serum alongside a chromatogram (dark blue trace) that shows absorbance at 280nm representing the passage of serum through the column. The 'JC-1' and 'OX24' columns contain monoclonal antibody to FB and FH respectively and therefore capture and remove FB and FH from serum. Fractions (black arrows) containing serum following passage of the JC-1 and OX24 column were collected and analysed to confirm the depletion of FB and FH by Western blotting. Serum (1:100), before and after FB and FH depletion, and purified FB and FH were separated on 6% SDS-PAGE under non-reducing conditions. Incubation with goat anti-FH (1:14000) and goat anti-FB (1:2000) primary antibody and rabbit anti-goat HRP (1:3000) as a secondary antibody was used to detect FB and FH. FB and FH were detected in serum (NHS). Fractions of depleted serum(ΔBΔHNHS) show no evidence of FB and only traces of FH. A pair of bands visible at ~30kDa are likely to be FHR1.

2.4.11 Purification of FB and FH from immunoaffinity column

A 1ml HiTrap NHS HP column with bound antibody to FB and a 1ml HiTrap NHS HP column with bound antibody to FH, generated in section 2.4.6 were used to purify FB or FH from serum. Freshly prepared serum was loaded onto a HiTrap NHS HP column with bound antibody using an ÄKTA purifier, equilibrated with 5 column volumes of binding buffer (PBS). The column was washed with a further 5 column volumes of binding buffer before elution of protein using an elution buffer (0.1M glycine-HCl, pH 2.7) (Figure 20). The eluted protein was collected in 500µL fractions and immediately neutralised with 80µL 1M Tris, pH 8. Fractions containing eluted protein were pooled, gel-filtered into PBS and concentrated.

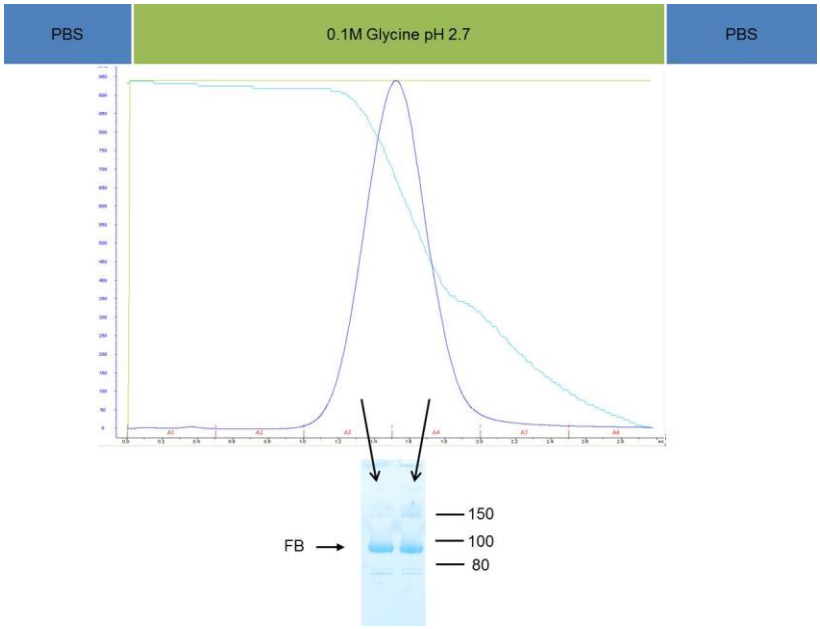

Figure 20 Purification of FB from serum

Representative chromatogram of elution from the 'JC-1' column'. Cyan line indicates conductivity. As it falls, demonstrating the transition of PBS to 0.1M Glycine pH 2.7 in the column. Chromatogram (blue trace) is a measure of absorbance at 280nm and the peak shown is evidence of protein elution. Elution fractions corresponding to the peak were separated on 4-15% gradient SDS-PAGE under non-reducing conditions. Bands at <100kDa can be seen, at the size expected for FB.

2.4.12 Purification of 402Y FH from Serum

A 1ml HiTrap NHS HP column with bound antibody to 402Y FH (MBI6), generated in section 2.4.7 was used to purify 402Y-specific FH from serum in the study of a FH/FHR3 hybrid protein. Freshly prepared serum was loaded onto a HiTrap NHS HP column with bound antibody on an ÄKTA purifier, equilibrated with 5 column volumes of binding buffer (PBS). The column was washed with a further 5 column volumes of binding before elution of protein using an elution buffer (PBS containing 0.05% diethylamine, pH 11.5) (Figure 21).

The eluted protein was collected in 500µL fractions and immediately neutralised with 80µL 1M Tris, pH 7. Fractions containing eluted protein were pooled and gel-filtered into PBS and concentrated for testing in functional assays.

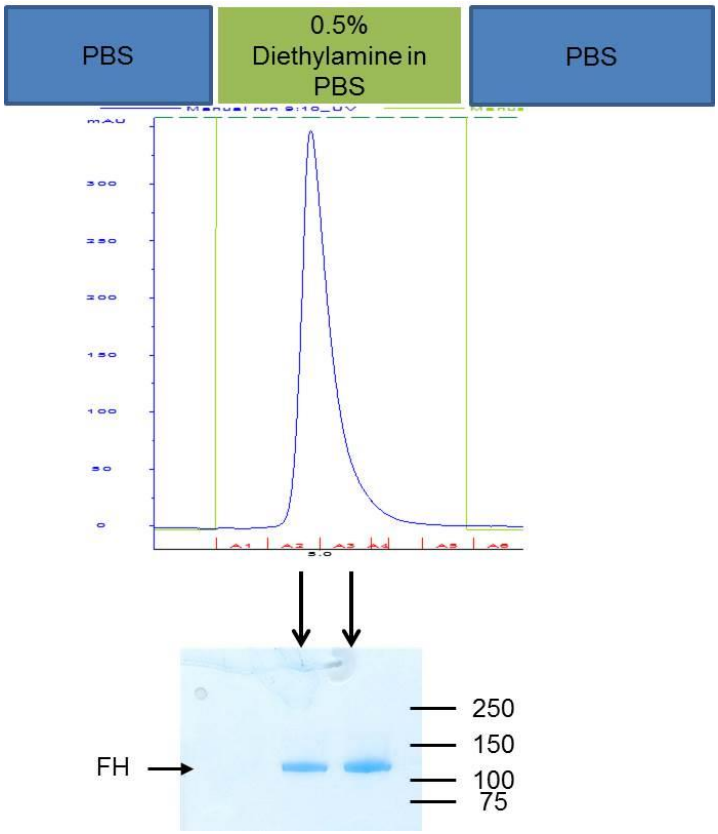

Figure 21 Purification of 402Y-specific FH

Representative chromatogram of elution of FH from the 'MBI-6'column. Donor control serum was loaded onto the column. UV chromatogram (blue trace) a measure of absorbance at 280nm and the peak shown is evidence of protein elution. Elution fractions corresponding to the peak were separated on 4-15% gradient SDS-PAGE under non-reducing conditions. Bands between 100 and 150kDa can be seen, consistent with FH.

2.4.13 Heparin Affinity Chromatography

Heparin chromatography was used to determine the differences in heparin binding between a hybrid FH/FHR3 protein and WT FH. HiTrap Heparin HP (GE Healthcare) is a prepacked column containing Heparin Sepharose™ High Performance. FH or hybrid FH/FHR3 protein purified from serum was loaded onto a 1ml HiTrap Heparin HP following equilibration with a binding buffer (10mM sodium phosphate, pH 7). The column was washed following sample loading with 5 ml of binding buffer before elution of protein using an elution buffer (10mM sodium phosphate, pH 7 and 1M NaCl) on a gradient. The salt concentration at peak elution was determined from the chromatogram.

2.4.14 Quantification of Protein

The absorbance of protein (A_{280}) was determined using a NanoDrop 8000 (Thermo Scientific) at 280nm.

The concentration of a protein was determined using a formula (Figure 22) based on A_{280} and extinction coefficient of a protein determined using www.Expasy/Protparam. Extinction coefficient values for different proteins are shown (Appendix 4, Table 58).

$$\text{Concentration (M)} = \frac{\text{Absorbance at 280nm } (A_{280})}{\text{Extinction coefficient } (M^{-1}.cm^{-1})}$$

Figure 22 Formula to determine concentration of a protein

2.4.15 Buffer Exchange

Following protein purification, size-exclusion chromatography (as described in section 2.4.3) was used to gel-filter purified proteins into a suitable buffer for functional studies. Alternatively, Vivaspin concentrators (Sartorius) were used to buffer exchange samples.

2.4.16 Concentration of Protein Samples

Concentration of purified protein samples in the correct buffer to an adequate concentration for functional studies was performed using Vivaspin concentrators.

2.5 Structural and Functional Analysis of Expressed Proteins

2.5.1 NMR Studies

Purified samples of SCR1-2 (WT and R83S) expressed by me using ^{15}N-ammonium sulphate as the sole nitrogen source were freeze-dried by Dr. Andy Herbert and resuspended at 20mM potassium phosphate pH 7 with added 10% D_2O. The sample was loaded onto a Bruker Avance III spectrometer fitted with a TCI cryogenic probe for nuclear magnetic resonance (NMR) spectroscopy. Two-dimensional $_1$H-$_{15}$N-heteronuclear single quantum coherence (HSQC) spectra were acquired at 37°C and analysed by Dr Andy Herbert.

In this analysis, resonance assignments were determined where possible by comparison with the previously assigned WT FH SCR1-2 spectrum (Hocking *et al.*, 2008). Chemical shift differences between the WT FH SCR1-2 and R83S FH SCR1-2 proteins were determined using the CcpNmr Analysis Suite of Programs. Normalized log values of the chemical shift differences were entered into the B factor field of the Protein Data Bank file (2RLP) and plotted onto cartoon representations of the representative structure of WT FH SCR1-2 using Pymol.

2.5.2 Immobilisation of C3b for SPR Studies

The surface of a CM5 chip can be activated for standard amine coupling by the addition of N-hydroxysuccinimide (NHS) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), allowing covalent binding of a ligand containing primary amino groups. This allows the analysis of protein-protein interactions between an analyte and the bound ligand using surface plasmon resonance (SPR).

The amount of ligand to be bound as measured by resonance units (RU) was determined by the ratio of molecular mass of the ligand (C3b) and the analyte (FH SCR1-4) as a multiple of the maximum number of RU to be measured during flow of the analyte. For binding studies, maximal RU chosen was 100, so ~600 RU of C3b was immobilised.

C3b was immobilised to a CM5 biosensor chip by standard amine coupling on a Biacore X100 (GE Healthcare). C3b (CompTech) was diluted to 5µg/ml in an immobilisation buffer (50mM sodium acetate pH 5). The surface of a CM5 biosensor chip (GE Healthcare) was prepared for standard amine coupling by the injection of a freshly prepared mixture of NHS and EDC (both GE Healthcare) in equal volumes over both flow cell surfaces for 540 seconds

at 10µL/min. Serial injections of 5µg/ml C3b were performed onto flow cell 2 only for 10-20 seconds at a time at 10µL/min until the desired amount of C3b was detected on the surface of flow cell 2. The active sites were then blocked by an injection of ethanolamine (GE Healthcare) for 540 seconds at 10µL/min (Figure 23).

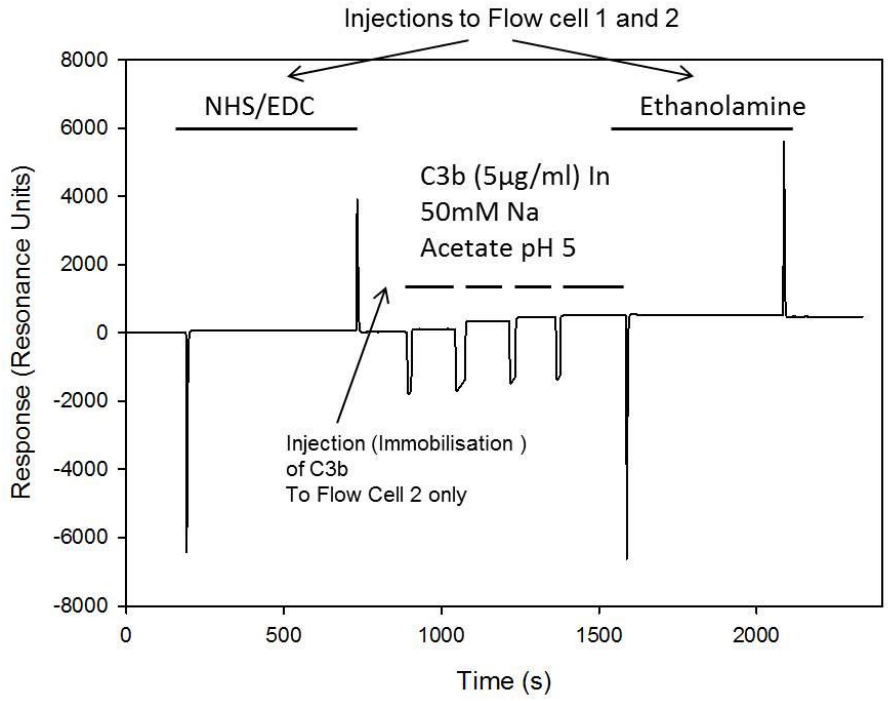

Figure 23 Immobilisation of C3b by standard amine coupling

Blank substracted sensorgram (Flow cell 2 – Flow cell 1) showing activation of surface of CM5 chip by the addition of NHS/EDC over flow cells 1 and 2. This is followed by the flow of C3b on flow cell 2 only and results in an incremental rise in baseline, due to immobilisation of C3b on the surface of the CM5 chip. Finally, injection of ethanolamine over both flow cells blocks active sites.

2.5.3 Testing Affinity of FH SCR1-4 to C3b

Wildtype and mutant FH SCR1-4 were gel-filtered into a HEPES-buffered running buffer (HBS-EP+) (10mM HEPES-buffered saline, 3mM EDTA and 0.05% surfactant P20) and concentrated. Wildtype and R83S FH SCR1-4 samples were injected over both flow cells for 90 seconds followed by a dissociation time of 600 seconds. Flow cells were regenerated using 2 x 45 second injections of 1M NaCl (Figure 24). A steady state was achieved within seconds of the injection of FH SCR1-4. A range of at least 5 different concentrations of FH SCR1-4 including at least one duplicate injection was performed. In experiments comparing R53C, D90G, G69E, Q81P, D130N, S159N, A161S and M162V and wildtype, FH SCR1-4 samples were injected over both flow cells for 45 seconds followed by a dissociation time of 100 seconds. The steady-state response was plotted against concentration of FH SCR1-4. An

affinity curve was generated using Biacore T200 Evaluation software (GE Healthcare) and the affinity constant (K_D) determined for mutant proteins and compared to wildtype performed on the same immobilised C3b.

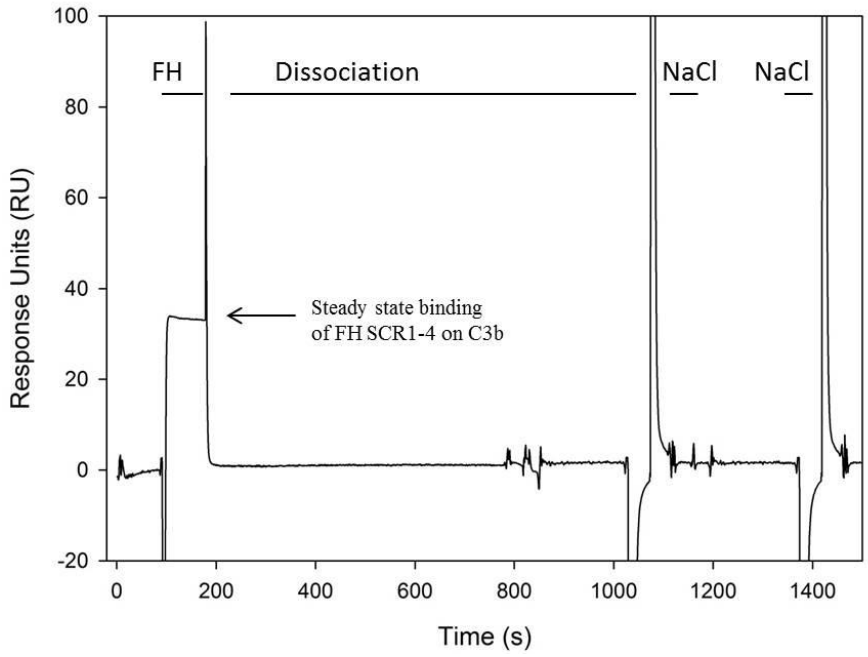

Figure 24 Initial single cycle of binding of FH SCR1-4 to immobilised C3b

FH SCR1-4 is injected over the surface of immobilised C3b. A senogram shows the rapid association and acheivement of steady state binding between FH SCR1-4 and C3b. At the end of FH injection, there is rapid dissocation. The spike at the end of FH injection is an artefact of refractive index changes of different buffers. Due to the rapid dissocation to baseline, the period of dissociation and the number of regneration steps was reduced to shorten assay time.

2.5.4 Testing Decay Activity in Real-time Using SPR

In initial experiments testing R83S, R53C and D90G, FB (CompTech) and FD (CompTech) were buffer exchanged into a HEPES-buffered running buffer supplemented with magnesium (HBS-P+Mg) (HEPES-buffered saline containing 0.5% (v/v) surfactant P20 and 1mM MgCl2) and concentrated. A mixture of 500µM FB and 60µM FD in HBS-P+Mg was injected over both flow cells of immobilised C3b on the surface of a CM5 biosensor chip for 120 seconds, forming an on-chip C3bBb. C3bBb was allowed to decay for 210 seconds. FH SCR1-4 (wildtype or mutant), also in HBS-P+Mg was then injected for 45 seconds and acceleration of C3bBb decay was visualised in real time. The surface was regenerated by the injection of 2µM FH SCR1-4 for 45 seconds followed by 1M NaCl for 45 seconds.

In experiments testing decay activity of G69E, Q81P, D130N, S159N, A161S and M162V vs wildtype, FB and FD (CompTech) were diluted to 250µM FB and 30µM FD in HBS-P+Mg and was injected over both flow cells to form an on-chip C3bBb.

In experiments testing decay activity of all variants except R83S vs wildtype, sensograms were normalised from baseline prior to injection of FB and FD (0%) to the binding stability reading (100%) after injection of FB/FD. Overlaying sensograms from mutant and wildtype injections of FH SCR1-4 are shown and representative of decay acceleration for at least 2 different experiments. A representative sensogram is shown in Figure 25.

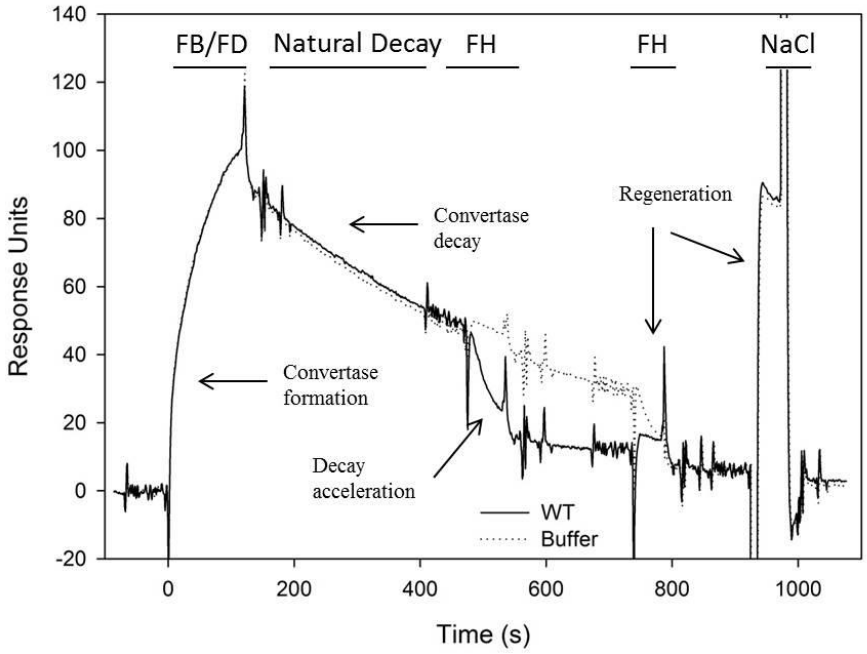

Figure 25 Decay of on-chip C3 convertase

C3bBb was formed by the injection of FB and FD in a HEPES-buffered running buffer supplemented with magnesium onto immobilised C3b on the surface of CM5 biosensor chip on Biacore X100. Sensograms for wildtype (solid line) and a buffer (dotted line) are shown in overlay. Following 210 seconds of natural dissociation of C3bBb, the first injection of WT FH accelerates C3bBb decay whereas buffer does not. At the second (regeneration) injection of FH, remaining C3bBb following initial buffer injection completely decays.A further regeneration step takes place with the injection of NaCl.

2.5.5 Testing Co-Factor Activity of FH in the Fluid Phase

A mixture of 3µL C3b (5.68µM), 4.5µL FI (114nM) and 5µL FH SCR1-4 (750nM) or 5µL of FH or FH/FHR3 (150nM) in a final volume of 15µL PBS were incubated for 30 minutes before the reaction was terminated by the addition of 15µL Laemmli 2x reducing sample buffer (Sigma). A positive and negative control was set up by the addition of 5µL of FH (6.45µM) (CompTech) and 5µL of PBS respectively. The products of reaction were

visualised following 10% SDS-PAGE and staining with Coomassie blue or by transfer to nitrocellulose membrane and Western blotting. For Western blotting (Appendix 3 - Table 57), the α- and β-chains of C3b were detected using rabbit polyclonal C3 primary antibody (Abcam) (1:5000) and goat anti-rabbit HRP secondary antibody (Abcam) (1:5000) followed by chemoluminescence. Evidence of co-factor activity was visualised by the loss of the α'-chain and appearance of the α_1-chain (Figure 26).

In the study of the R83S mutant protein, a mixture of 3µL C3b (5.68µM), 4.5µL FI (114nM) and 5µL FH (750nM) in a final volume of 15µL PBS were incubated for 15, 30, 60 and 120 minutes before the reaction was terminated by the addition of 15µL 2x Laemmli 2x reducing sample buffer. Co-factor activity was visualised following 10% SDS-PAGE and Western blotting by the loss of the α'-chain and appearance of the α_1-chain. The image was captured using BioSpectrum 500 Imaging System (UVP) and the intensity of the bands quantified using VisionWorks®LS Analysis Software (UVP) and normalised to the β-chain.

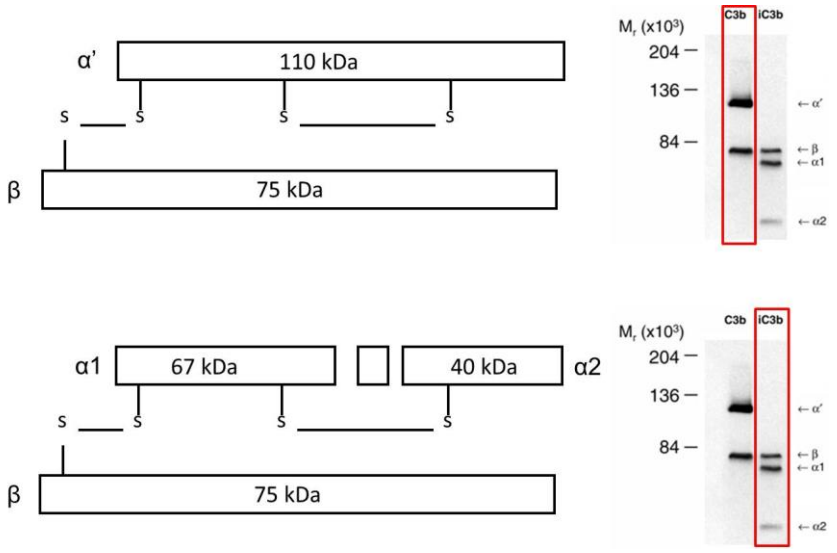

Figure 26 Inactivation of C3b in a fluid phase co-factor assay

In this represenative figure, C3b formed of α'- and β-chain are held together by disulphide bonds. In the presence of FI and a co-factor, FH, inactivation of C3b to iC3b via proteolytic cleavage of the α'-chain occurs, forming α_1 and α_2 chains. Under reducing conditions, the α- and β-chains are separated on 10% SDS-PAGE and transferred onto nitrocellulose membrane. Bands are visualised using rabbit anti-goat (1:5000) and goat anti-rabbit–HRP (1:5000) as primary and secondary antibody respectively follwed by enhanced chemoluminescence. Loss of the α'-chain and appearance of the α_1 and α_2 chains indicate intact co-factor activity of FH.

2.5.6 Generation of C3b-coated Sheep Erythrocytes

Sheep erythrocytes in Alsever's solution (TCS Biosciences) were washed 3 times in complement fixation diluent (CFD) (Oxoid) by centrifugation at 2000rpm for 5 minutes.

Washed sheep erythrocytes were sensitised by incubation with rabbit anti-sheep RBC stroma (Sigma) (1:4000) at a final concentration of 2% erythrocytes in CFD for 30 minutes at 37°C.

Sensitised sheep erythrocytes (EA) were then washed 3 times in CFD by centrifugation at 2000rpm for 5 minutes and resuspended at a final concentration of 2%. C3b was then deposited on the surface of EA by the addition of 1 volume of 4% ΔBΔHNHS and 6µg/ml *Ornithodoros moubata* complement inhibitor (OMCI) (Kind gift from Varleigh Immuno Pharmaceuticals) in CFD and an equal volume of 2% EA and incubated for 20 minutes at 37°C (Figure 27). EA-coated with C3b (EA-C3b) were then washed 3 times in AP buffer (5mM sodium barbitone, pH 7.4, 150mM NaCl, 7mM $MgCl_2$ and 10mM EGTA) and resuspended at a final concentration of 2% for decay and co-factor experiments.

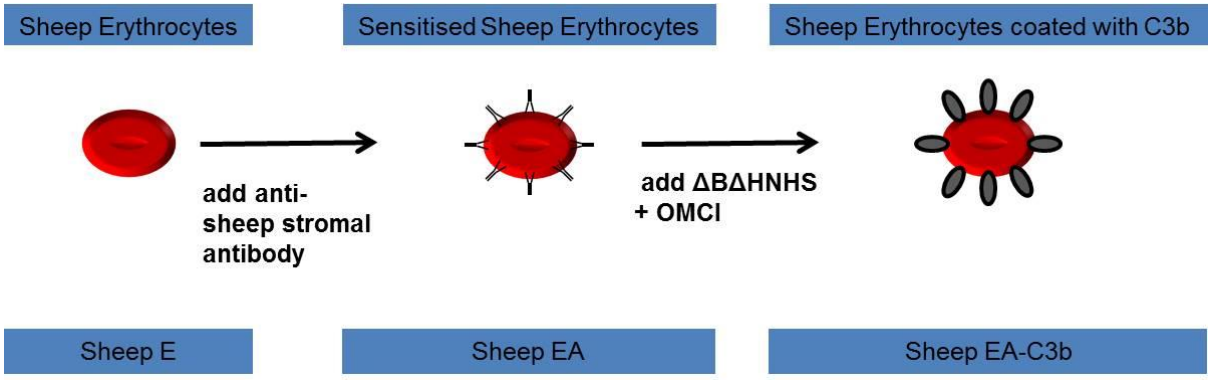

Figure 27 Generation of sheep EA-C3b

Sheep E is sensitised by the addition of Sheep E and anti-sheep RBC stroma in a complement fixation diluent that contains calcium and magnesium ions permitting classical pathway activity. The cells are washed and sensitised sheep E (sheep EA) are added to ΔBΔHNHS and OMCI. OMCI is added to prevent cell lysis by serum. C3b deposits on the surface of Sheep E by the classical pathway. The depletion of FB from serum stops C3bBb formation at this stage. Cells are then washed into AP buffer for functional tests. Subsequent testing in both AP buffer (containing EGTA that chelates calcium ion) and PBS supplemented with EDTA (that chelates calcium and magnesium ions) prevents further classical pathway activation in the remainder of the protocol.

2.5.7 Testing Decay Activity of FH on the Surface of Sheep Erythrocytes

C3bBb was formed on the surface of sheep erythrocytes by the addition of 50µL of 40µg/ml FB and 0.2µg/ml FD diluted in AP buffer to 50µL of EA-C3b for 15 minutes at 37°C. C3bBb formation was then stopped by the addition of PBS containing 250µM EDTA pH 7.4 at a 1:20 dilution. C3bBb decay was then commenced by the addition of a concentration range of FH (wildtype or mutant) in triplicate, diluted in 50µl PBS for 15 minutes at 25°C. Lysis was then instigated as measure of remaining of C3bBb by the addition of 4% ΔBΔHNHS in PBS and incubation for 20 minutes at 37°C (Figure 28). To determine the amount of lysis, cells

were pelleted at 1200rpm for 3 minutes and 100µL of supernatant was sampled and the absorbance at 420nm (A_{420}) measured on Multiskan Ascent® (Agilent). No lysis controls for blank subtraction were set up by the addition of no serum. Maximal lysis was determined by A_{420} from the addition of PBS instead of FH. Mean readings of experiments in triplicate were calculated. Total protection from lysis for each concentration of FH was determined by 1-[mean A_{420} (FH) – mean A_{420} (blank)]/A_{420} (PBS) expressed as a percentage. A sigmoidal curve was fitted using SigmaPlot and used to determine the concentration at which FH gave 50% protection from lysis (IC_{50}).

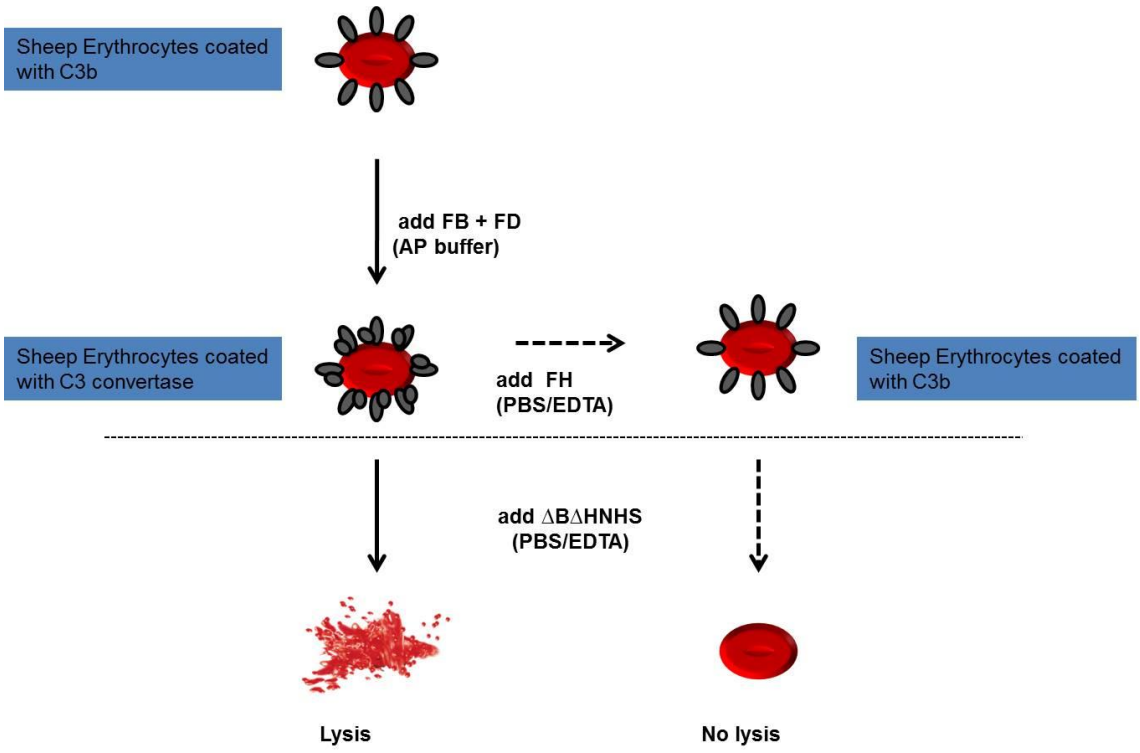

Figure 28 Decay activity on surface of sheep erythrocytes

C3bBb is formed by the addition of FB and FD on the surface of sheep EA-C3b in AP buffer, permitted by the presence of magnesium. Lysis can then be initiated by the addition of ΔBΔHNHS (diluted in PBS containing EDTA) from which C3 is cleaved via preformed C3bBb on the surface of sheep erythrocytes. Lysis occurs following the subsequent formation of C3bBbC3b and activation of terminal pathway and MAC formation. EDTA chelates both calcium and magnesium ions preventing further formation of C3bBb via AP. Lysis is therefore dependent the amount of C3bBb present prior to the addition of ΔBΔHNHS. Decay activity of FH can be tested on the surface of Sheep EA-C3b with formed C3bBb. This is added to FH that binds to C3b and sialic acids on sheep E. FH decays C3bBb and protects sheep E from lysis.

2.5.8 Testing Co-Factor Activity of FH on the Surface of Sheep Erythrocytes

Co-factor activity of FH was determined by the addition of 50µL of a concentration range of FH (wildtype or mutant) in triplicate, and 2.5µg/ml FI in AP buffer to 50µL of EA-C3b. In the experiments for R83S and R53C, co-factor activity was allowed to occur for 8 minutes at

25°C (Figure 29). In the experiments for the remainder of mutants, co-factor activity was allowed to occur for 15 minutes. After the incubation period, cells were washed 3 times in 200µL of AP buffer followed by pelleting for at 1200rpm 3 minutes. After the final wash, cells were resuspended in 50µL AP buffer. C3bBb was formed on the surface of sheep erythrocytes containing intact C3b by the addition of 50µL of 40µg/ml FB and 0.2µg/ml FD to 50µL of EA-C3b for 15 minutes at 37°C. Lysis was then instigated as measure C3bBb that was able to form by the addition of 4% ΔBΔHNHS and incubation for 20 minutes at 37°C. To determine the amount of lysis, cells were pelleted for 3 minutes at 1200rpm and 100 µL of supernatant was sampled and the absorbance as 420nm measured (A_{420}). No lysis controls for blank subtraction were set up by the addition of no serum. Maximal lysis was determined by A_{420} from the addition of PBS instead of FH. Mean readings of experiments in triplicate were calculated. Total protection from lysis for each concentration of FH was determined by 1-[mean A_{420} (FH) – mean A_{420} (blank)]/A_{420} (PBS) expressed as a percentage. A sigmoidal curve was fitted using SigmaPlot and used to determine the concentration at which FH gave 50% protection from lysis (IC_{50}).

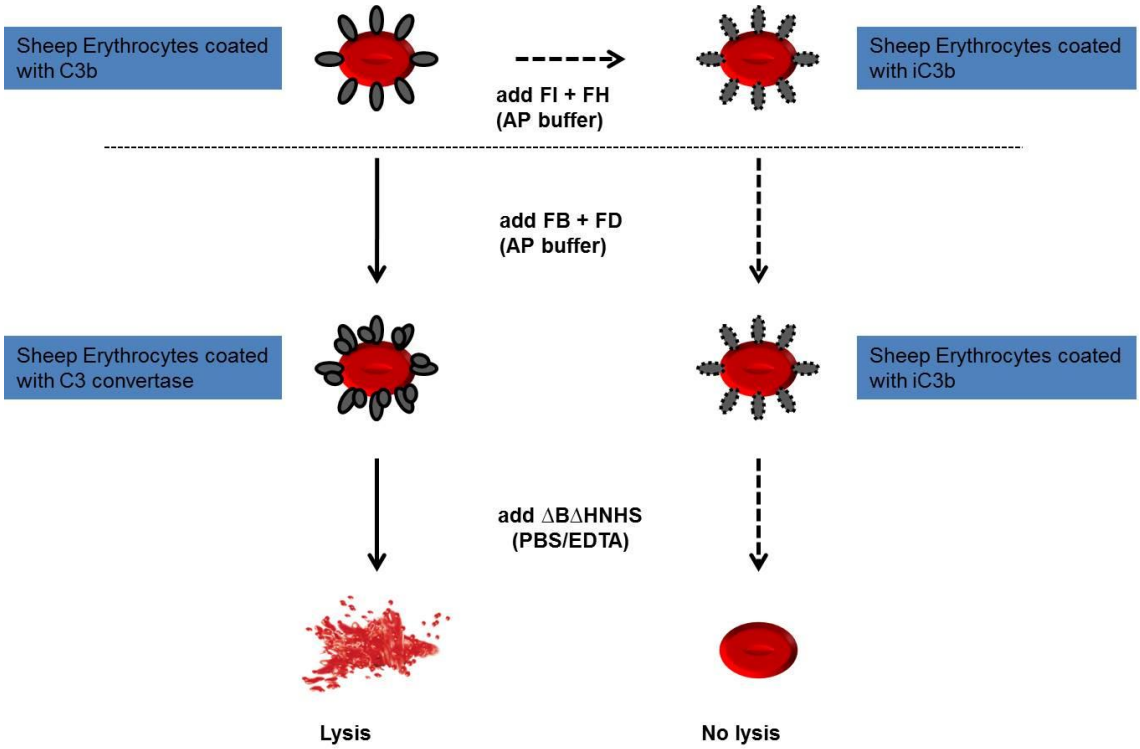

Figure 29 Co-factor activity on surface of sheep erythrocytes

C3bBb is formed by the addition of FB and FD on the surface of sheep EA-C3b in AP buffer. Lysis as before can then be initiated by the addition of ΔBΔHNHS. Co-factor activity of FH can be tested on the surface of Sheep EA-C3b. Sheep EA-C3b is added to FI and FH. FH acts as a co-factor for the FI-mediated proteolytic cleavage of C3b into iC3b and prevents C3bBb formation. In turn, this protects sheep E from lysis.

Chapter 3 – Complement Abnormalities in Cohorts of MPGN and C3G

3.1 Introduction

MPGN and C3G are rare diseases that associate with abnormalities of the AP. Included in these cohorts are the distinct phenotypes, IC-MPGN, DDD and C3GN that are characterised by their appearances on light microscopy, electron microscopy and immunofluorescence. These patients share clinical features that include proteinuria, haematuria and renal failure (Servais *et al.*, 2012; Medjeral-Thomas *et al.*, 2014b). Renal failure is progressive and 40% of patients develop ESRD at 10 years (Cansick *et al.*, 2004; Servais *et al.*, 2012; Medjeral-Thomas *et al.*, 2014b). Recurrence in transplantation is common in all subtypes, ranging from 30-40% in MPGN to 80-90% in DDD (Braun *et al.*, 2005). There are no universally effective treatments for these conditions.

The role of the AP has been described in patients with MPGN/C3G for many years. Uncontrolled activation of AP in association with C3 nephritic factor (Spitzer *et al.*, 1969) and FH deficiency (Levy *et al.*, 1986) in MPGN has been reported for over 30 years. More recently, functional studies of familial rare genetic variants in *CFH* (Licht *et al.*, 2006) and *C3* (Martinez-Barricarte *et al.*, 2010; Chauvet *et al.*, 2015) have also been reported.

Genetic variants in *CFH*, *CFI*, *CD46*, *C3* and *CFB* have been described in several cohorts of MPGN/C3G and includes rare genetic variants and common polymorphisms (Servais *et al.*, 2012; Bu *et al.*, 2015; Iatropoulos *et al.*, 2016). Autoantibodies to FH have been described and characterised in cohorts of MPGN/C3G (Goodship *et al.*, 2012; Blanc *et al.*, 2015). Furthermore, studies of several large families with C3G have led to the discovery of abnormal FHR proteins, arising due to genomic abnormalities within a region of high sequence homology (Malik *et al.*, 2012; Tortajada *et al.*, 2013; Zhang *et al.*, 2013b; Chen *et al.*, 2014) and includes *CFHR5* nephropathy (Gale *et al.*, 2010). Studies of these abnormal FHR proteins have suggested a role for FHRs on surface bound C3b resulting in the de-regulation of FH (Goicoechea de Jorge *et al.*, 2013; Tortajada *et al.*, 2013).

Further cohort studies to determine the prevalence of these acquired and genetic abnormalities of AP are required. Ongoing study of these abnormalities might improve our understanding of the factors that determine disease onset and progression. This may allow a targeted approach to therapies in MPGN and C3G.

3.1.1 Chapter Aims

In this chapter, I will describe my analysis of two cohorts of patients with MPGN/C3G.

In a prospective study of a paediatric (RaDaR) cohort recruited from 2011 until July 2014, DNA, serum and biopsies were collected. In this study,

1. I co-ordinated the central review of pathological samples by an expert pathologist (Prof Terry Cook) with a special interest in C3G;
2. I analysed the clinical data that was provided by the RaDaR registry;
3. I analysed the sequencing and copy number variation data that was undertaken by the Northern Molecular Genetics Service and
4. I analysed the autoantibody data provided by Dr. Kevin Marchbank (and performed the screening for autoantibodies against FHR3 and FHR4).

In a retrospective cohort of patients referred to the Newcastle Complement Genetics Service since 2001,

1. I analysed the autoantibody data performed by Dr. Kevin Marchbank and
2. I analysed the sequencing data that was undertaken by the Northern Molecular Genetics Service.

Overall, my aim was to describe the prevalence of acquired and genetic abnormalities of AP in these cohorts of MPGN/C3G, in which I predict these abnormalities to be present.

3.2 Complement Abnormalities in a Prospective Paediatric Cohort of MPGN and C3G

3.2.1 Clinical Characteristics of a Prospective Paediatric Cohort (RaDaR)

Patients from 10 paediatric centres were recruited into the National Study of MPGN and C3G, a part of RaDaR. A diagnosis from renal biopsy was made by pathologists at local centres. Eighty-three patients were recruited between 2011 and July 2014. Central pathology review was performed by Professor Terry Cook of available histology, immunofluorescence and electron microscopy. Three patients were excluded from any further analysis at this stage as no form of glomerulonephritis could be identified on the pathological specimens available. A diagnosis of DDD, C3GN or IC-MPGN was made in 64 patients. In a further 8 patients, there was immune-complex deposition. Light microscopy review of these patients identified features of mesangial expansion and cellular proliferation but insufficient double contouring of glomerular basement membrane for a diagnosis of MPGN. These cases have been included in this data analysis but treated as a separate group called immune-complex glomerulonephritis (IC-GN) (Figure 30). The analysis of patients was performed for 4 sub-groups – IC-GN, IC-MPGN, DDD and C3GN.

Patients from all groups presented throughout childhood, ages ranging from 2 years to 15 (Table 19). The typical presentation of patients in all sub-groups was hypoalbuminaemia (Figure 31), proteinuria (Figure 32), and haematuria. Patients with DDD and C3GN presented with lower estimated glomerular filtration rate (eGFR) than IC-MPGN ($P<0.05$) (Figure 33).

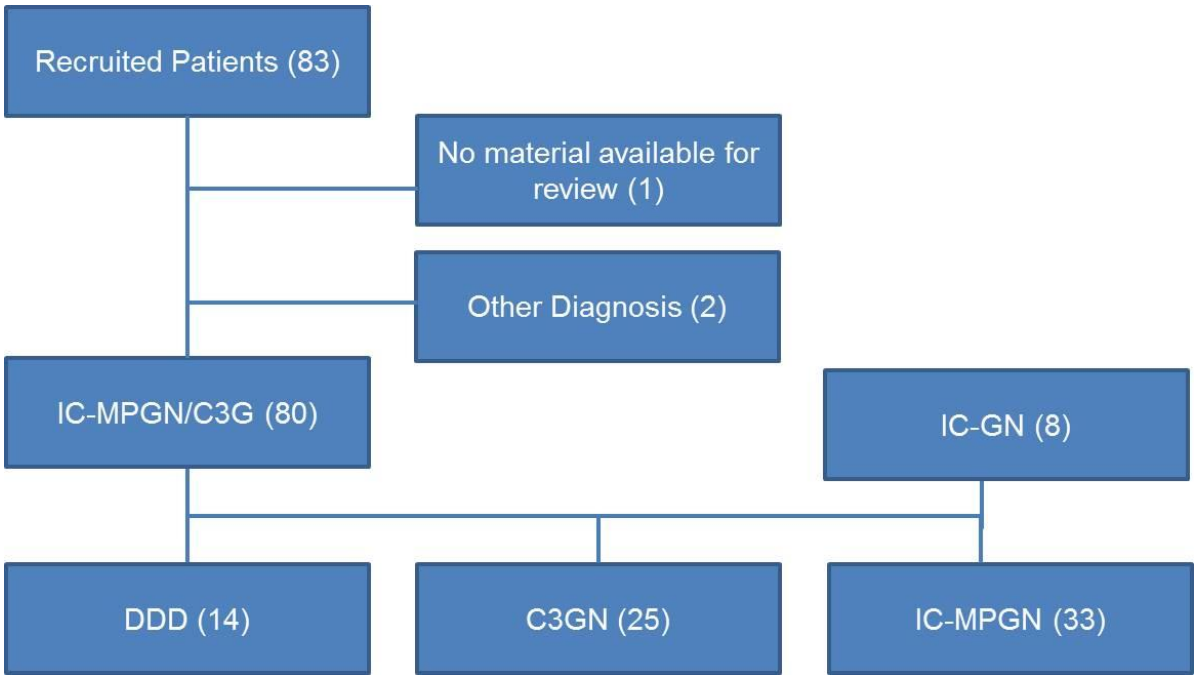

Figure 30 Pathological classification of patients in RaDaR cohort

	IC-GN	IC-MPGN	DDD	C3GN
Number of patients	8	33	14	25
Median age at presentation (range)	8 (2-10)	9 (2-15)	9.5 (4-15)	9 (4-15)
Median albumin at presentation (n)(g/L)	34 (7)	25 (31)	30 (14)	26 (22)
Median P:Cr (n) (mg / mmol creatinine)	332.3 (5)	546.9 (18)	775.5 (8)	402.0 (17)
Number of patients with documented haematuria (%)	6 (75.0)	20 (60.1)	10 (71.4)	13 (52.0)
Median eGFR (ml/min/1.73m^2) at presentation (n)	95.5 (4)	125.5 (24)	74 (7)	96 (18)

Table 19 Clinical characteristics of RaDaR cohort

n = number of patients for which data available, P:Cr = protein to creatinine ratio, Cr = creatinine, % = percentage of total number of patients, eGFR= estimated glomerular filtration rate, determined by Schwartz formula.

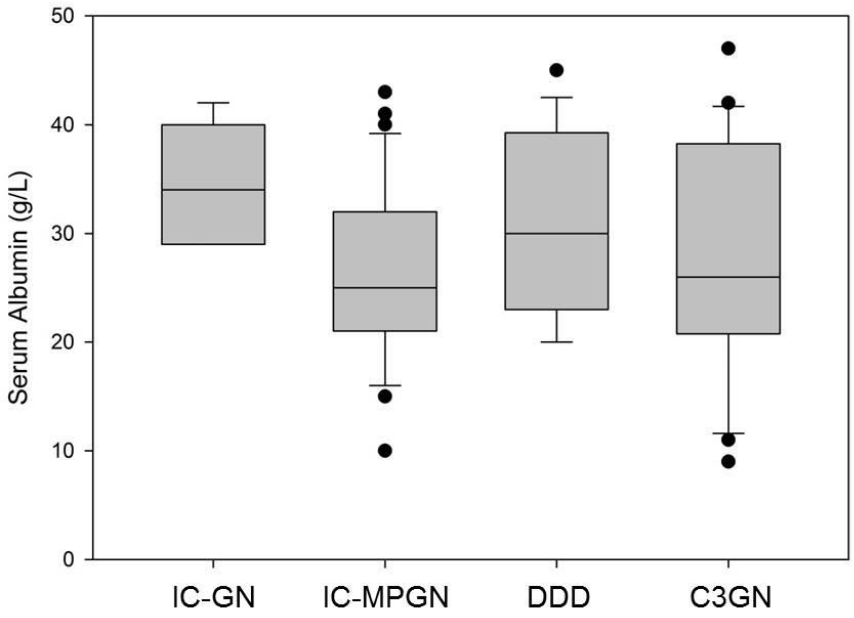

Figure 31 Serum albumin at presentation in RaDaR cohort

A lower limit of normal serum albumin is 35g/L. The differences between groups were not statistically significant.

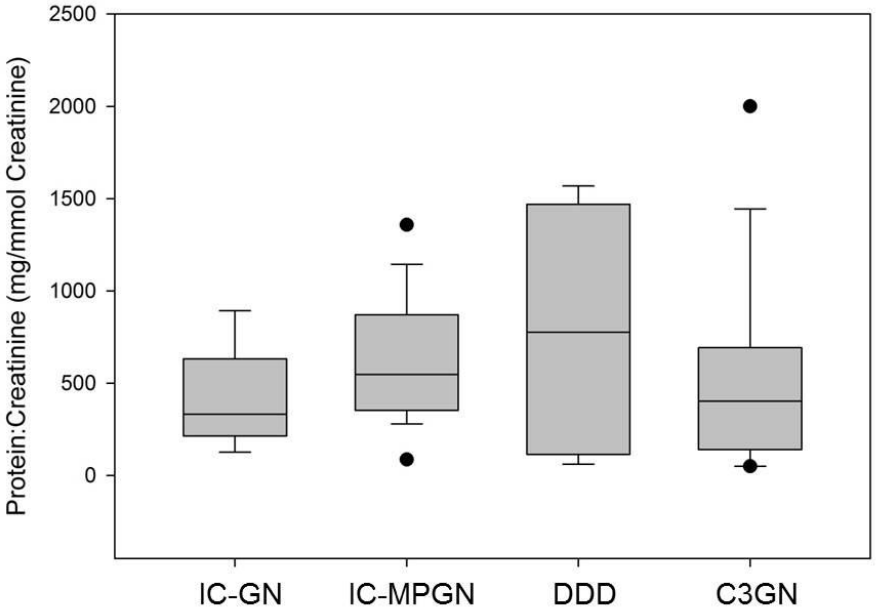

Figure 32 Urinary protein:creatinine at presentation in RaDaR cohort

An upper limit of normal urinary protein:creatinine is 20mg/mmol creatinine. The differences between groups were not statistically significant.

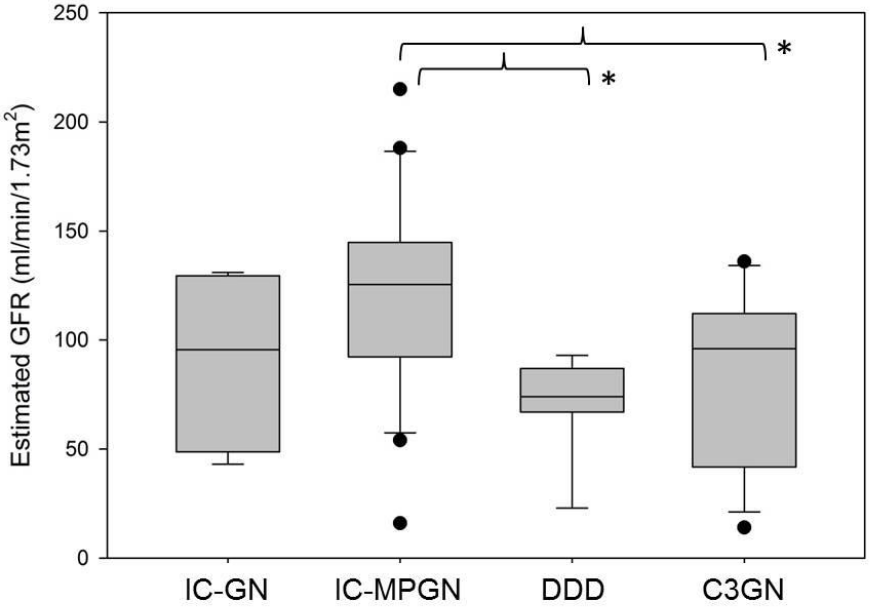

Figure 33 Estimated glomerular filtration rate at presentation in RaDaR cohort

*Normal estimated glomerular filtration rate (eGFR) is >90ml/min/1.73m^2. eGFR determined by Schwartz formula. eGFR was significantly lower in DDD and C3GN compared to IC-MPGN *= P<0.05.*

3.2.2 Complement Levels in RaDaR Cohort

At the time of first investigation, 83.6% of patients had a low C3 level. C3 levels were lowest in patients with DDD (Figure 34). Over 70% of patients with immune-complex disease (IC-GN and IC-MPGN) had a low C4, a higher proportion compared to patients with C3 glomerulopathy (DDD and C3GN) (Figure 35). C3 and C4 levels for individual patients can be seen in Figure 36. Patients with low C4 were more likely to have a form of immune-complex disease than C3 glomerulopathy (Odds Ratio 6.6, P=0.0009) (Table 20).

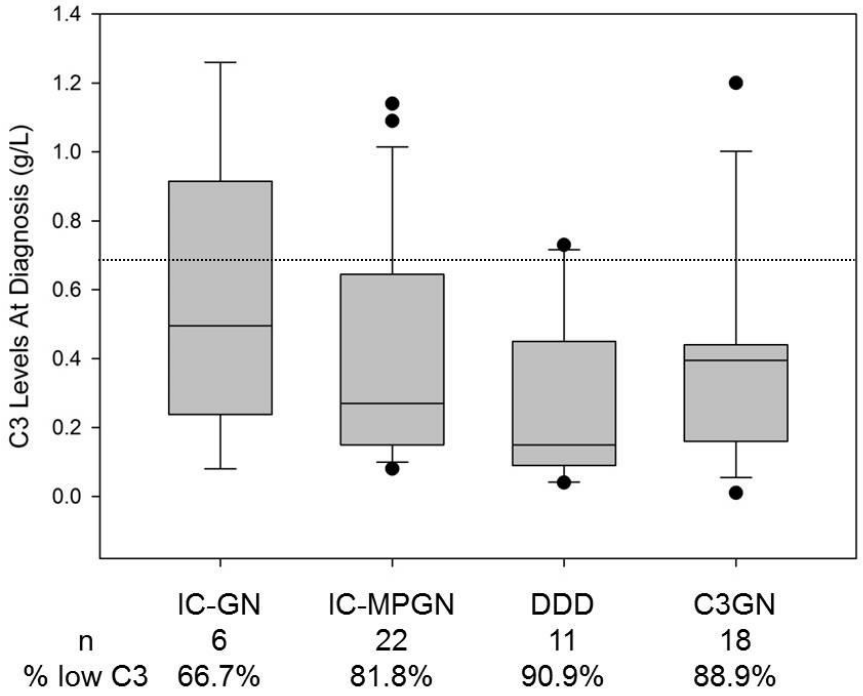

Figure 34 C3 levels in RaDaR cohort at diagnosis

Dotted line indicates lower limit of normal C3 (0.68g/L). n = number of patients in sub-group with data available. The differences between groups were not statistically significant.

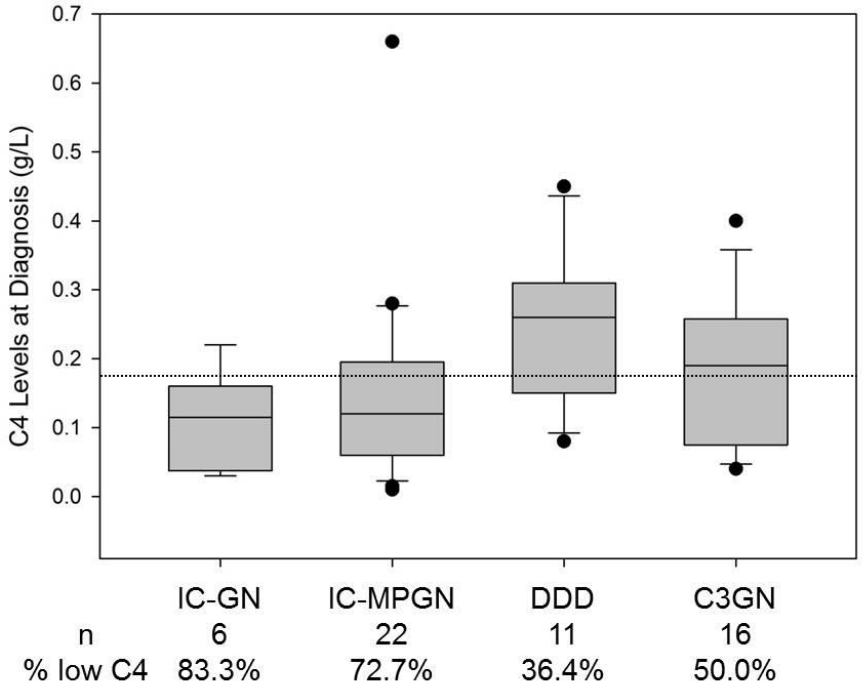

Figure 35 C4 Levels in RaDaR Cohort at diagnosis

Dotted line indicates lower limit of normal C4 (0.18g/L). n = number of patients in sub-group with data available. The differences between groups were not statistically significant..

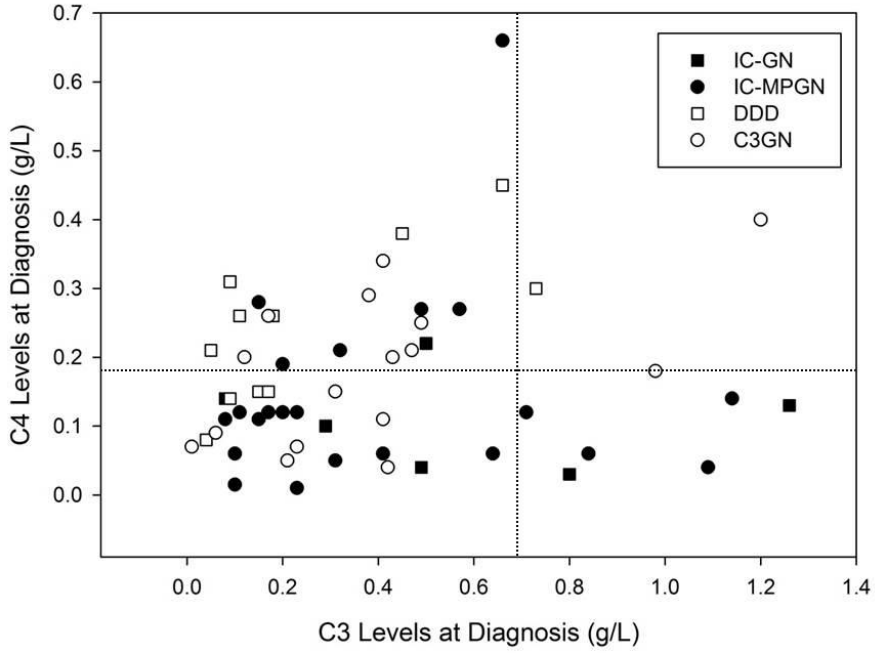

Figure 36 C3 and C4 levels plotted for individual patients according to disease sub-group at diagnosis

Dotted lines indicate lower limits of normal complement levels. Filled markers are forms of immune-complex disease. Open markers are forms of C3 glomerulopathy.

	Immune-Complex Disease	C3 Glomerulopathy
Number of patients with Low C4 (%)	32 (82.1)	11 (40.7)
Number of patients with normal C4 (%)	7 (17.9)	16 (59.3)

Table 20 Frequency of patients with low or normal C4 Levels in RaDaR cohort at diagnosis

Patients with low C4 were more likely to have immune-complex disease than C3 glomerulopathy (Odds Ratio 6.6 P=0.0009).

C3 and C4 levels were measured on samples at time of recruitment into the RaDaR cohort in the laboratory at Newcastle Hospitals NHS Foundation trust. These samples were taken at a median duration of 23.4 (IQR 3.0 – 48.1) months from initial diagnosis (Table 21). This duration was used as a measure of initial follow-up time in this study. Follow-up in the patients with DDD was slightly less than in patients in other sub-groups but not statistically significant. C3 and C4 levels at diagnosis and follow-up are summarised in Figure 37 and Figure 38. Median C3 levels increased from diagnosis to follow-up in all sub-groups. This increase was statistically significant in IC-GN, IC-MPGN and C3GN. Fewer patients at follow-up in all sub-groups had low C3, though almost three-quarters of patients with DDD still had low C3 at this time.

	Number of Patients	Median Follow Up (months)	IQR Follow Up (months)
All	80	23.4	3.0 – 48.1
IC-GN	8	27.8	3.2 - 57.4
IC-MPGN	33	35.5	4.3 - 52.9
DDD	14	10.5	1.1 - 44.3
C3GN	25	20.0	3.3 - 40.5

Table 21 Time since diagnosis at time of recruitment of patients into RaDaR

Follow-up times between groups was not statistically significant. IQR = interquartile range.

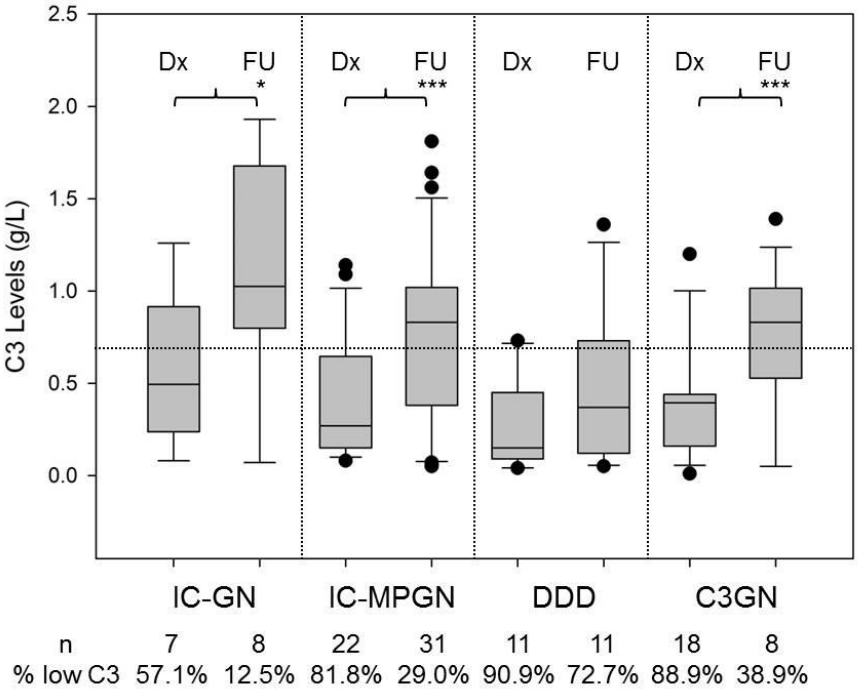

Figure 37 C3 levels at diagnosis and follow-up

*Dotted lines indicate lower limits of normal complement levels. n = number of patients in sub-group with data available. C3 levels were significantly higher at follow-up (FU) compared to diagnosis (Dx) in IC-GN, IC-MPGN and C3GN. *=P<0.05 ***=P<0.001.*

C4 levels were slightly higher at follow-up compared to diagnosis in the two immune-complex sub-groups, though half of patients continue to have low C4 levels. None of the differences in C4 levels between diagnosis and follow-up were statistically significant.

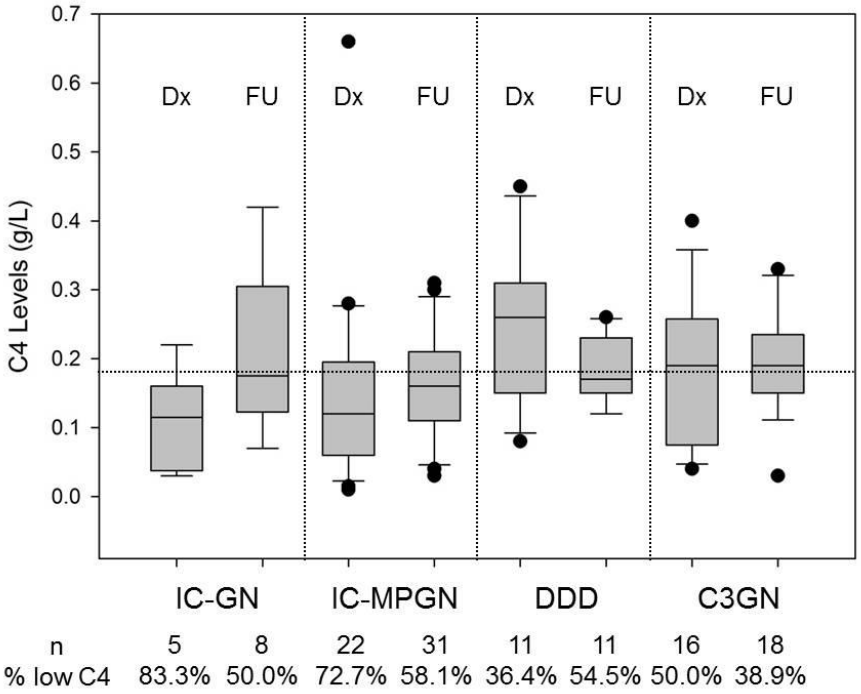

Figure 38 C4 levels at diagnosis and follow-up

Dotted lines indicate lower limits of normal complement levels. n = number of patients in sub-group with data available. The differences between groups were not statistically significant.

3.2.3 C3 Nephritic Factor in RaDaR cohort

There was a poor return of data for C3 nephritic factor at time of diagnosis from the clinical teams. It was available in only 27 cases (33.8%). Using this data only, an estimate of prevalence of C3 nephritic factor in this cohort was determined (Figure 39). The prevalence of C3 nephritic factor in DDD was 66.7%, 28.6% in IC-MPGN and 33.3% in C3GN. A C3 nephritic factor at the time of diagnosis was not detected in the available results from patients with IC-GN.

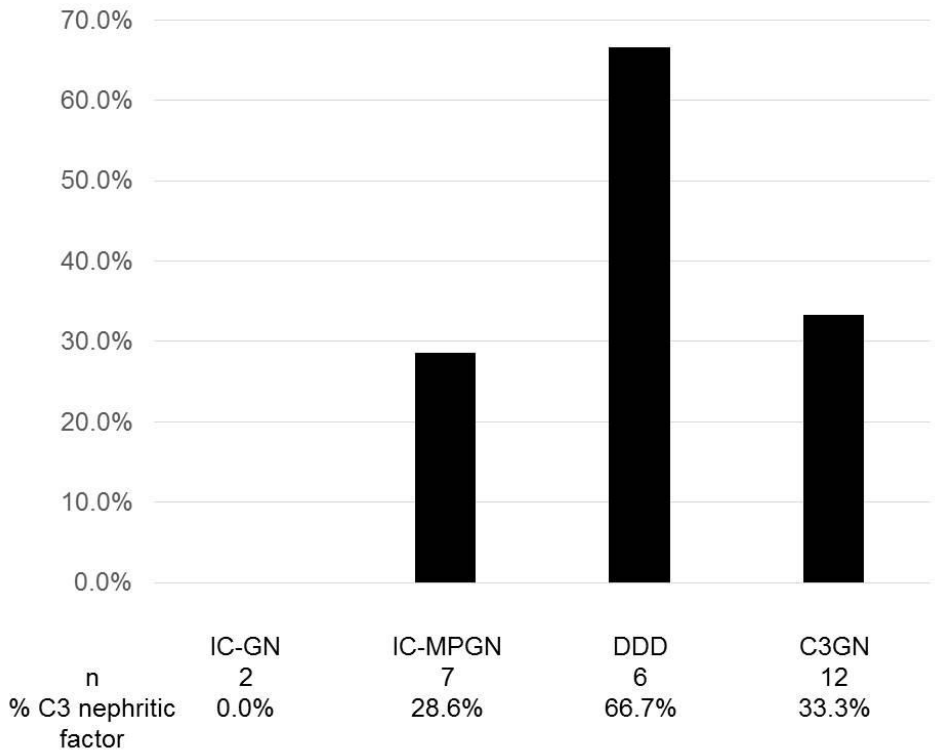

Figure 39 C3 nephritic factor in RaDaR cohort at diagnosis

The prevalence of C3 nephritic factor in this cohort was 42.1%, and highest in the DDD group. n = number of patients in sub-group with data available. The differences between groups were not statistically significant.

C3 and C4 levels for individual patients are shown in Figure 40 for patients with available C3 nephritic factor data at diagnosis. In this limited dataset, 18/21 (85.7%) patients had low C3 levels and was comparable with the figure of 83.6% of patients described in section 3.2.2. All patients with a low C3 nephritic factor at diagnosis had a low C3 level. C3 levels were lower in patients with a C3 nephritic factor at diagnosis compared those without (P=0.039) (Figure 41). C4 levels were lower in patients without C3 nephritic factor though this difference did not reach statistical significance (Figure 42).

In patients in whom C3 nephritic factor data was available, the association of C3 levels with C4 levels and the presence of a C3 nephritic factor is shown (Figure 43). Low C3 levels were explained by low C4 levels or C3 nephritic factor in 11 patients. Low C3 levels were not explained by low C4 levels or C3 nephritic factor in 5 patients. A further two patients (1 IC-MPGN and 1 C3GN) with low C3 levels had both low C4 levels and C3 nephritic factor. In the 3 patients that had normal C3 levels, 1 (IC-GN) had low C4 levels and 2 (both C3GN) had normal C4 levels (Figure 43).

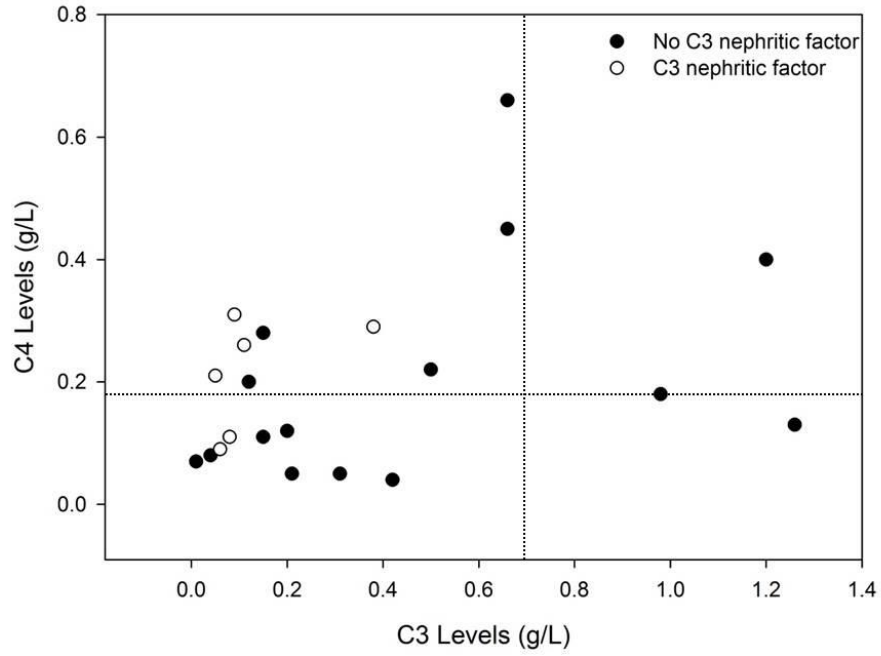

Figure 40 C3 vs C4 levels for individual patients according to C3 nephritic factor status

Dotted lines indicate lower limits of normal complement levels.

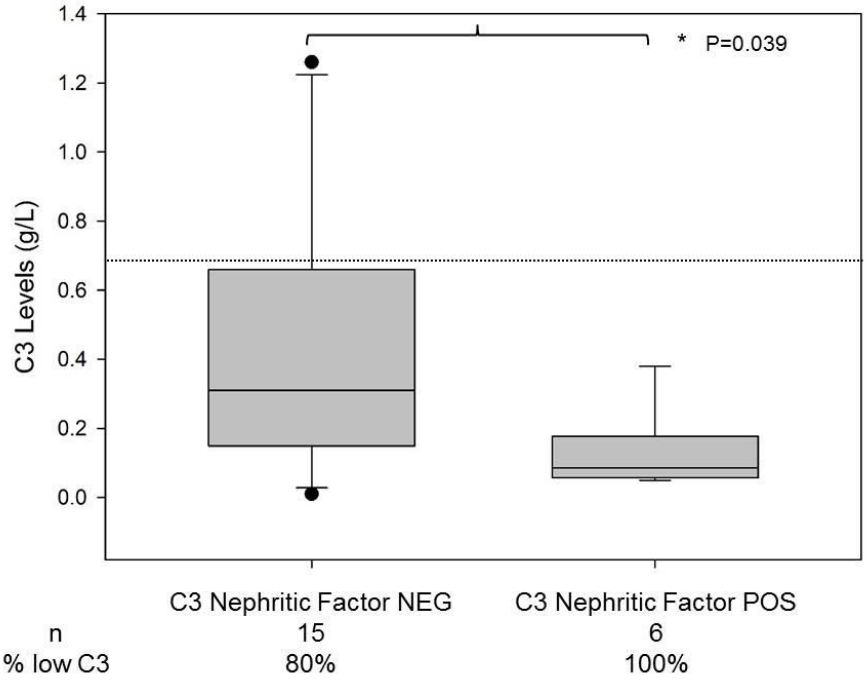

Figure 41 C3 levels according to C3 nephritic factor status

Dotted lines indicate lower limits of normal complement levels. n = number of patients in sub-group with data available. C3 levels were lower if C3 nephritic factor was present (P=0.039).

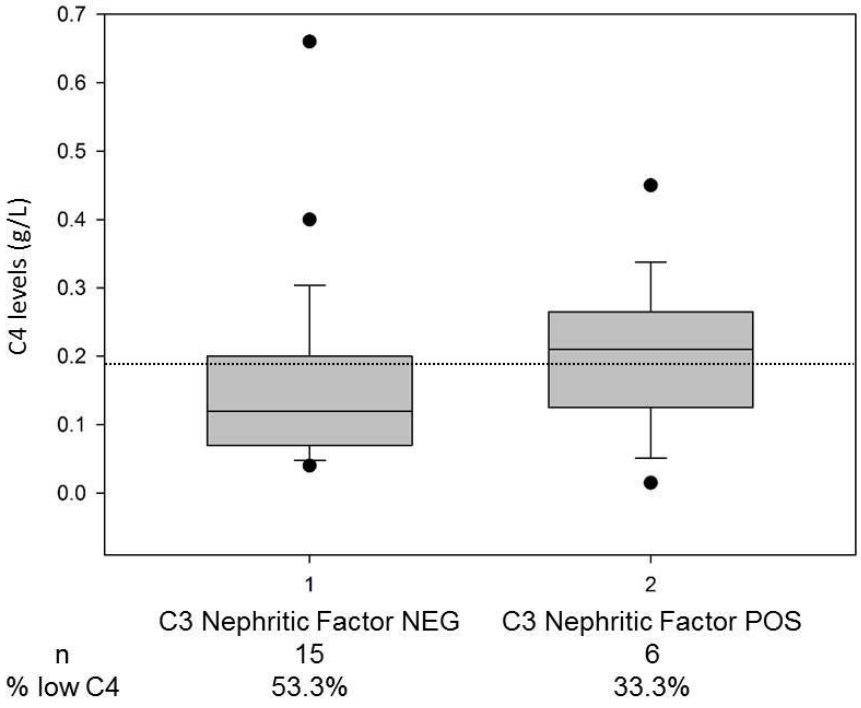

Figure 42 C4 levels according to C3 nephritic factor status

Dotted lines indicate lower limits of normal complement levels. n = number of patients in sub-group with data available. The differences between groups were not statistically significant.

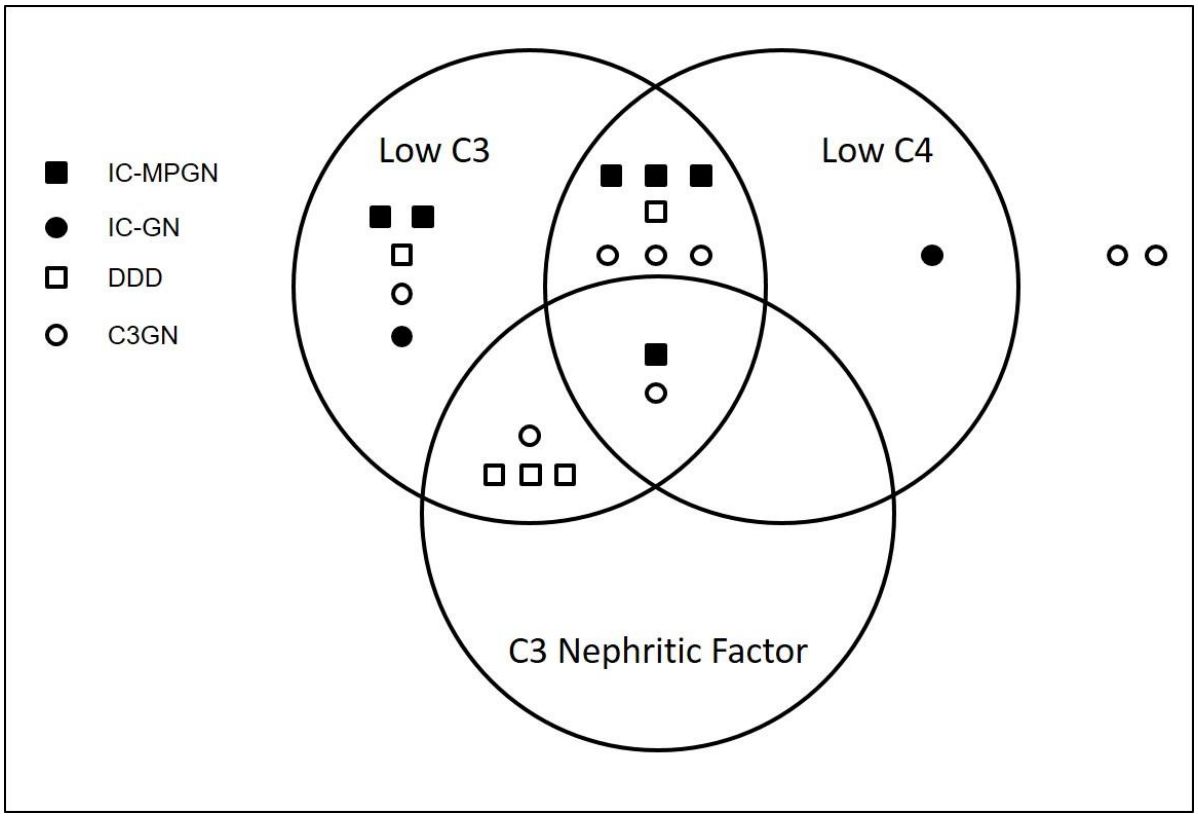

Figure 43 Patients with low C3, low C4 and C3 nephritic factor in RaDaR cohort.

3.2.4 Autoantibodies to FH in RaDaR cohort

Samples of serum at the time of recruitment were screened for the presence of autoantibodies to FH by Dr. Kevin Marchbank. These samples were taken at a median follow up time of 23.4 (IQR 3.0 – 48.1) months after diagnosis. 13/78 (16.7%) of patients were positive for autoantibodies to FH (Figure 44).

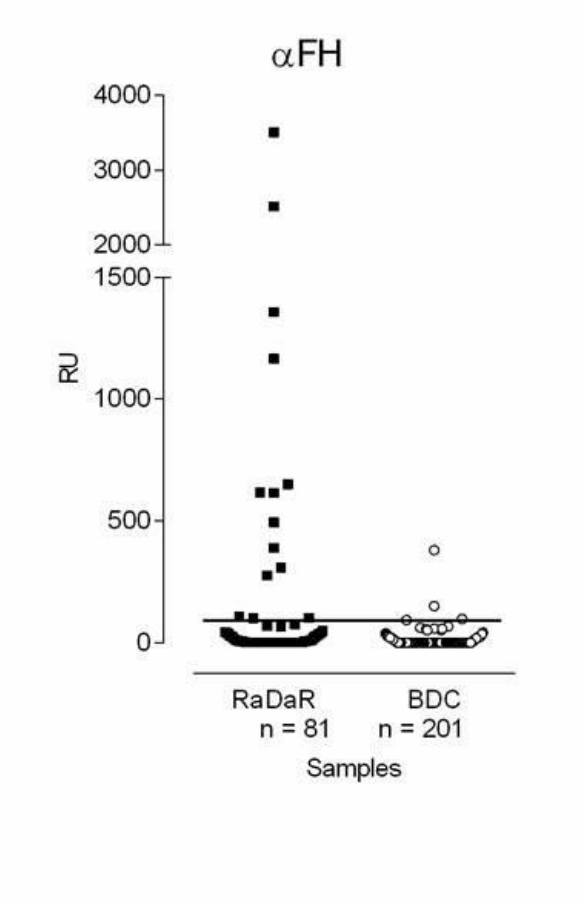

Figure 44 Raw data for screening for autoantibodies to FH

Data and Figure prepared by Dr. Kevin Marchbank. RU = response units titrated to standard published in (Goodship et al., 2012), BDC = blood donor control, line indicates 97.5th percentile. Screening was performed in an initial 81 patients, but analysis is of 78 patients that had a diagnosis of IC-MPGN/C3G following central pathology review.

Binding studies to short fragments of FH were performed by Dr. Kevin Marchbank in patients that were positive for autoantibodies to FH. Binding to FH SCR1-7, SCR8-15, SCR16-18 and SCR19-20 was tested (Figure 45). Predominant binding of each autoantibody to FH is summarised in Figure 46. Patients with autoantibodies to FH in this cohort predominantly bound to FH SCR1-7.

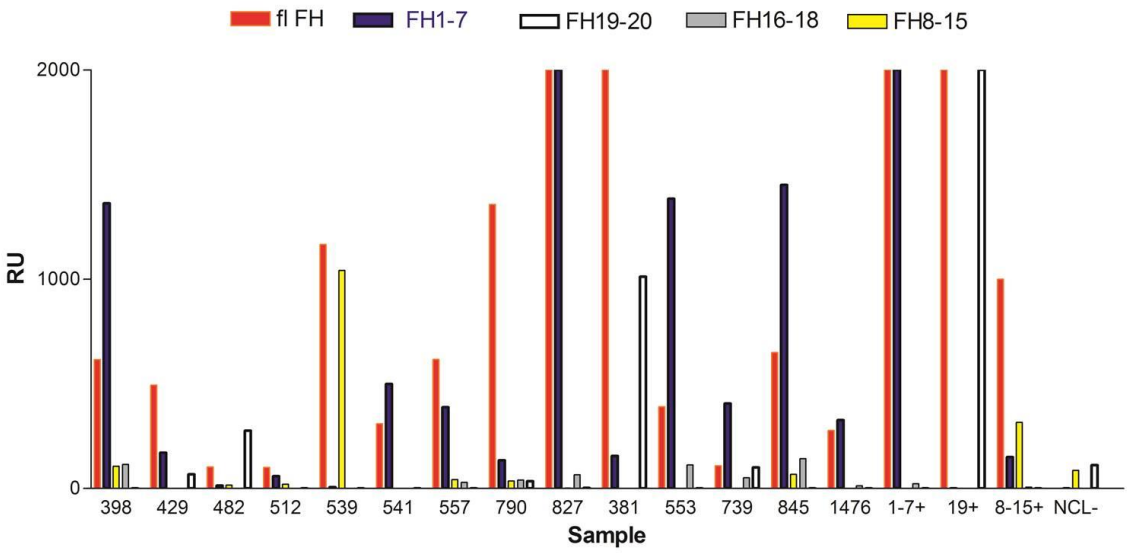

Figure 45 Epitope binding characteristics of autoantibodies to FH in RaDaR cohort

Data and Figure prepared by Dr. Kevin Marchbank. Positive and negative controls for binding to each fragment are shown at the right of the figure. 14 patients with autoantibodies to FH are indicated. Epitope binding is shown for patient 1476 – not included in analysis as excluded due to no central pathology review. RU = response units.

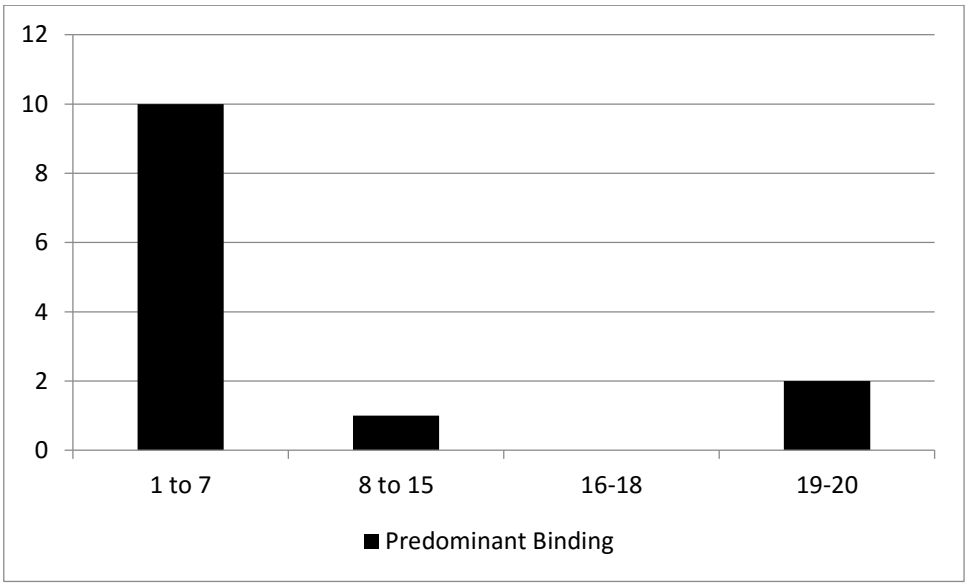

Figure 46 Predominant binding of autoantibodies to FH

Epitope binding in patients with autoantibodies to FH was tested against short SCR fragments (1-7, 8-15, 16-18 and 19-20). The predominant reactivity was to SCR1-7 in 10 cases out of 13.

The characteristics of the 13 patients with autoantibodies to FH are summarised in Table 22. C3 and C4 levels were compared from time of diagnosis and follow-up. C3 nephritic factor was identified in 2 patients (15.4%) but data for most patients was not available.

Patient	Disease	Predominant SCR Binding	C3 at Dx	C4 at Dx	C3 at FU	C4 at FU	C3 Nephritic Factor
381	C3GN	19 to 20	↓	↓	↓	↓	Not Known
398	DDD	1 to 7	↓	↔	NR	NR	P
429	IC-MPGN	1 to 7	NR	NR	↓	↓	P
482	DDD	19 to 20	NR	NR	↓	↔	Not Known
512	IC-MPGN	1 to 7	↓	↔	↓	↔	Not Known
539	IC-GN	8 to 15	NR	NR	↔	↔	N/A
541	C3GN	1 to 7	↓	↔	NR	NR	Not Known
553	IC-MPGN	1 to 7	↓	↓	↔	↓	Not Known
557	C3GN	1 to 7	NR	NR	↔	↓	N/A
739	IC-GN	1 to 7	↓	↓	↔	↓	Not Known
790	DDD	1 to 7	↓	↓	↓	↓	Not Known
827	IC-MPGN	1 to 7	↔	↓	↔	↓	N/A
845	IC-GN	1 to 7	↔	↓	↔	↓	N/A

Table 22 Disease association and complement profile of patients with autoantibodies to FH in RaDaR cohort

SCR = short consensus repeat, Dx = diagnosis, FU = follow-up, NR = no result, ↓ = below normal range, ↔ = within normal range, Not known = C3 levels were low but no C3 nephritic factor data available, P = positive, N/A = C3 levels were normal, C3 nephritic factor testing not applicable in the clinic.

3.2.5 Autoantibodies to other complement components in RaDaR cohort

Screening of patient serum at time of recruitment was also performed for autoantibodies to FI, CD46, CD35, CD55, CD59, FHR1, FHR2, FHR3, FHR4 and FHR5 for 81 serum samples available in the RaDaR cohort. Screening for antibodies against C3b and FB was performed in 80 samples. I performed screening for autoantibodies against FHR3 and FHR4. Screening for all other autoantibodies was performed by Dr. Kevin Marchbank. The raw data was summarised by Dr. Kevin Marchbank and presented in Figure 47 to Figure 49.

Data was plotted in response units using a previously designated positive control for screening against FI, CD46, CD35, CD55 and CD59 (Kavanagh *et al.*, 2012; Watson *et al.*, 2015). A minimum threshold for positive autoantibodies was determined by the 97.5th percentile of values from screening of at least 100 blood donor controls for the same protein. In a small number of patients, a value above the cut-off was noted. A specific antibody response to these proteins was not demonstrated in Western blot analysis by Dr. Kevin Marchbank (data not shown). Therefore, no autoantibodies were identified against other complement regulatory components (FI, CD46, CD35, CD55 and CD59) (Figure 47).

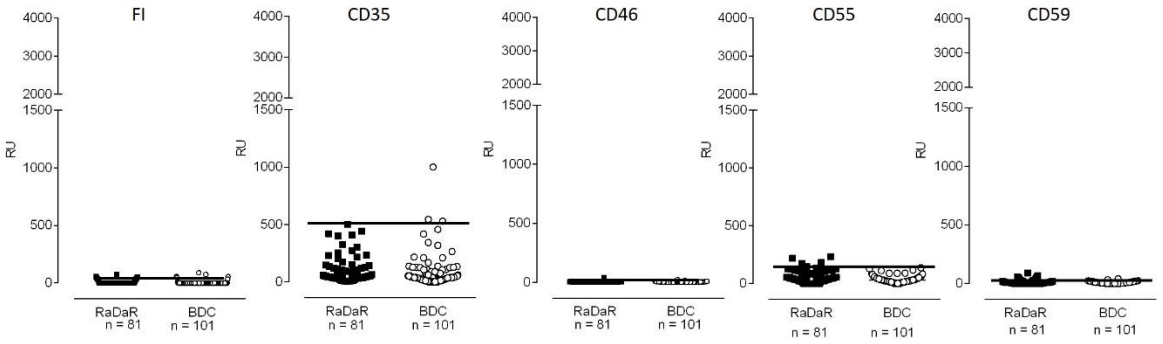

Figure 47 Screening for auto-antibodies to complement regulatory proteins

Data and figure generated by Dr. Marchbank. Autoantibodies to FH were identified, but not to FI, CD46, CD35, CD55 or CD59. BDC = blood donor control, line indicates 97.5th percentile, the minimum threshold for identifying an autoantibody. Positive findings were not confirmed in western blot testing.

Screening for autoantibodies was performed against the FHR proteins. FHR proteins were expressed in mammalian cell lines by Dr. Kevin Marchbank. Data is shown as optimal density readings and not converted to response units due to the lack of a positive control. A minimum threshold for positive autoantibodies was determined by the 97.5th percentile of values from screening of at least 100 blood donor controls for the same protein (Figure 48). Screening for autoantibodies was also performed against the complement components C3b and FB. Data is shown as optimal density readings and not converted to response units due to

the lack of a positive control. A minimum threshold for positive autoantibodies was determined by the 97.5th percentile of values from screening of at least 100 blood donor controls for the same protein (Figure 49).

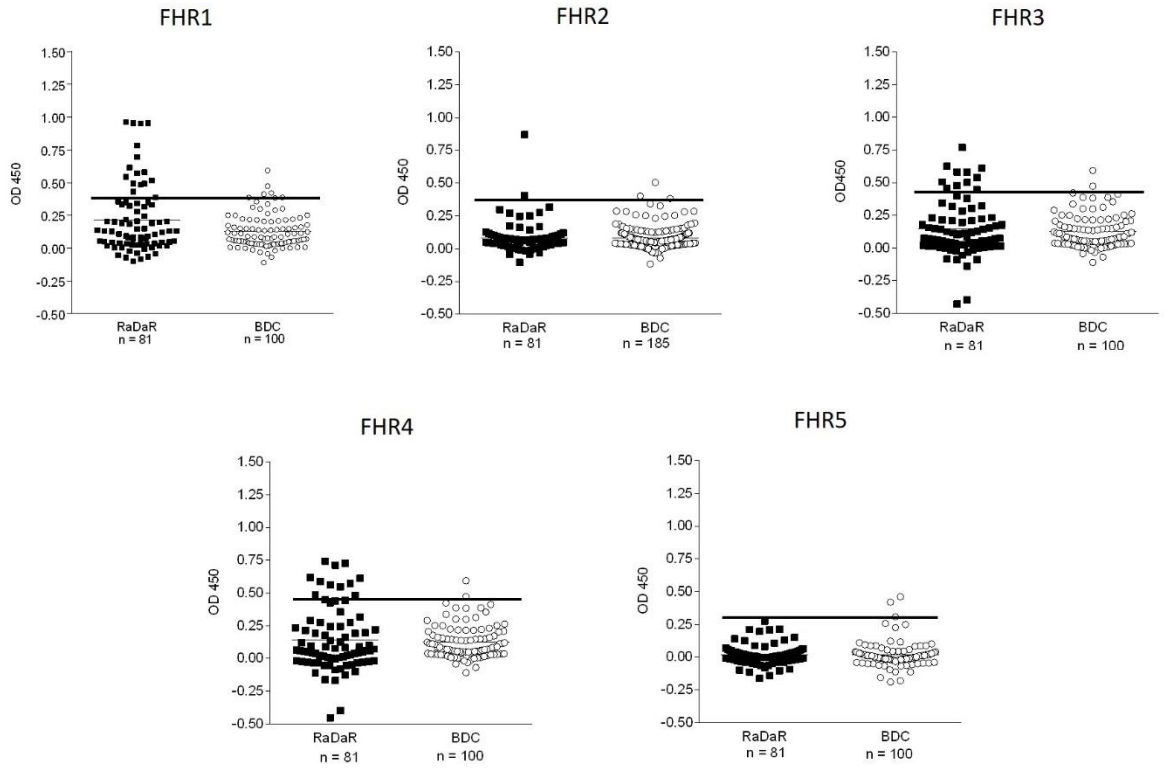

Figure 48 Screening for autoantibodies to FHR proteins

Data and figure generated by Dr. Marchbank. BDC = blood donor control, line indicates 97.5th percentile, the minimum threshold for identifying an autoantibody. Autoantibodies were identified in patients against FHR1-4, but not FHR5. Further confirmation of the specificity of the antibody response is required.

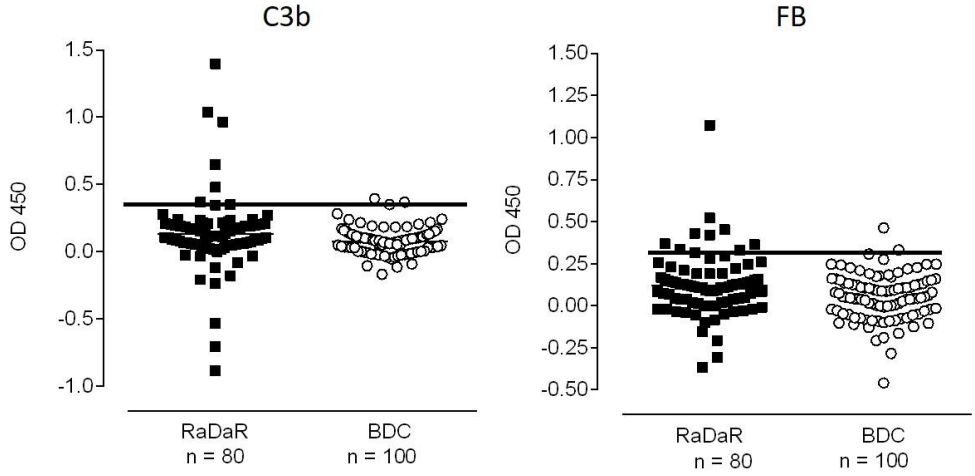

Figure 49 Screening for autoantibodies to C3b and FB.

Data and figure generated by Dr. Marchbank. BDC = blood donor control, line indicates 97.5th percentile, the minimum threshold for identifying an autoantibody. Autoantibodies were identified in patients against C3b and FB. Further confirmation of the specificity of the antibody response is required.

Most of the autoantibody data was generated and presented in raw form by Dr. Kevin Marchbank. I have undertaken the analysis of this data. The minimum threshold to define positivity for an autoantibody to a protein was the 97.5th percentile of values measured in blood donor controls. I have identified all patients that fulfil this minimal criterion as patients who may have autoantibodies to either the FHR proteins, C3b or FB.

Patients with autoantibodies to the FHR proteins are summarised in Figure 50. There were 22 patients that positive for autoantibodies to FHR1, FHR2, FHR3 or FHR4 during the initial screening protocol. No patients had autoantibodies to FHR5. There were 16 patients who had cross-reactivity to different FHR proteins and FH, of which 6 were to FHR1, FHR3 and FHR4 and a further 6 patients were to at least two of FHR3, FHR4 or FH. One patient had cross-reactivity to FHR1 and FHR2 and none of the patients had cross-reactivity to FH and FHR1.

Patients with autoantibodies to C3b and FB are compared to those with C3 nephritic factor identified at diagnosis (Figure 51). Three patients had an autoantibody to C3b. Eight patients had an autoantibody to FB. Of patients identified with a C3 nephritic factor, 2 also have an autoantibody to FB.

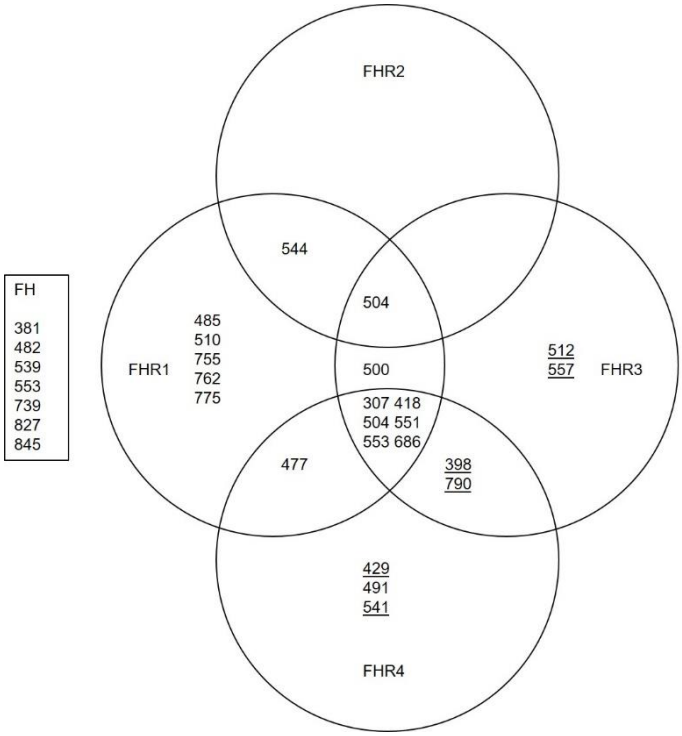

Figure 50 Patients with autoantibodies to FHR proteins

Patients with autoantibodies to FHR1, FHR2, FHR3 and FHR4 identified in the initial screening are shown. Numbering is taken from RaDaR cohort. Underlined and in box are patients with autoantibodies to FH. Cross-reactivity to different FHR proteins and FH is observed in 16 of the 22 patients.

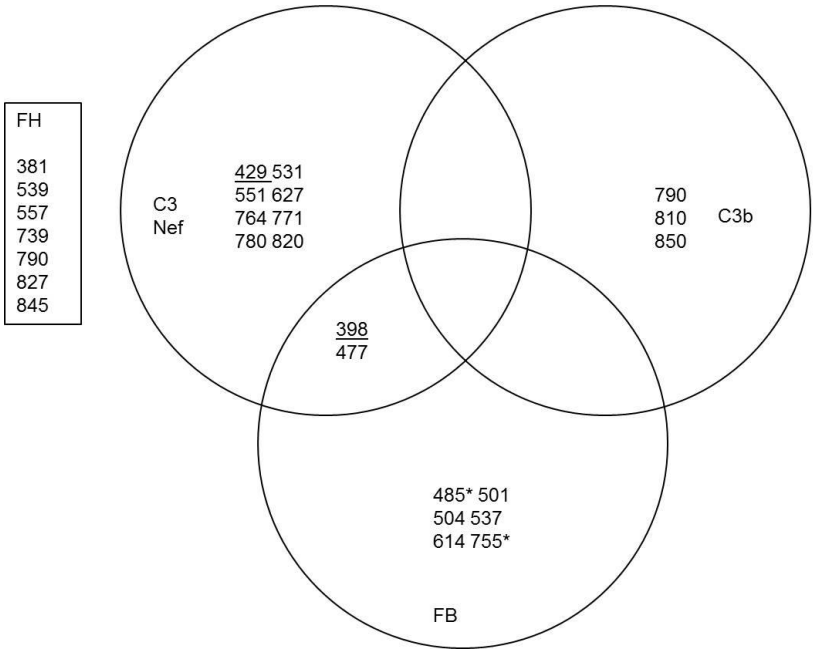

Figure 51 Patients with autoantibodies to C3b and FB

*Patients with autoantibodies to C3b and FB in the initial screening are shown. Numbering is taken from RaDaR cohort. Patients that had a detectable C3 nephritic factor at diagnosis are also shown. Underlined and in box are patients with autoantibodies to FH. Confirmation of specificity of these autoantibodies using western blot is an important next step. *C3 nephritic factor was negative for patient 485 and 755 but not available in all other patients with autoantibodies to C3b or FB.*

3.2.6 Genetic Studies in RaDaR cohort

3.2.6.1 Rare Genetic Variants

Patients were screened for genetic variants in *C3*, *CFB*, *CFH*, *CFI* and *CD46* in the Northern Molecular Genetics Service in Newcastle. I undertook the analysis of the genetic sequencing data. Rare genetic variants were defined as a minor allele frequency of <1% in the exome variant server database (evs.gs.washington.edu/).

In total, 71 patients in the RaDaR cohort were screened for genetic variants. Rare genetic variants were identified in 6 patients. Four patients had 1 variant and 2 patients had 2 variants (Figure 52). The overall prevalence of patients with rare genetic variants was 8.5%. Three variants were in *CFH*, 3 in *C3*, 1 in *CFB* and 1 in *CFI* (Figure 52). A further 9 patients had genetic variants in CFH with a minor allele frequency of 1-5% in the exome variant server database (Appendix 6, Table 60).

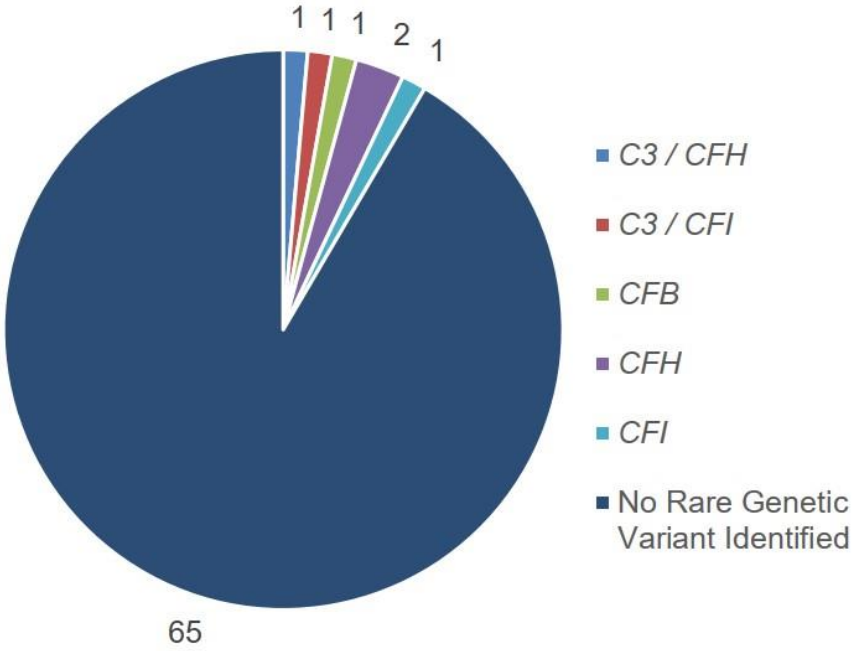

Figure 52 Proportion of RaDaR cohort with a rare genetic variant

Rare genetic variant defined as <1% in evs.gs.washington.edu/.

Patient	Disease	Gene	Base pair change	Amino acid change	Frequency in control cohort[1]	C3 (0.68-1.38 g/L)	C4 (0.18-0.60 g/L)	FH (0.35-0.59 g/L)	FI (38-58 mg/L)	C3 nephritic factor	Autoantibody to FH
491	C3GN	*CFI*	1657C>T	P553S	0.14%	1.22	0.2	0.74	73	Not done	Negative
527	IC-MPGN	*CFH*	1949G>T	G650V	0.023%	1.03	0.16	0.54	63	Not done	Negative
501	IC-MPGN	*CFH*	1001G>C	G334A	N/A	1.09[2] 0.92	0.04[2] 0.11	0.44	47	Not done	Negative
827	IC-MPGN	*CFB*	621A>C	E207D	N/A	0.71[3] 0.81	0.12[3] 0.07	0.46	58	Not done	Positive
686	IC-MPGN	*C3* *CFH*	1855G>A 2675C>T	K155Q A892V	0.22% 0.023%	0.23[3] 0.1	0.12[3] 0.11	0.67	55	Not known	Negative
771	DDD	*C3* *CFI*	4594C>T 355G>A	R1532W G119R	0.0077% 0.085%	0.11[3] 0.58	0.26[3] 0.22	0.52	36	Positive	Negative

Table 23 Rare genetic variants identified in RaDaR cohort

[1] Control population taken from evs.gs.washington.edu/ C3 and C4 levels at diagnosis at >[2]6 months or [3]<6 months prior follow up. All other complement levels are at follow up, N/A = C3 levels were normal, C3 nephritic factor testing not done in the clinic, Not known = C3 levels were low, no C3 nephritic factor data available, Normal ranges of complement levels in brackets.

Of the 6 patients with rare genetic variants, 4 had IC-MPGN, 1 had DDD and 1 had C3GN (Table 23). C3 and C4 levels at diagnosis were available for 4 of these patients. The three patients with IC-MPGN all had low C4 levels, and only 1 had low C3 levels at diagnosis. One patient with DDD had low C3 and normal C4 levels at diagnosis. One patient had a rare genetic variant and an autoantibody to FH. One patient had a rare genetic variant and C3 nephritic factor.

3.2.6.2 Copy Number Variation

Patients were screened for copy number variation in the *CFH* and *CFHR* genes in the Northern Molecular Genetics Service in Newcastle. I undertook the analysis of the data. The allele frequency of the *CFHR3/1* deletion in each sub-group and in patients with autoantibodies to FH are summarised in Table 24 and Table 25 respectively. The *CFHR3/1* gene was deleted in a total 9.0% of alleles and was protective against cases in the RaDaR cohort (P=0.01). The protective effect was observed only in the IC-MPGN group (P=0.02) and not in patients with IC-GN and DDD.

The allele frequency of the *CFHR3/1* gene deletion was 4.5% in patients who developed an autoantibody to FH and lower than in controls but this difference was not statistically significant. There was no evidence of copy number variation in the remainder of the *CFHR* genes (Data reported by the Northern Molecular Genetics Service in Newcastle and not shown).

CFHR3/1 deletion	del/del	del/+	+/+	Frequency of risk allele	Odds Ratio (95% CI)	P (vs control)
All	0	13	59	0.090	0.47 (0.26-0.85)	0.01
Ig-GN	0	2	6	0.125	0.67 (0.15-2.99)	0.60
Ig-MPGN	0	3	26	0.052	0.26 (0.08-0.83)	0.02
DDD	0	4	9	0.143	0.85 (0.29-2.52)	0.78
C3GN	0	4	18	0.091	0.47 (0.17-1.33	0.16
Control				0.175		

Table 24 Allele frequency of CFHR3/1 copy number in disease sub-group

CFHR3/1 gene is deleted (del) or present (+). Control cohort was taken from (Moore et al., 2010). The allele frequency of the CFHR3/1 deletion was 175 out of 1000.

CFHR3/1 deletion	del/del	del/+	+/+	Frequency of risk allele
Anti-FH POS	0	1	10	0.045
Anti-FH NEG	0	12	48	0.10
Fisher's Exact test, P=0.37				

Table 25 Allele frequency of CFHR3/1 copy number and autoantibodies to FH

CFHR3/1 gene is deleted (del) or present (+). Autoantibodies to FH (anti-FH) were either positive (POS) or negative (NEG).

3.2.6.3 Risk Polymorphisms

Patients were genotyped for common risk polymorphisms in *C3*, *CFB*, *CFH* and *CD46* in the Northern Molecular Genetics Service in Newcastle. I undertook the analysis of genetic sequencing data. The genotypes and allele frequency of disease associated SNPs are summarised (Appendix 5, Table 59). Due to testing of 10 SNPs, a value of P<0.005 was regarded as significant. Only one SNP had a P<0.005, *C3* R102G that has previously been associated with DDD.

In patients who had C3 nephritic factor testing, the allele frequency of the SNP, *C3* 102G was higher in patients who were C3 nephritic factor positive, but this did not reach statistical significance (P=0.18) (Table 26).

	G/G	G/C	C/C	Frequency of risk allele
C3 NEF Neg	2	10	21	0.212
C3 NEF Pos	2	9	10	0.310
Fisher's Exact test, 1 tail, p=0.18				

Table 26 Allele frequency C3 R102G in patients with C3 nephritic factor

The allele frequency of C3 102G was 1.46 times higher in patients with C3 nephritic factor compared to those without C3 nephritic factor (P=0.18).

3.2.7 Associations of Low Complement Levels in RaDaR cohort

C3 levels were significantly lower in patients with C3 nephritic factor as described in section 3.2.3. There were no differences in C3 levels in patients who had an autoantibody to FH or those who had a rare genetic variant compared to those who had neither, either at diagnosis or at follow-up (Figure 53).

The pattern of C4 levels in the presence of an autoantibody to FH or those who had a rare genetic variant was similar to the pattern of C3 levels. One difference was that patients who had an autoantibody to FH had lower C4 levels at follow-up compared to those who did not (P=0.02) (Figure 54).

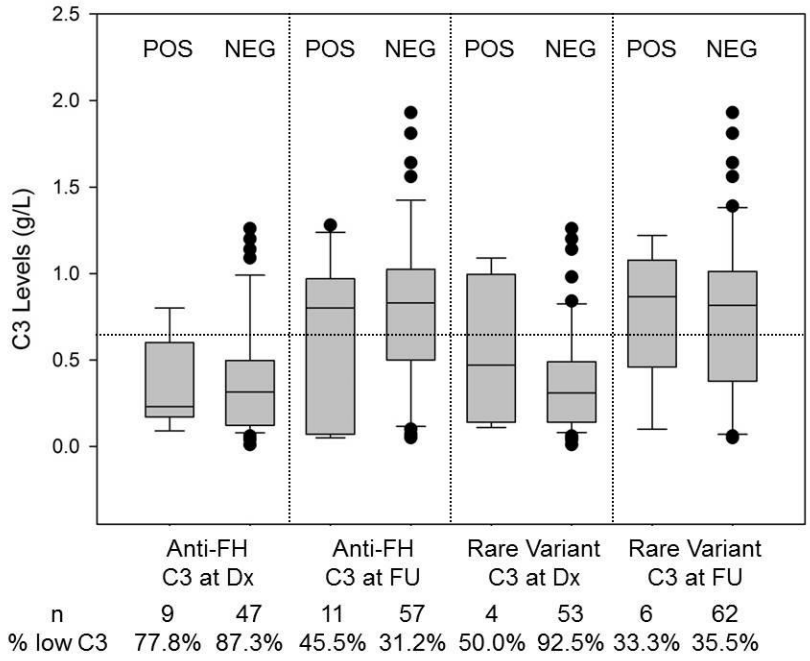

Figure 53 C3 levels in patients with autoantibody to FH or rare genetic variant

Dotted line indicates lower limit of normal C3 (0.68g/L). n = number of patients in sub-group with data available. C3 levels between patients who were either positive (POS) or negative (NEG) for an autoantibody to FH (anti-FH) or a rare genetic variant, were not statistically significant at either diagnosis (Dx) or at follow-up (FU).

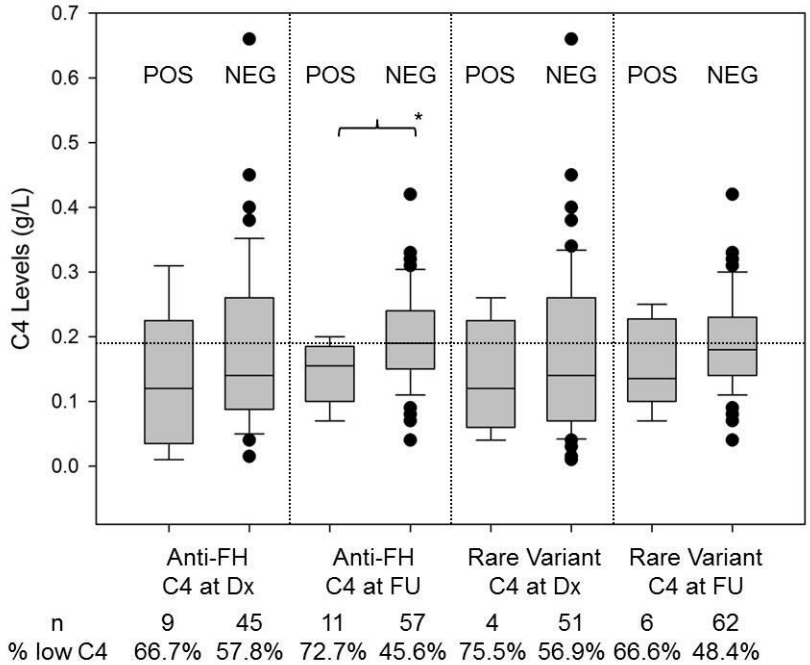

Figure 54 C4 levels in patients with autoantibody to FH or rare genetic variant

*Dotted line indicates lower limit of normal C4 (0.18g/L). n = number of patients in sub-group with data available. C4 levels between patients who were either positive (POS) or negative (NEG) for an autoantibody to FH (anti-FH) or a rare genetic variant, were not statistically significant, except at FU for those with autoantibodies to FH. *P<0.05.*

Complotypes have been defined by the SNPs *C3* R102G, *CFB* R32Q and *CFH* I62V. Patients with the alleles comprising the most active complotype (*C3* 102GG, *CFB* 32RR and *CFH* 62VV) were compared to those with the least active complotype (*C3* 102RR, *CFB* 32QQ and *CFH* 62II). In-vitro assays demonstrated a 6-fold difference in AP activity between these complotypes (Heurich *et al.*, 2011). Comparisons were made based upon the number alleles carried pertaining to the most active complotype (Table 27 and Table 28). There were no differences in complement levels between patients with the most active and less active complotypes in this cohort.

	At Diagnosis			At Follow up			P (Follow-up vs Diagnosis)
Risk alleles	n	Median C3 (g/L)	IQR	n	Median C3 (g/L)	IQR	
5 or 6	15	0.42	0.11-0.66	21	0.740	0.30-0.97	0.02
4	20	0.250	0.09-0.49	23	0.760	0.18-0.99	0.011
2 or 3	15	0.380	0.20-0.80	20	0.970	0.55-1.09	0.008

Table 27 C3 levels according to number of risk alleles of an active complotype

n=number of patients with data available. A complotype defined by C3 R102G, CFB R32Q and CFH I62V associates with alternative pathway activity. C3 levels between patients with the most active complotype (5 or 6 risk alleles) did not differ from those with the least active complotypes (4 risk alleles and 2 or 3 risk alleles). No patients had the least active complotypes of 0 or 1 risk alleles.

	At Diagnosis			At Follow up			P (Follow-up vs Diagnosis)
Risk alleles	n	Median C4 (g/L)	IQR	n	Median C4 (g/L)	IQR	
5 or 6	15	0.140	0.07-0.26	21	0.150	0.11-0.21	0.91
4	20	0.110	0.06-0.20	23	0.170	0.12-0.23	0.06
2 or 3	14	0.185	0.12-0.26	20	0.200	0.15-0.26	0.273

Table 28 C4 levels according to number of risk alleles of an active complotype

n=number of patients with data available. A complotype defined by C3 R102G, CFB R32Q and CFH I62V associates with alternative pathway activity. C4 levels between patients with the most active complotype (5 or 6 risk alleles) did not differ from those with the least active complotypes (4 risk alleles and 2 or 3 risk alleles). No patients had the least active complotypes of 0 or 1 risk alleles.

3.3 Study of a Retrospective Cohort Study of MPGN and C3G

3.3.1 Recruitment and Study Protocol

Patients were referred to Prof Tim Goodship or Dr. David Kavanagh and screening for abnormalities in AP was performed on a case by case basis. The diagnosis was recorded on the Newcastle Complement Genetics Service Patient Registry. 243 patients were entered onto the clinical database with a diagnosis of MPGN, DDD or C3GN (Figure 55). Biopsies were not reviewed and the diagnosis was based upon the clinical information provided. Based upon the clinical discussion between a clinician and an expert in complement and renal disease, a decision was made to undertake either screening for rare genetic variants in *C3 CFB*, *CFH*, *CFI* and *CD46* and/or screening for autoantibodies to FH. Sequencing of each gene became available incrementally over several years. Complement levels were measured in the laboratory at Newcastle Hospitals NHS Foundation trust at time of referral. The diagnoses included MPGN type 1 and type 3, DDD, C3GN and unspecified MPGN. I performed a retrospective analysis of the available data.

3.3.2 Characteristics of Cohort

In total, 222 patients went on to have screening for rare variants in at least one gene or autoantibodies to FH. 52.3% were male. This was a cohort of childhood and early adulthood (Figure 56). Many patients had low C3 levels at time of referral, and the median C3 level was lowest at time of referral in cases of DDD (Figure 57). Median C4 levels were within the normal range (Figure 58).

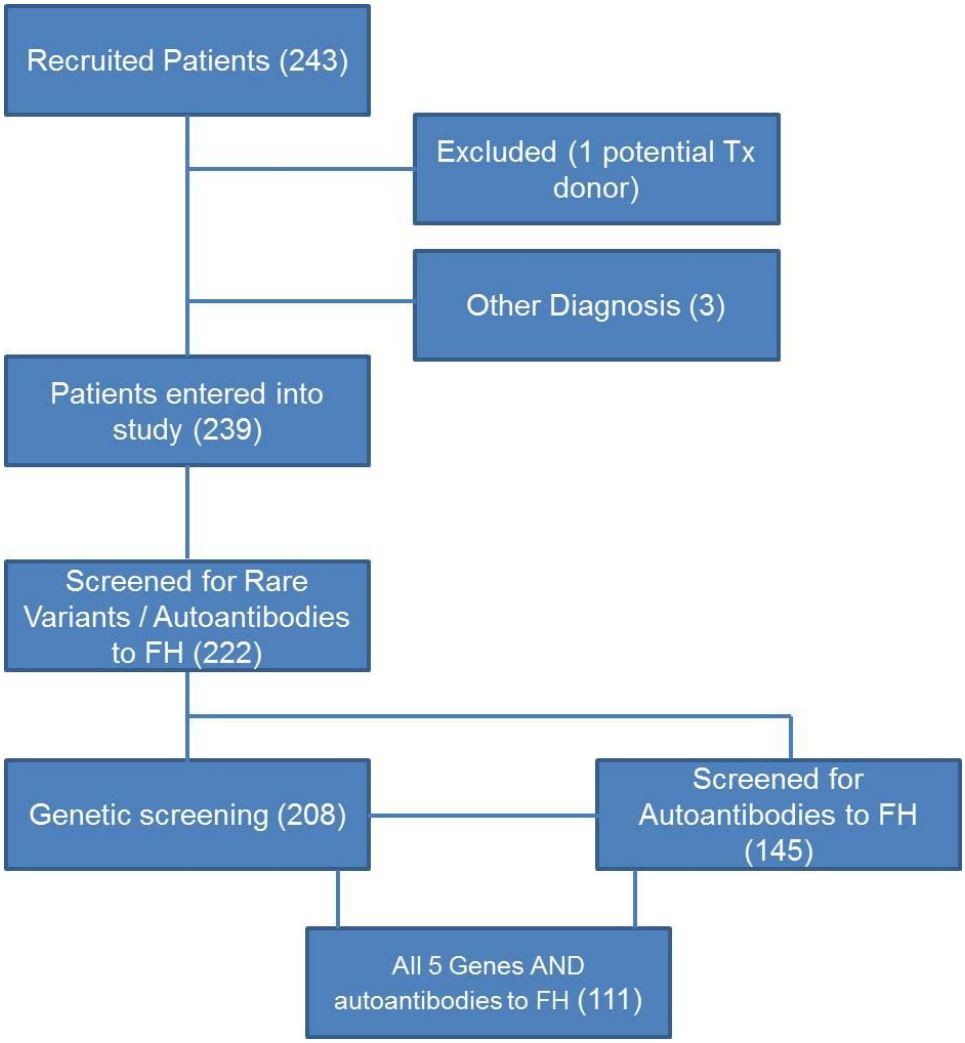

Figure 55 Patients referred to Newcastle with a diagnosis of MPGN or C3G

Patients were screened for autoantibodies to FH (antiFH) or a rare genetic variant in up to 5 genes – C3, CFB, CFH, CFI and CD46. Tx = transplant.

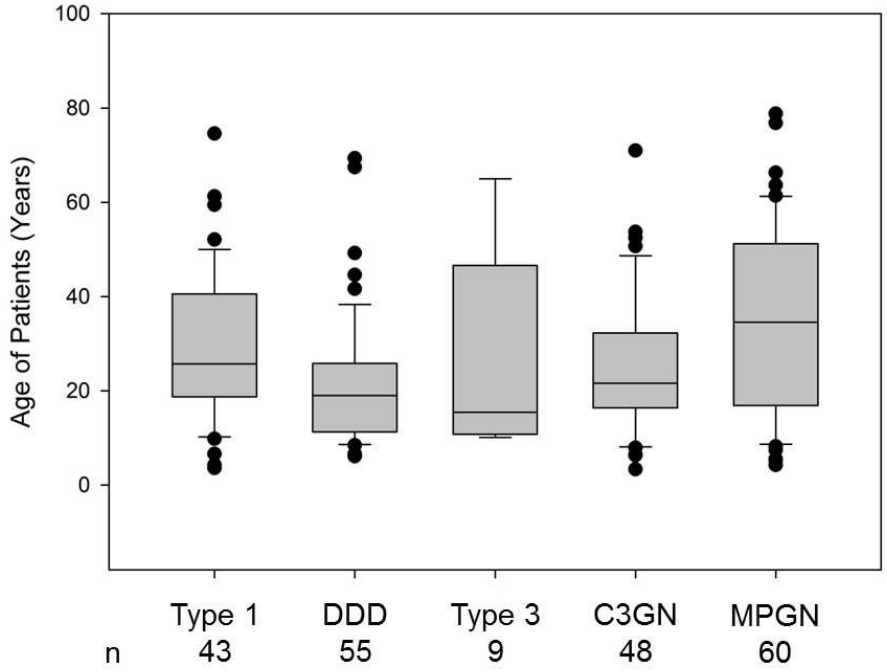

Figure 56 Age at referral of Newcastle cohort

n = number of patients in sub-group with data available.

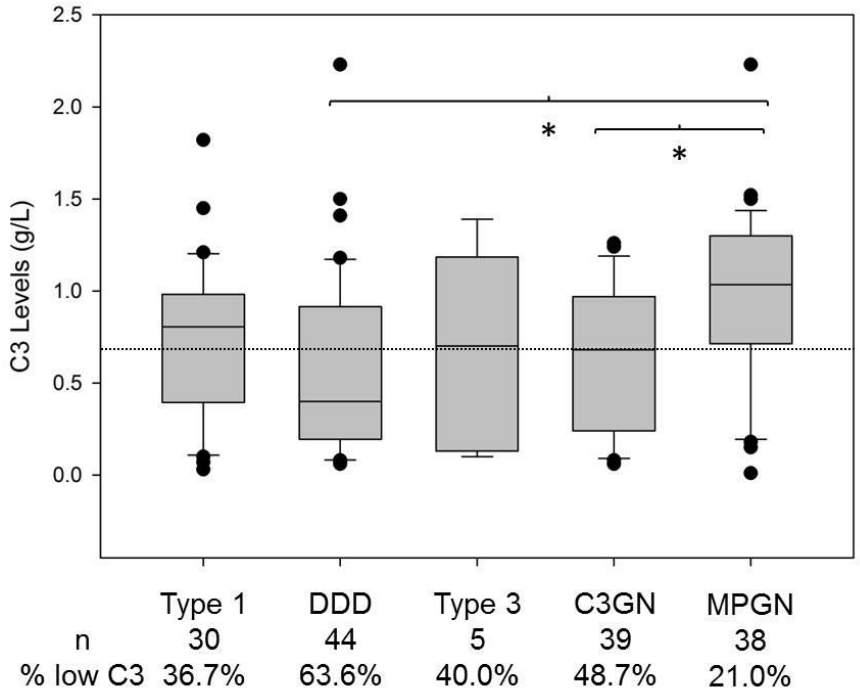

Figure 57 C3 levels at referral of Newcastle cohort

*Dotted line indicates lower limit of normal C3 (0.68g/L). n = number of patients in sub-group with data available. C3 levels were lower in patients with DDD and C3GN compared to MPGN. *P<0.05.*

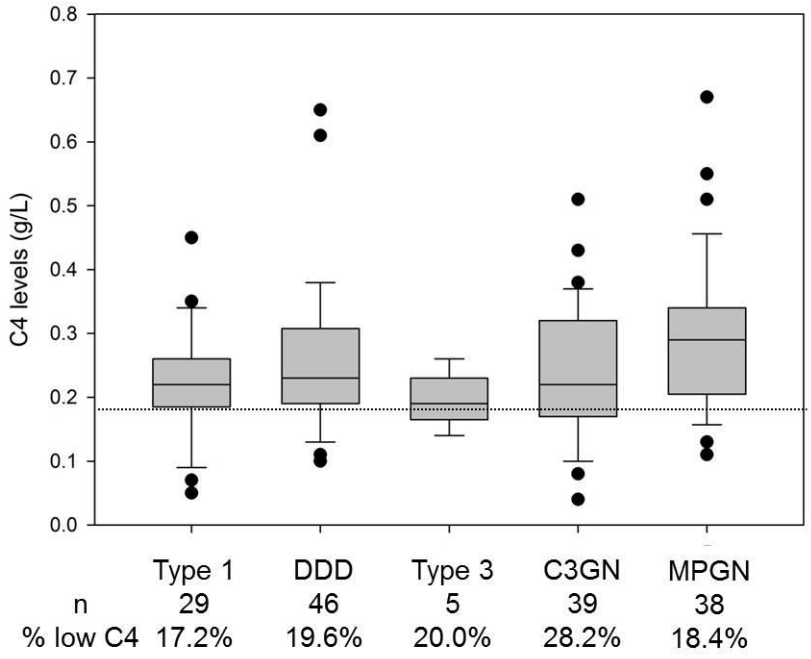

Figure 58 C4 levels at referral of Newcastle cohort

Dotted line indicates lower limit of normal C4 (0.18g/L). n = number of patients in sub-group with data available. There were no statistically significant differences between groups.

3.3.3 Screening for Autoantibodies to FH

Serum from patients was screened for the presence of autoantibodies to FH by Dr. Kevin Marchbank. Screening was performed in 145 patients. An autoantibody to FH was identified in 21 cases (14.5%). There were no differences in C3 or C4 levels at the time of referral between patients who had an autoantibody to FH and those who did not (Table 29), though median C3 levels were below normal in patients with an autoantibody to FH. C3 nephritic factor data was not available. An association of low C3 levels with C3 nephritic factor cannot be excluded.

	Anti-FH POS	Anti-FH NEG	
C3 levels	0.55 (0.30-0.99)	0.81 (0.28-1.05)	P=0.375
C4 Levels	0.30 (0.19-0.34)	0.22 (0.19-0.30)	P=0.095

Table 29 Complement levels in patients screened for autoantibodies to FH in Newcastle cohort

Complement levels were not statistically different if patients had autoantibodies to FH or not.

3.3.4 Screening for Rare Genetic Variants

Screening for rare genetic variants in *C3*, *CFB*, *CFH*, *CFI* and *CD46* was performed in the Newcastle cohort of patients in the Northern Molecular Genetics Service. A total of 208 patients had screening of at least 1 gene. 161 patients had all 5 genes screened. *C3* was screened in a total of 165 patients, *CFI* in 192, *CD46* in 188, and *CFH* was screened in a total of 199 patients. The differences in testing reflect the availability of testing for each gene during the history of the genetics service. There were a total of 29 different variants were identified in 28 individuals and two families in this cohort.

The prevalence of rare genetic variants was highest in *C3* and *CFH*. A rare variant was identified in 6.7% and 6.0% of patients screened in *C3* and *CFH* respectively (Table 30). Rare variants in *CFB* and *CFI* were less frequently identified (3.7 and 1.6%) and one variant was identified in *CD46*. An estimate of the overall prevalence of patients with rare genetic variants in *C3*, *CFB*, *CFH*, *CFI* and *CD46* in this cohort was 17.9%.

	C3	*CFB*	*CFH*	*CFI*	*CD46*	Anti-FH
Number of patients with rare genetic variants or autoantibody to FH (anti-FH)	11	5[1]	12[2]	3[3]	1	21
Number of patients screened	165	161	199	192	188	145
Prevalence of abnormality	6.7%	3.1%	6.0%	1.6%	0.5%	14.5%

Table 30 Prevalence of rare genetic variants and autoantibodies to FH in Newcastle cohort

A total of 29 different rare genetic variants were identified in 28 patients and 2 families following screening in the 5 genes, C3, CFB, CFH, CFI and CD46. [1]4 patients had two variants in CFB, [2]one patient had two variants in CFH, [3]2 patients had 2 variants in 2 genes (C3/CFI and CFH/CFI).

3.3.5 Clinical Significance of Autoantibodies to FH and Rare Genetic Variants

In 111 patients, screening was undertaken for autoantibodies to FH and rare genetic variants in *C3*, *CFB*, *CFH*, *CFI* and *CD46*. C3 levels were lower in patients who had a rare genetic variant or an autoantibody to FH compared to those who had neither abnormality but this difference was not statistically significant (Figure 59). The proportion of patients with low C3 in each group was similar. C3 nephritic factor data was not available in this cohort, so their importance in explaining many of the cases of low C3 cannot be excluded.

C3 and C4 levels are shown in Figure 60 for the patients who were screened for a rare genetic variant in *C3*, *CFB*, *CFH*, *CFI* and *CD46*, and for an autoantibody to FH. Approximately half of patients had low C3 levels, regardless of the presence or not of a rare genetic variant or an autoantibody to FH. In total, 28 patients had a low C3 level despite having neither abnormality. In 8 patients, this was associated with low C4 levels. Again, the association of low C3 levels with a C3 nephritic factor cannot be excluded.

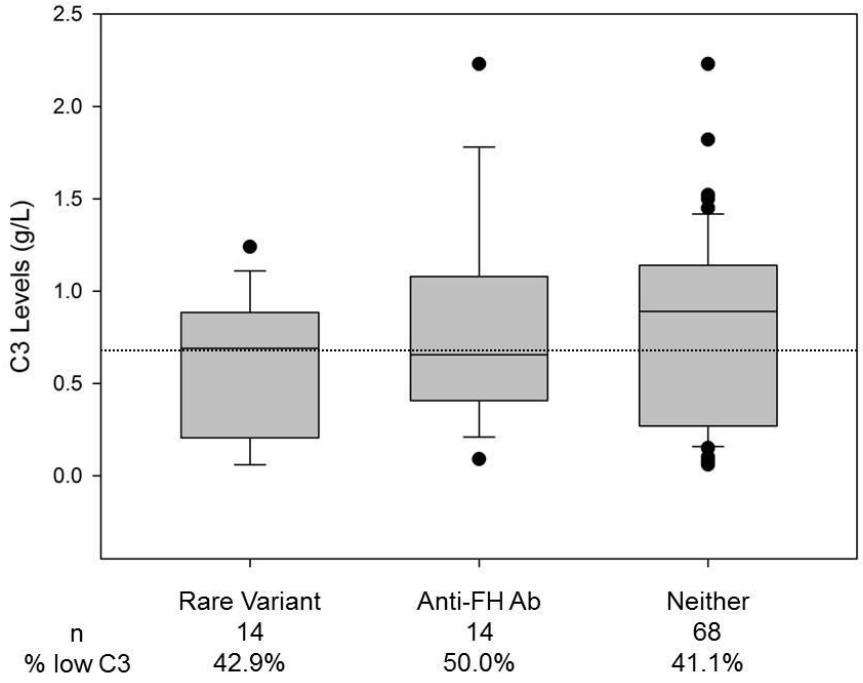

Figure 59 C3 levels in patients with rare variant or autoantibodies to FH in Newcastle cohort

Dotted line indicates lower limit of normal C3 (0.68g/L). n = number of patients in sub-group with data available. The differences between groups were not statistically significant.

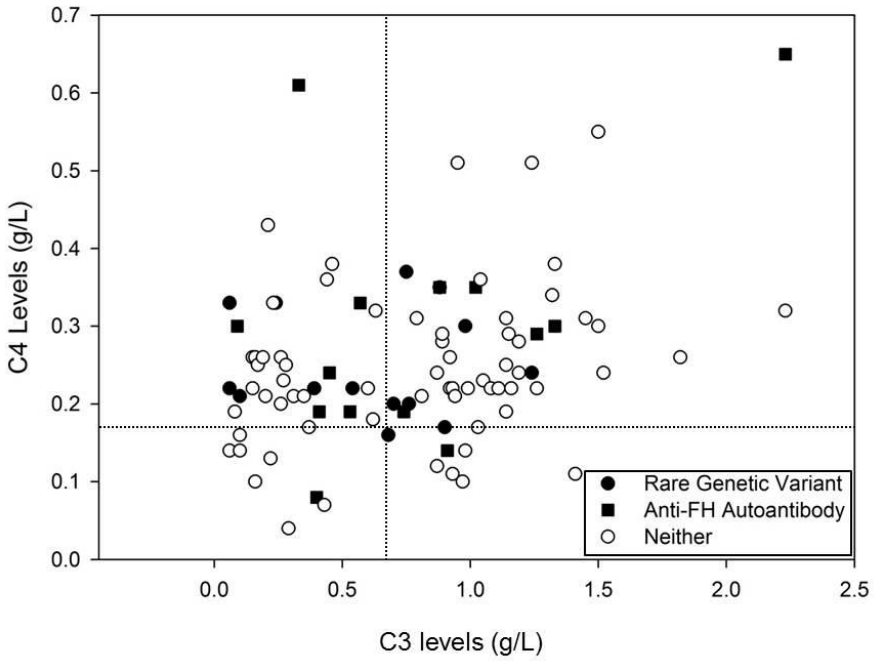

Figure 60 Complement levels in patients screened for a rare variant or autoantibodies to FH

Dotted lines indicates lower limit of normal C3 (0.68g/L) or C4 (0.18g/L). Patients' C3 levels were spread evenly within and below normal range, regardless of a rare genetic variant or autoantibody to FH.

In this group of 111 patients, 27 patients could be positively identified as CKD stage 5 (pre-dialysis, on dialysis or transplanted). The proportion of patients with a rare genetic variant or autoantibodies to FH was compared to the remaining patients in whom CKD stage 5 had not been positively identified. Furthermore, 17 patients could be positively identified as having disease recurrence in renal transplant. Again, the proportion of patients with a rare genetic variant or autoantibodies to FH was compared to the remaining patients in whom disease recurrence in renal transplant had not been positively identified. The likelihood of a rare genetic variant or autoantibody to FH was not significantly higher in a group of patients with either confirmed CKD stage 5 (Table 31) or disease recurrence in a renal transplant (Table 32).

Complement levels at time of referral in patients with disease recurrence in renal transplant can be seen in 14 individuals (Figure 61). Three patients had either an autoantibody to FH, a rare genetic variant or both. One patient with low C3 did not have an autoantibody to FH or a rare genetic variant. Data for C3 nephritic factor was not available. In total, 10 patients have normal C3 (and are unlikely to have a functionally significant C3 nephritic factor), no rare genetic variant in *C3*, *CFB*, *CFH*, *CFI* and *CD46* and no autoantibody to FH.

CKD 5 Confirmed?	Yes	No	CKD 5 Confirmed?	Yes	No
Rare Variant	6	12	anti-FH Ab	3	12
No Rare Variant	21	72	No anti-FH Ab	24	72
% with Variant	22.2%	14.3%	% with anti-FH Ab	11.1%	14.3%
OR (95% CI)	1.71 (0.57-5.12)			0.75 (0.20-2.88)	
P value	0.33			0.68	

Table 31 Rare genetic variants or autoantibodies to FH in patients with CKD5

Patients with confirmed chronic kidney stage 5 (CKD5) did not have a significantly higher percentage (%) of patients with a rare genetic variants or autoantibody to FH (anti-FH Ab) compared to the remainder of patients.

Recurrence in Tx Confirmed?	Yes	No	Recurrence in Tx Confirmed?	Yes	No
Rare Variant	3	15	anti-FH Ab	3	12
No Rare Variant	14	79	No anti-FH Ab	14	82
% with Variant	17.6%	16.0%	% with anti-FH Ab	17.6%	12.7%
OR (95% CI)	1.13 (0.29-4.41)			1.46 (0.37-5.86)	
P value	0.86			0.59	

Table 32 Rare genetic variants or autoantibodies to FH in patients with disease recurrence in renal transplant

Patients with confirmed disease recurrence in a renal transplant (Tx) did not have a significantly higher percentage (%) of patients with a rare genetic variants or autoantibody to FH (anti-FH Ab) compared to the remainder of patients.

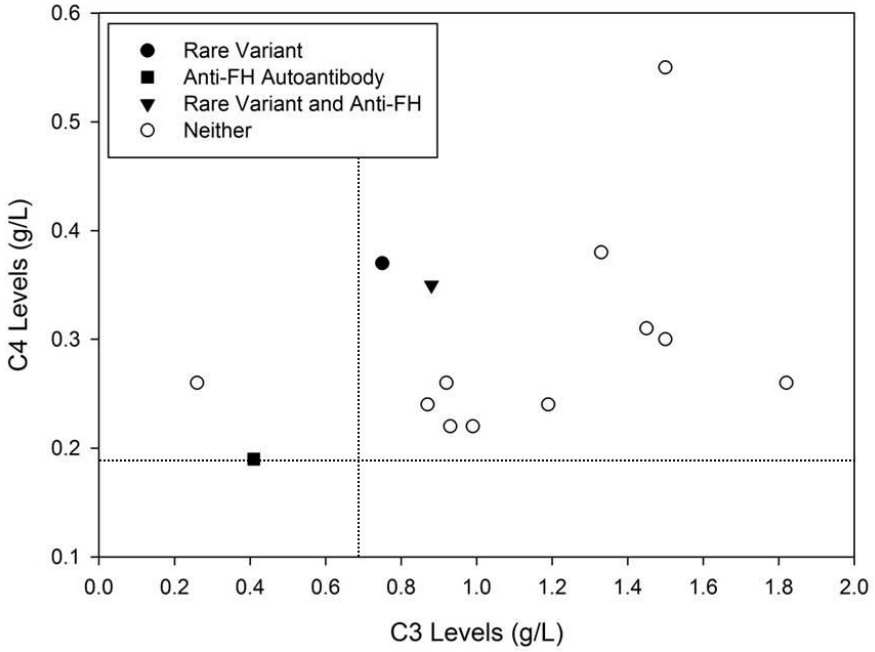

Figure 61 Complement abnormalities in patients with disease recurrence in transplant

Dotted lines indicates lower limit of normal C3 (0.68g/L) or C4 (0.18g/L). In total, 10 patients had disease recurrence in transplant, and at time of referral, had normal C3 and no evidence of autoantibodies to FH or a rare genetic variant.

3.4 Discussion

C3 nephritic factor is the prototypal abnormality of AP leading to overactivation of AP activity by stabilising C3bBb and consumption of C3. It is consistently the most prevalent finding in cohorts of MPGN/C3G. In this chapter, I have analysed the data following screening of a prospective and a retrospective cohort of MPGN/C3G for autoantibodies to FH and rare genetic variants in *C3, CFB, CFH*, *CFI* and *CD46*. I have also analysed data following screening of a prospective cohort of MPGN/C3G for autoantibodies to other complement components and SNPs that have been associated with MPGN/C3G in previous studies.

3.4.1 C3 Nephritic Factor in MPGN/C3G

C3 nephritic factor data from this study was available only from the clinical data provided, though screening in the prospective cohort is now underway. The available data from referring centres in the prospective study confirms the prevalence of C3 nephritic factor in this cohort of patients, ranging from 28.6% in cases of IC-MPGN to 66.7% in cases of DDD, although no patients with IC-GN had a C3 nephritic factor. The prevalence of C3 nephritic factor in the prospective cohort is only slightly lower than in recent relatively large cohorts of MPGN/C3G identifying 44.4-54% in cases of MPGN type 1 and C3GN and up to 86.4% in DDD (Servais *et al.*, 2012; Iatropoulos *et al.*, 2016). The clinical importance of a C3 nephritic factor remains unclear given that neither of these studies demonstrates it as a predictor of poor outcomes.

C3 nephritic factor and low C3 are strongly associated at diagnosis. Given that C3 levels tend to be higher during follow-up, an explanation might be that C3 nephritic factor does not constantly remain detectable in patients with MPGN/C3G (Servais *et al.*, 2012). This may be due to treatments that generally include immunosuppressive agents. One important factor is that testing for C3 nephritic factor is neither simple nor consistent as described in a number of larger studies that describe heterogeneity in C3 nephritic factor testing (Paixao-Cavalcante *et al.*, 2012; Zhang *et al.*, 2012). The relationship of clinical course and treatments in conjunction with systematic screening and study of different C3 nephritic factors in a prospective study may be helpful.

In patients who had undergone C3 nephritic factor testing, patients who were positive for C3 nephritic factor had a higher prevalence of the SNP, C3 R102G compared to those who were

negative for C3 nephritic factor (31% vs 21.1%) though this association was not statistically significant. These data are comparable with the statistically significant association of C3 nephritic factor with the SNP C3 R102G that was observed in an earlier study in which the prevalence of C3 R102G was 32.7% in patients positive for C3 nephritic factor compared to an expected prevalence of 20% in the population (Finn and Mathieson, 1993). Confirmation of this association may be possible in a larger sample.

3.4.2 Autoantibodies to Complement Proteins in MPGN/C3G

Screening for autoantibodies to FH was performed in both cohorts of this study. In my prospective cohort, autoantibodies to FH were identified in 16.7% of patients. Epitope binding studies were performed demonstrating a predominance of binding to a FH fragment (FH SCR1-7) that comprises the N-terminal regulatory domain of FH. Screening for autoantibodies to FH is now well established as part of the investigation of aHUS and protocols followed by Dr. Kevin Marchbank are part of the standardised protocols followed by research laboratories globally (Watson *et al.*, 2014). The prevalence of autoantibodies in this cohort is a little higher than that of previous cohorts of both MPGN/C3G (11%) (Blanc *et al.*, 2015) and aHUS (6.3-9.2%) (Dragon-Durey *et al.*, 2005; Moore *et al.*, 2010) and similar to my retrospective cohort of MPGN/C3G (14.5%).

The predominant binding to the N-terminal domain of FH in this study is consistent with previous reports in cohorts of MPGN/C3G (Goodship *et al.*, 2012; Blanc *et al.*, 2015) though studies demonstrating the ability of these autoantibodies to impair regulation have not yet been performed for the autoantibodies identified in my study. This predominant binding to the N-terminal domain of FH contrasts with those autoantibodies to FH in patients with aHUS that bind predominantly to the C-terminal domain of FH (Jozsi *et al.*, 2007). This is in keeping with impairment of FH regulatory function in the fluid-phase and cell-surface respectively and consistent with the paradigm of loss of fluid phase complement regulation in MPGN/C3G.

A further difference between autoantibodies in MPGN/C3G, and in patients with aHUS is the difference in association of autoantibodies to FH with the *CFHR3/1* deletion. No patients with autoantibodies to FH in this cohort had the *CFHR3/1* deletion in homozygosity, an observation that is consistent with previous studies (Blanc *et al.*, 2015) and is contrary to that observed for cohorts of aHUS. Autoantibodies to FH from patients with aHUS cross-react

with the C-terminal domain in FHR1 that is structurally very similar. This FHR1 C-terminal domain would be absent in patients that have the *CFHR3/1* deletion in homozygosity and also in a small number of patients that have the *CFHR3/1* deletion and *CFHR4/1* deletion on different alleles (Moore *et al.*, 2010) and may be important in pathogenesis. Patients with MPGN/C3G generally do not have *CFHR1* deletion, and the autoantibodies in these patients are not directed against the C-terminal domain of FH, suggesting that autoantibodies to FH in MPGN/C3G and aHUS have a different aetiology.

In a childhood cohort of MPGN/C3G, 41% of patients with autoantibodies to FH also had C3 nephritic factor (Blanc *et al.*, 2015). From the data available, this association was observed in 15.4% of patients in my prospective childhood of MPGN/C3G cohort. C3 levels were low in a further 6 patients in whom C3 nephritic factor may have been positive. The role of these autoantibodies in disease remains to be determined, though purification of IgG for functional studies as previously performed (Goodship *et al.*, 2012; Blanc *et al.*, 2015) and serial analysis in a prospective study may be of benefit.

The presence of autoantibodies to other complement proteins is less well established. Autoantibodies to C3b and FB were identified in 11 patients in this cohort. Given the prevalence of C3 nephritic factor, an autoantibody that binds to and stabilises C3bBb, it might be expected that there would be cross-reactivity of autoantibodies to either C3b or FB. This association was observed in 2 cases in which autoantibodies to FB associate with C3 nephritic factor, though C3 nephritic factor data was not available for all patients so this association might be greater. Patients with autoantibodies to C3b and FB (Chen *et al.*, 2011), C3b (Jozsi *et al.*, 2014) and FB have been previously described (Strobel *et al.*, 2010; Zhang *et al.*, 2012). Autoantibodies in two of these studies were able to stabilise C3bBb and further analysis did not identify C3 nephritic factor (Strobel *et al.*, 2010; Chen *et al.*, 2011). In these studies, there was evidence of complement overactivation that would support the association of these autoantibodies with disease. Further studies of the autoantibodies to C3b and FB identified in my cohort are required.

Autoantibodies to at least one of 4 FHR proteins (FHR1-4) were found in 22 patients. No patients had an autoantibody to FHR5. A role for autoantibodies to FHR proteins in MPGN/C3G has not yet been described. The dominant feature of these autoantibodies is the cross-reactivity to several FHR proteins that were expressed in mammalian cell lines. Cross reactivity of a monoclonal antibody response might have occurred between SCRs of high sequence homology such as that observed between SCR1 and 2 of FHR1, FHR2 and FHR5

(Goicoechea de Jorge *et al.*, 2013) and also of SCR5 or FHR1 and SCR20 of FH (Moore *et al.*, 2010). Cross reactivity to these proteins was observed in only one patient, between FHR1 and 2.

Sequence homology between SCRs 3 and 4 of FHR3 and corresponding SCRs of FHR4 are also high (Jozsi *et al.*, 2005). This might explain the high degree of cross-reactivity between autoantibodies in 8 patients to these proteins. However, of these 8 patients, 6 also demonstrated cross-reactivity with FHR1 where sequence homology is not as high. A further 4 patients with autoantibodies to FHR3 or FHR4 had cross-reactivity to FH. The possibility of a non-specific polyclonal response cannot be ruled out and further experiments to confirm the specificity of these autoantibodies to individual FHR proteins are required.

3.4.3 Rare Variants in *CFH* in 2 Cohorts of MPGN/C3G

There were a total of 13 different rare genetic variants in *CFH* identified in this chapter. Complement levels are summarised for these variants in 16 patients (Table 33). 2 variants (R232X and W1096R) resulted in low FH levels and should be considered significant. The variant W1096R, identified in a case of familial DDD was the only variant inherited in homozygosity in this cohort, resulting in complete FH deficiency. The R232X variant was inherited in heterozygosity and resulted in partial FH deficiency. Both variants associated with low C3 levels. The variant, C431S identified in my cohort had normal FH levels, but has previously been described in association with low FH levels and is likely to be significant (Dragon-Durey *et al.*, 2004). The introduction of a stop codon in the variant E1172X is highly likely to be functionally significant and may not be secreted. Assuming it is secreted, it would be as a truncated protein that lacks most of an important C-terminal recognition domain. FH levels were not available in this patient.

Of the remaining 9 variants, functional studies have only been performed in one, T956M that was found in 3 patients. No functional effect was observed in haemolytic assays (Szarvas *et al.*, 2016). All others are variants of uncertain significance (VUS) (Kavanagh and Anderson, 2012). Three of these VUS lead to amino acid substitutions within the regulatory domain, including the familial variant R83S that associates with low C3 (Figure 62). The variant R83S (and S199G) is at interface of FH and C3b and a mutant protein may have impaired binding to C3b resulting in loss of regulatory function, consistent with the paradigm of complement dysregulation in MPGN/C3G.

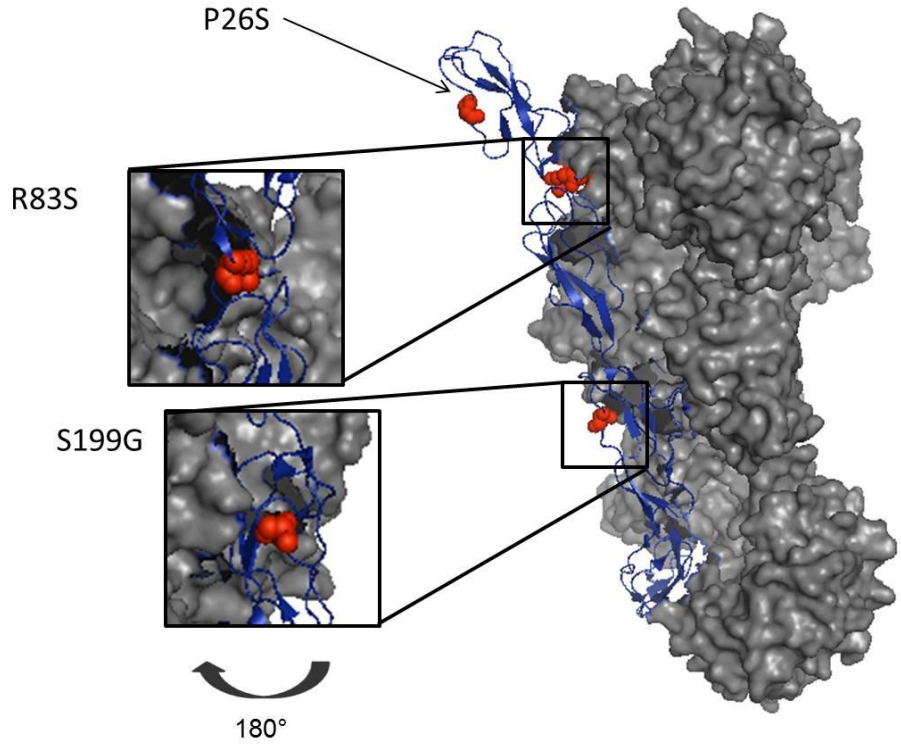

Figure 62 Rare genetic variants in CFH in the N-terminal functional domain identified in chapter 3

P26S, R83S and S199G (red spheres) are modelled on the co-crystal structure of FH SCR1-4 (blue ribbon) and C3b (grey) using Pymol. The variants R83S and S199G are at interface of FH and C3b and a mutant protein may have impaired binding to C3b resulting in loss of regulatory function, consistent with the paradigm of complement dysregulation in MPGN/C3G.

Disease	SCR	Amino Acid Change	CKD5	NEF	ANTI-FH	C3	C4	FH	FI
C3GN	1	P26S	NK	NK	N	↔	↓	↔	↔
MPGN1	1	R83S[1]	Y	Y	N	↓	↔	↔	↔
MPGN	4	S199G[2]	T	NK	NK	NK	NK	NK	NK
C3GN	4	R232X	NK	NK	N	↓	↔	↓	↔
IC-MPGN	6	G334A	NK	N	N	↔	↓	↔	↔
C3GN	7	C431S	Y	NK	N	↔	↔	↔	↔
MPGN1	10	V609I	T	NK	Y	NK	NK	NK	NK
MPGN3	10	V609I	NK	NK	NK	NK	NK	NK	NK
IC-MPGN	11	G650V	NK	N	N	↔	↓	↔	↔
MPGN1	15	H878Y	NK	NK	NK	↔	↔	↔	↔
IC-MPGN	15	A892V[3]	NK	N	N	↓	↓	↔	↔
C3GN	16	T956M	NK	NK	N	↔	↓	↔	↔
MPGN1	16	T956M[4]	NK	NK	N	↓	↓	↔	↔
MPGN1	16	T956M	NK	NK	NK	NK	NK	NK	NK
DDD	18	W1096R[1,5]	NK	NK	Y	↓	↔	↓	↔
MPGN1	20	E1172X[2]	T	NK	NK	NK	NK	NK	NK

Table 33 Patients with rare genetic variants in CFH identified in chapter 3

MPGN1 = MPGN Type 1, MPGN3 = MPGN Type 3, SCR = short consensus repeat, CKD5 = chronic kidney disease stage 5, NEF = C3 nephritic factor, anti-FH = autoantibodies to FH, NK = not known, ↔ within normal range, ↓ below normal range, T = transplanted, [1]familial, [2]2 variants in same patient, [3]also has K155Q in C3, [4]also has P553S in CFI, [5]homozygous.

3.4.4 Rare Variants in *C3* in 2 Cohorts of MPGN/C3G

Genetic screening revealed 11 different rare genetic variants in *C3* in this chapter (Table 34). All were inherited in heterozygosity. Most of the previous studies of rare genetic variants in *C3* were identified in cases of aHUS (Fremeaux-Bacchi *et al.*, 2008; Martinez-Barricarte *et al.*, 2015; Schramm *et al.*, 2015). Familial variants in DDD (Martinez-Barricarte *et al.*, 2010) and C3GN (Chauvet *et al.*, 2015) have also been shown to be functionally significant. Functionally significant variants in *C3* that have been studied are usually due to impaired binding to FH, CD46 or CR1. As a result, there is impairment of FI-mediated inactivation of C3b. In one study of 23 variants, 4 of the variants that did not affect show a clear functional defect were not in the FH binding surface (Schramm *et al.*, 2015). Rarely, a mutant C3 protein has enhanced binding to FB and C3bBb formation (Lhotta *et al.*, 2009) or the formation of C3bBb that is resistant to decay (Martinez-Barricarte *et al.*, 2010).

Nine variants in *C3* identified in this chapter are shown in a co-crystal structure of C3b with FH SCR1-4 (Figure 63). One of these, T1383N (reported as T1361N without the signal sequence) (Schramm *et al.*, 2015) has been previously studied and was shown to have no effect on binding to any of FH, CD46 or CR1. Loss of binding to FH, an important regulator of C3b in the fluid phase would be in keeping with the paradigm of loss complement regulation in the fluid phase. However, none of the 9 variants, including T1383N were at the interface directly opposing FH SCR1-4. One other variant, R1532W however is near the interface with FB and may lead to enhanced FB binding and C3bBb formation. This patient also had a C3 nephritic factor. Functional studies of these variants should be performed to determine whether any of these variants in *C3* have a functional defect.

Two variants previously studied, Y832X and C1136W were shown to result in impaired secretion. Two variants in this study, a splice site change (689-3C>G) and the introduction of a stop codon (F1246X) both associate with very low levels of C3. How these variants associate with would fit with a complement overactivation model of disease is unclear. It is possible that these variants, although functionally significant are not associated with the pathogenesis of disease.

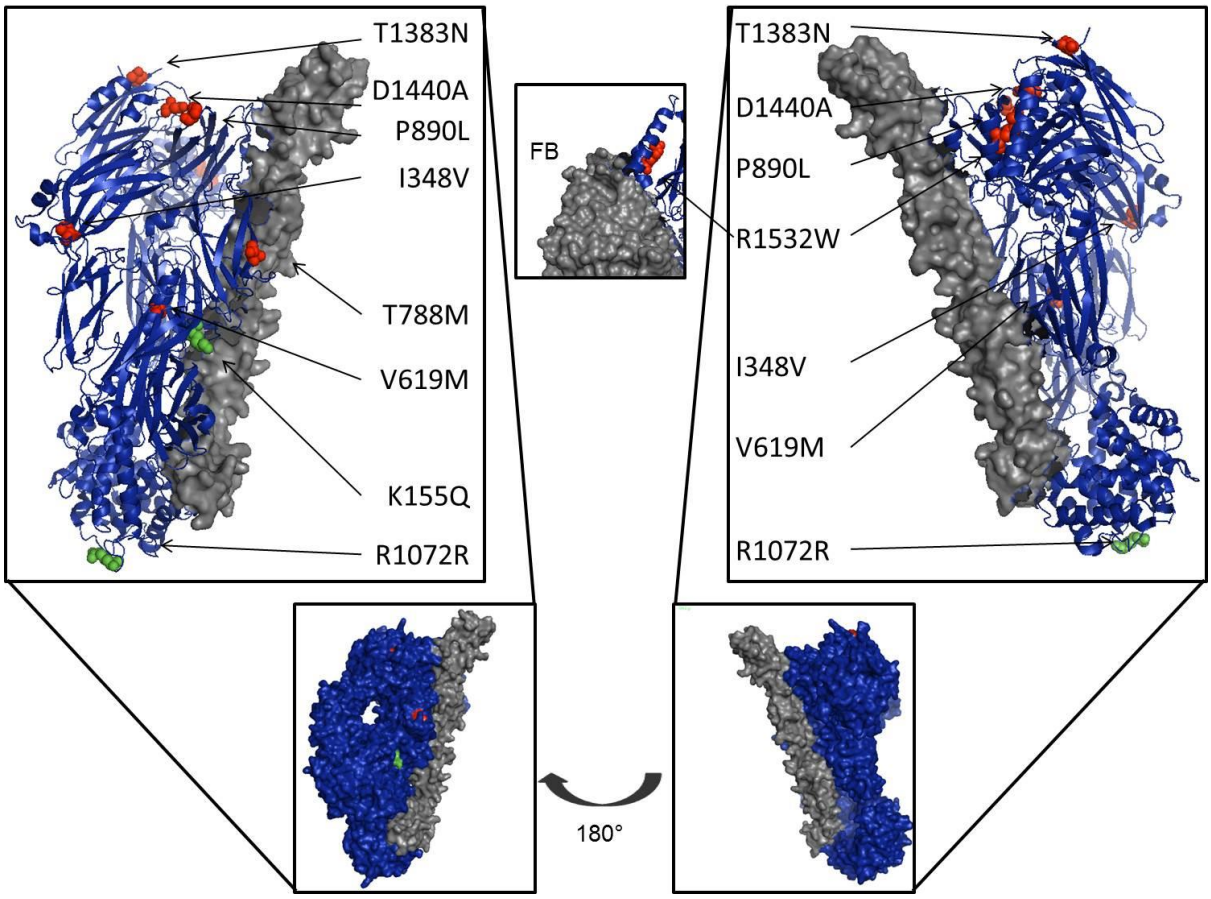

Figure 63 Rare genetic variants in C3 identified in chapter 3

Rare variants in C3 found in <1% (green sphere) and <0.1% (red sphere) of exome variant server cohort are shown on the co-crystal structure of FH SCR1-4 (grey) and C3b (blue ribbon) using Pymol. None of the variants are in the binding domain with FH. Centre inset shows C3b on the co-crystal structure with FB (grey). The variant, R1532W is near the binding domain near to FB and may lead to enhanced FB binding and C3bBb formation.

Disease	Amino Acid Change	CKD5	NEF	Anti-FH	C3	C4	FH	FI
IC-MPGN	K155Q[1]	NK	N	N	↓	↓	↔	↔
C3GN	K155Q	NK	NK	NK	NK	NK	NK	NK
MPGN1	689-3C>G	NK	N	N	↓	↔	↔	↔
MPGN3	I348V	NK	NK	N	↔	↔	↔	↔
MPGN	V619M	NK	NK	NK	NK	NK	NK	NK
MPGN1	T788M	R	NK	N	↔	↔	↔	↔
DDD	P890L	NK	NK	N	↔	↔	↔	↔
DDD	R1072R	5	NK	N	↓	↔	↔	↔
C3GN	F1246X	NK	NK	N	↓	↔	↔	↔
C3GN	T1383N	T	N	N	↔	↔	↔	↔
DDD	D1440A	T	N	P	↓	↓	↔	↔
DDD	R1532W[2]	NK	P	N	↓	↔	↔	↔

Table 34 Patients with rare genetic variants in C3 identified in chapter 3

MPGN1 = MPGN type 1, MPGN3 = MPGN type 3, CKD5 = chronic kidney disease stage 5, NEF = C3 nephritic factor, anti-FH = autoantibodies to FH, [1]Also has A892V in CFH, NK = not known, ↓ below normal range, ↔ within normal range, R=recurrence in transplant, T = transplanted, [2]also has G119R in CFI.

3.4.5 Rare Variants in *CFB* in 2 Cohorts of MPGN/C3G

Genetic screening revealed 7 different rare genetic variants in *CFB* in this chapter (Table 35). All variants were inherited in heterozygosity. Functional studies of rare genetic variants in *CFB* have been performed in variants reported in aHUS (Goicoechea de Jorge *et al.*, 2007; Marinozzi *et al.*, 2014). Six of the rare variants identified in this study are shown in Figure 64 alongside several gain-of-function mutations previously described but they do not share a similar position on FB. Nonetheless, two variants, E207B and V669L are in close proximity to C3b and may have enhanced binding to C3b. Functional studies of these variants should be performed. A 7th variant results in the introduction of a stop codon. Assuming that this leads to a secreted protein, it would be truncated. Analysis of patient serum for evidence of a

truncated protein would be an initial step before determining whether such a protein could be result in complement activation and be associated with MPGN/C3G.

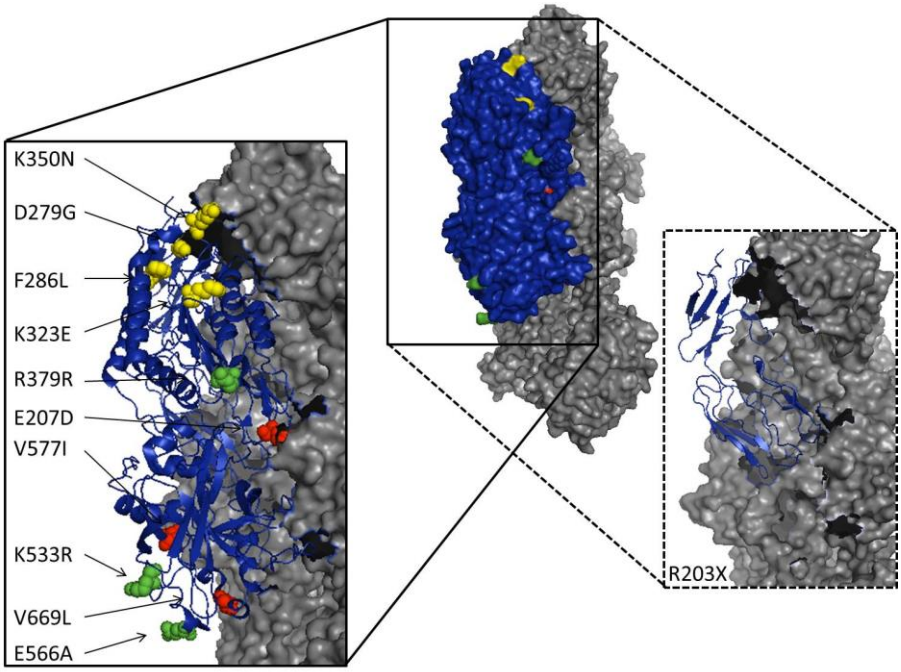

Figure 64 Rare genetic variants in CFB identified in chapter 3

Rare variants in CFB found <1% (green sphere) and <0.1% (red sphere) of exome variant server cohort are shown on the co-crystal structure of CFB (blue ribbon) and C3b (grey) using Pymol. Previously studied gain-of-function mutants are also shown (yellow sphere). Variants in this study do not cluster with the previously studied variants, though E207D and V669L are at an interface with C3b that might lead to enhanced C3b binding and C3bBb formation that would be consistent with a model of complement overactivation in MPGN/C3G. If expressed, the R203X protein would be truncated and is also modelled.

Disease	Amino Acid Change	CKD5	NEF	ANTI-FH	C3	C4	FH	FI
C3GN	R203X	Y	Y	N	↓	↔	↔	↔
IC-MPGN	E207D	NK	NK	Y	↔	↓	↔	↔
MPGN1	R379R and E566A	Y	NK	NK	NK	NK	NK	NK
C3GN	R379R and E566A	NK	NK	NK	NK	NK	NK	NK
DDD	K533R and E566A	NK	Y	NK	↓	↔	↔	↔
C3GN	V577I and V669L	NK	NK	N	↔	↔	↔	↔

Table 35 Rare genetic variants in CFB identified in chapter 3

CKD5 = chronic kidney disease stage 5, NEF = C3 nephritic factor, anti-FH = autoantibodies to FH, ↓ below normal range, ↔ within normal range, NK = not known, [1 2 3] denote 2 rare variants in same patient.

3.4.6 Rare Variants in *CFI* and *CD46* in 2 Cohorts of MPGN/C3G

None of the 3 variants identified in *CFI* in this chapter were novel and have all been described in aHUS cohorts (Table 36). The G119R variant is functionally significant and has been associated with low FI levels in AMD cohorts (Kavanagh *et al.*, 2015) though normal levels have been reported in cases of aHUS (Fremeaux-Bacchi *et al.*, 2013) as observed in this case. R317W has been shown to have impaired co-factor activity and associated with normal FI levels (Kavanagh *et al.*, 2007) though this was not observed in a subsequent study (Nilsson *et al.*, 2010). P553S is associated with normal FI levels (Bienaime *et al.*, 2010). No functional data is available.

There was 1 rare genetic variant in *CD46* in this chapter (Table 36). The D185G variant identified in this chapter has not previously studied, but the D185N variant has been previously studied. Functional studies showed impairment of C4b binding and inactivation of C4b; C3b binding however, and C3b inactivation by FI was not affected (Fremeaux-Bacchi *et al.*, 2006).

Disease	Gene	Amino Acid Change	CKD5	NEF	ANTI-FH	C3	C4	FH	FI
DDD	*CFI*	G119R[1]	NK	P	N	↓	↔	↔	↔
C3GN	*CFI*	P553S	NK	NK	N	↔	↔	↔	↔
MPGN1	*CFI*	P553S[2]	NK	NK	N	↓	↓	↔	↔
MPGN	*CFI*	R117W	NK	NK	NK	NK	NK	NK	NK
MPGN	*CD46*	D185G	NK	NK	NK	NK	NK	NK	NK

Table 36 Rare genetic variants in CFI* and *CD46 identified in chapter 3

MPGN1 = MPGN type 1, CKD5 = chronic kidney disease stage 5, NEF = C3 nephritic factor, anti-FH = autoantibodies to FH, NK = not known, ↓ below normal range, ↔ within normal range, [1] also has R1532W in C3, [2] also has T956M in CFH.

3.4.7 Rare Variants in 2 Cohorts of MPGN/C3G

Rare genetic variants were identified in both cohorts of MPGN/C3G in this chapter. The prevalence of rare genetic variants patients in the prospective cohort (8.2%) is low, and After allowing for the possibility that some of the variants identified might not be causative in

disease (discussed further in next section), it makes it difficult to justify routine screening for rare genetic variants in *C3*, *CFB*, *CFH*, *CFI* and *CD46* in a prospective paediatric cohort. The prevalence of patients with a rare genetic variant in the retrospective cohort was 17.9%, higher than in the prospective cohort and may be due to selection bias of patients with particular clinical characteristics that may include persistent complement activation as evidenced by low C3 or adverse outcomes including recurrence in renal transplantation. Additional clinical data is required to address these important issues.

Nonetheless, the prevalence of rare genetic variants in my retrospective cohort is comparable to the prevalence recently reported in an Italian cohort in whom 18% of patients had a rare genetic variant in one or more of the genes, *C3*, *CFB*, *CFH*, *CFI* and *CD46 (Iatropoulos et al., 2016)*. The prevalence of rare genetic variants would probably be higher in the French cohort if screening for *C3* and *CFB* was included (17.9% in the genes *CFH*, *CFI* and *CD46*) (Servais *et al.*, 2012) and is higher in an American cohort (43.3% in *C3*, *CFB*, *CFH*, *CFI* and *CD46*) (Bu *et al.*, 2015). These differences may reflect cohort selection and importantly, these prevalence data precede any functional analysis. The true prevalence of functionally rare genetic variants in cohorts of MPGN/C3G is likely amongst the lower prevalence rates and low compared to prevalence rates of the acquired abnormalities. Future studies to determine clinical significance of rare genetic variants are required.

3.4.8 Limitations of Study

Several important limitations need to be addressed. The functional importance of many abnormalities identified in these cohorts, both acquired and genetic is yet to be established. Furthermore, this data will need be correlated with clinical outcomes and the characteristics of complement abnormalities during follow up, especially C3 nephritic factor and autoantibodies to FH. The prospective study of a RaDaR cohort and the inclusion of central pathology review is a rich resource for ongoing study that may address these important questions. Further clinical information from the retrospective study of the Newcastle cohort including a more comprehensive assessment of pathology and disease classification will contribute to this analysis.

3.5 Summary

In this chapter, I have described a prospective and a retrospective cohort of patients with MPGN/C3G. Screening for rare genetic variants in *C3*, *CFB*, *CFH*, *CFI* and *CD46* in these cohorts reveals a low prevalence of abnormalities. Compared to the prevalence rate of C3 nephritic factor identified in this prospective cohort, and the prevalence rate of other autoantibodies identified in both the prospective and retrospective cohorts, it is clear that the predominant abnormalities in these cohorts are acquired, suggesting that MPGN/C3G is largely an autoimmune phenomenon.

The rate of detection for rare genetic variants in the prospective paediatric cohort is particularly low at 8.2% of the cohort, and pending functional testing, the prevalence of functionally significant variants in this cohort maybe even lower. The prevalence of rare genetic variants was higher, at 17.9% in a retrospective cohort. These higher rates of rare genetic variants in this retrospective cohort and other published studies may be reflective of selection bias in a clinically referred cohort, and not representative of a prospective cohort.

Further longitudinal follow up of these cohorts and the characterisation of abnormalities, both acquired and genetic are required to improve our understanding of MPGN/C3G. Data from this study does not change the current paradigm of complement overactivation in the fluid phase as a hallmark of MPGN/C3G.

Chapter 4 – Structural and Functional Study of a Rare Genetic Variant in *CFH* in Familial MPGN

4.1 Introduction

A case of familial MPGN was first described in 1990 (Power *et al.*, 1990). Two family members had biopsy-proven MPGN (1:2 and 2:1) and progressed to ESRD. These two affected members and a third (2:4) also had APL. C3 nephritic factor was positive in all three patients with APL. At that time, no genetic studies had been undertaken. These clinical findings are summarised in Figure 65.

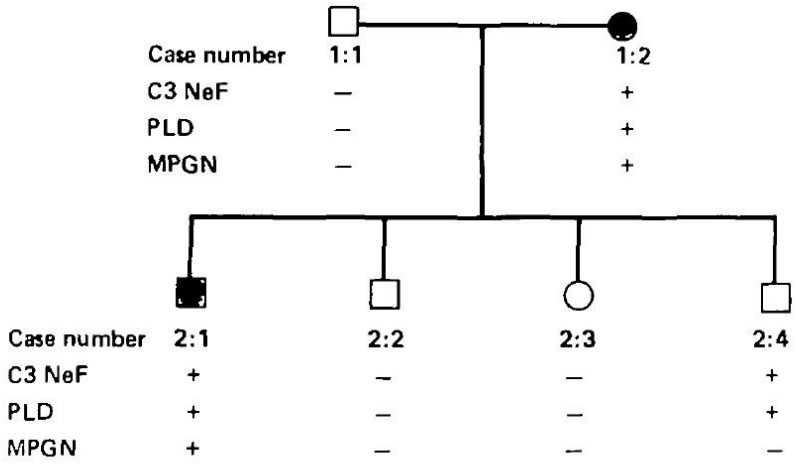

Figure 65 A case of familial MPGN, acquired partial lipodystrophy and C3 nephritic factor

Taken from (Power et al., 1990). Patient 1:2 and 2:1 had biopsy-proven MPGN, patient 1:2, 2:1 and 2:4 all had acquired partial lipodystrophy (abbreviated to PLD in this figure) and C3 nephritic factor (C3 NeF).

A number of novel rare genetic variants were identified in two cohorts of MPGN/C3G in chapter 3 of this thesis. The retrospective cohort study included members of the family described above. In an updated pedigree, patient 2.2 also developed ESRD. Screening for rare genetic variants in *C3*, *CFB*, *CFH*, *CFI* and *CD46* revealed a rare genetic variant in *CFH* that segregated with members of this family with the renal phenotype.

Of all of the novel rare genetic variants identified in chapter 3, this rare genetic variant identified in a familial case of MPGN was prioritised for functional study. It had a high likelihood of being pathogenic and it was in a functional domain of FH for which assays have

been previously described to study the structural (Hocking *et al.*, 2008) and functional (Pechtl *et al.*, 2011) consequences.

4.1.1 Chapter Aims

In this chapter I will therefore revisited this family to solve a long standing conundrum in the field complement mediated renal disease.

1. I will update clinical details and complement abnormalities of this family with MPGN in which a rare genetic variant in *CFH* was identified;
2. I will study the structural consequences of the genetic variant and
3. I will study the functional consequences of the genetic variant.

Overall, my aim is to solve the genetic basis of the disease in this family.

4.2 Clinical Description of a Case of Familial MPGN

4.2.1 Clinical Details

4.2.1.1 Patient 1:2

MPGN type 1 was diagnosed on renal biopsy (Figure 66) in 1975 following presentation with nephrotic syndrome and renal failure. She was aged 42. APL of the face was noted although the onset was not clear. C3 levels were low and sometimes undetectable with normal C4. She progressed to ESRD in 1984 and commenced haemodialysis. She died four months later due to complications of her renal failure.

4.2.1.2 Patient 2:1

MPGN type 1 was diagnosed on renal biopsy (Figure 66) in 1981 following presentation with dipstick albuminuria, haematuria, normal renal function and low C3 levels. He was aged 18. APL was noted although the time of onset was not clear. In 1985 he developed nephrotic syndrome and renal failure. He progressed to ESRD in 1988. He received a cadaveric renal transplant that failed after 7 years.

4.2.1.3 Patient 2:2

Initial presentation was with insulin-dependent diabetes mellitus aged 11. He had a spinal injury resulting in a complete T10 paraplegia aged 39. Renal failure and proteinuria developed and he progressed to ESRD aged 41. He did not have a renal biopsy and did not have APL. He died of sepsis aged 43.

4.2.1.4 Patient 2:3

Born in 1967, she remains healthy with no proteinuria, no haematuria and normal renal function. She does not have APL.

4.2.1.5 Patient 2:4

Born in 1969, he developed APL aged 8. He also has evidence of auto-immune diseases and developed arthritis of both knees aged 25 and multisystem symptoms with anti-nuclear

antibodies and double-stranded DNA antibodies aged 33. He does not however have evidence of renal disease. At latest follow up, his creatinine was 61μmol/L and he had normal urinalysis.

4.2.2 Biopsy Findings

Patients 1.2 and 2.1 had renal biopsies confirming MPGN type 1 (Figure 66). Both patients had light microscopy appearances of glomerular mesangial expansion, cellular proliferation and double contouring of glomerular basement membrane consistent with an MPGN pattern of injury. Electron microscopy revealed prominent sub-endothelial dense deposits.

In patient 1.2, reports from immunofluorescence studies describe localization of C3 and IgM in a coarsely granular pattern along capillary walls and in the mesangium. IgG, IgA and fibrin were present to a lesser extent. The immunofluorescence studies for patient 2.1 contained no glomeruli, but C3 was present in the arteriolar walls.

Patient 2.2 developed progressive renal failure. He was suspected of having MPGN type 1 due to the family history and low C3 levels. He did not have confirmatory renal biopsy.

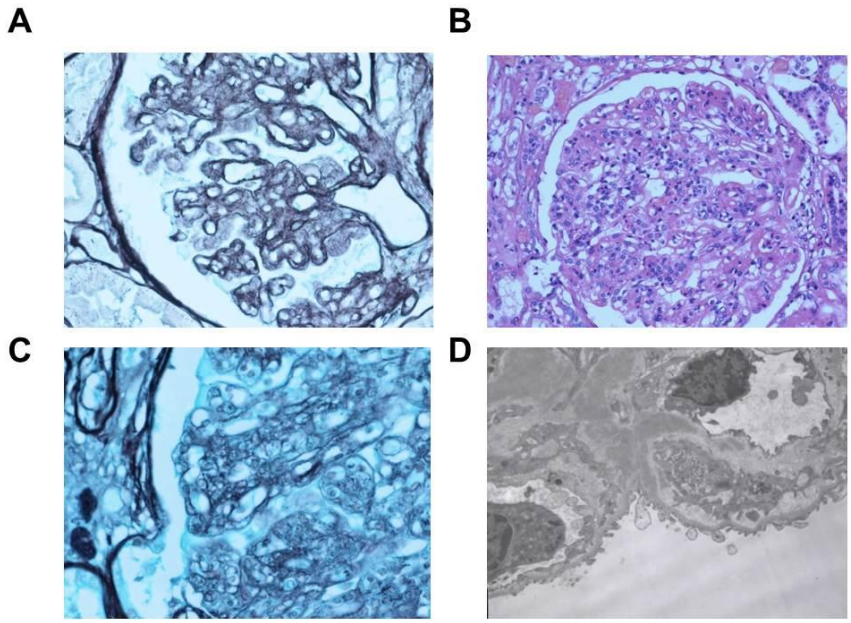

Figure 66 Biopsies of patients 1.2 and 2.1

Adapted from (Wong et al., 2014)(A-C) Light microscopy of renal biopsy from patient 1.2. (A) demonstrates layering of the glomerular basement membrane at initial diagnosis of MPGN (methenamine silver stain). (B) demonstrates diffuse global endocapillary proliferation and double layering of glomerular basement membrane (hematoxylin and eosin) in a post-mortem sample. (C) is a high power view and demonstrates part of the glomerular tuft on the right and Bowman's capsule and the beginning of proximal tubule on the left showing double layering of the glomerular basement membrane (methenamine silver stain) in a post-mortem sample. (D) Electron microscopy of renal biopsy from patient 2:1 demonstrating subendothelial and mesangial deposits.

4.3 Screening for Complement Abnormalities

Complement abnormalities are described below and summarised in Table 37.

4.3.1 Complement Levels

Patient 1:1 and 2:3 who were both healthy had no evidence of low C3 or C4. Patient 1:2, 2:1 and 2:4 all have had very low C3 levels. Patient 2:4 has persistently low C3 levels, whilst patient 2.1 subsequently had normal C3 levels. Patient 2:2 subsequently had slightly low C3 levels. There was no evidence of deficiency of FH, FI or CD46.

Patient	1:1	1:2	2:1	2:2	2:3	2:4
C3 (0.68-1.38 g/l)	N[1]	↓[1]	↓[1] / 0.73	N[1] / 0.61	N[1] / 1.87	↓[1] / 0.24
C4 (0.18-0.60 g/l)	N[1]	N[1]	N[1] / 0.19	N[1] / 0.18	N[1]	N[1] / 0.13
FH (0.35-0.59 g/l)	N/A	N/A	0.76	0.71	N/A	0.52
FI (38-58 mg/L)	N/A	N/A	58	62	N/A	59
C3 Nephritic Factor	-[1]	+[1]	+[1] / -	-[1] / -	-[1]	+[1] / +
Autoantibody to FH	N/A	N/A	-[2]	-	-	+
CFH genetic analysis	N/P	R83S	R83S	R83S	WT	WT
CFH haplotype	N/P	N/P	H3/H5	H3/H5	N/P	H3/H3
*CD46*gaggt haplotype	N/P	N/P	1 copy	1 copy	N/P	2 copies
*CD46*aaggt haplotype	N/P	N/P	0 copies	0 copies	N/P	0 copies
C3 R102G	N/P	N/P	R/V	V/V	R/R	R/V
C3 P314L	N/P	N/P	R/V	V/V	N/P	R/V

Table 37 Complement abnormalities in a family with MPGN

Adapted from (Wong et al., 2014). Complement levels, autoantibodies to complement components and genetic variants were measured and have been summarised. All measurements performed in 2009 unless stated. N – normal; [1] Historical measurements quoted in (Power et al., 1990); ↓ - reduced; N/A- not available. [2] Although a positive result was seen on ELISA, confirmatory western blot was negative. N/P- genetic analysis not performed; WT CFH genetic screening normal; R83S- variant present in heterozygosity; R- Reference sequence; V– Variant sequence.

4.3.2 Autoantibodies

C3 nephritic factor was present in patient 1:2, 2:1 and 2:4 when initially measured. Patient 2:2 was negative for C3 nephritic factor. Patient 2.1 subsequently was negative for C3 nephritic factor. Patient 2.4 remains positive for C3 nephritic factor.

Screening for autoantibodies to FH and subsequent characterisation was performed by Dr. Kevin Marchbank for patients 2:1, 2:2, 2:3 and 2:4. They were detected by ELISA. Confirmation of specificity for FH was confirmed on Western blot in patient 2:4 only. The autoantibodies were polyclonal in nature (Figure 67).

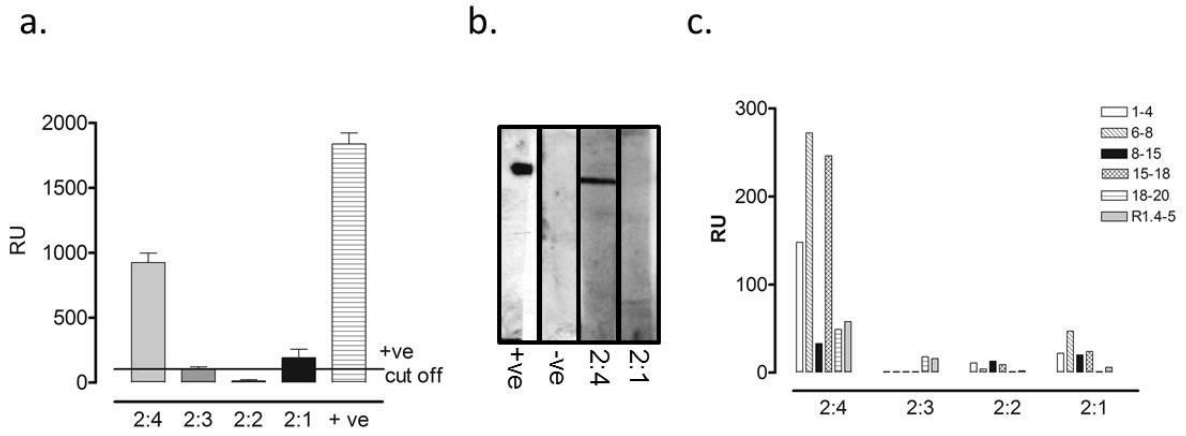

Figure 67 Autoantibodies to FH in familial case of MPGN

Figure generated by Dr. Marchbank and taken from supplementary data (Wong et al., 2014) RU=response units, –+ve = positive, –ve = negative. (a) The positive cut-off was derived from the 97.5th percentile of the autoantibody titre in controls (Goodship et al., 2012). (b) Purified FH was run on 10% SDS-PAGE and transferred to nitrocellulose. Strips were incubated with patient and control sera. Intervening irrelevant strips of the screening blot have been excised as indicated by T bar. Only individual 2:4, was positive. (c) Epitope mapping was undertaken using short fragments of FH and FHR1 using the same ELISA protocol as for purified FH. The autoantibody response for patient 2:4 was polyclonal in nature.

4.3.3 Genetic Screening

4.3.3.1 Identification of Rare Genetic Variants

All patients with the renal phenotype (1:2, 2:1 and 2:2) carried a rare genetic variant in *CFH* in heterozygosity. The rare variant c.249G>T that results in a non-synonymous substitution in the regulatory domain of FH, p.R83S was identified. The variant was not identified in patients 2:3 and 2:4.

No rare genetic variants were identified in *C3*, *CFB*, *CFI* or *CD46*.

R83S was predicted to be 'deleterious' and 'possibly damaging' in *in-silico* analysis, using Provean and Polyphen respectively.

4.3.2.2 Identification of Common Polymorphisms

Patients 2:1, 2:2, 2:3 and 2:4 were genotyped for SNPs in *C3*, *CFH* and *CD46*. The affected patients 2:1 and 2:2 carried the *CFH* haplotypes H3 and H5 that contain the DDD-associated SNP I62V. They also carried the DDD-associated SNPs in *C3,* R102G and P314L. No patient carried the $CD46_{aaggt}$ haplotype associated with C3GN and MPGN type 1. Patients 2:1 and 2:2 both carried one copy of the $CD46_{gaggt}$ associated with aHUS.

4.4 Structural Effects of the R83S Variant

4.4.1 Expression and Purification of WT and R83S SCR1-2 fragment of FH

The genetic variant described in this family of MPGN was a point mutation that resulted in an amino acid substitution within SCR1-2 of FH. In order to study the structural consequences of this, recombinant WT and R83S sequence variants in the pPICZαB vector were generated in *Pichia pastoris* in the setting of SCR1-2 FH using ^{15}N-ammonium sulphate as the sole nitrogen source in culture media. Figure 68 confirms successful introduction of R83S into the pPICZαB vector containing cDNA encoding SCR1-2 of FH (residues 19-142) (Hocking *et al.*, 2008).

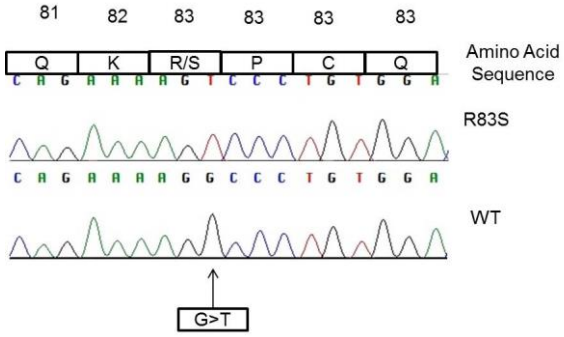

Figure 68 Sanger sequencing of maxiprep DNA to confirm introduction of point mutations – R83S

Site-directed mutagenesis was used to introduce the R83S variant into pPICZαB containing cDNA encoding SCR1-2 of FH (residues 19-142). The mutation was confirmed by Sanger sequencing.

Expressed protein was purified from supernatant using cation exchange. The protein was free of aggregates and degradation products following size exclusion and reverse phase chromatography (Figure 69).

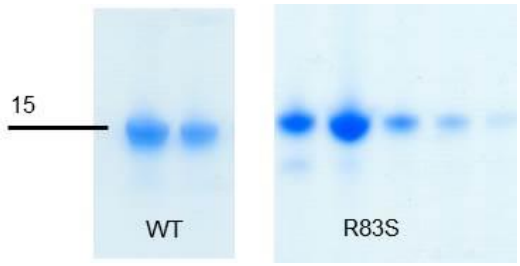

Figure 69 Purified FH SCR1-2 protein on SDS-PAGE – R83S

WT and R83S SCR1-2 fragments of FH expressed in Pichia pastoris using ^{15}N-ammonium sulphate has the sole nitrogen source in culture media were free of aggregates or degradation products following cation exchange, size exclusion and reverse phase chromatography. Following 4-15% SDS-PAGE and under non-reducing conditions, bands are visualised at ~15kDa the expected size of the protein.

4.4.2 NMR Spectroscopy

NMR spectroscopy was used to determine the functional consequences of the R83S mutant protein. The data was acquired at 37°C and two dimensional $^{1}H^{15}N$-HSQC spectra were generated and overlaid for analysis by Dr. Andy Herbert and summarised in Figure 70, generated by Dr. Andy Herbert. Data was compared with a previously assigned WT spectrum (Hocking *et al.*, 2008).

Spectra from both WT and R83S proteins demonstrated good chemical shift dispersion consistent with a well-structured protein. Furthermore, the differences between the two spectra were few, and localised to the intermodular interface between FH SCR1 and 2.

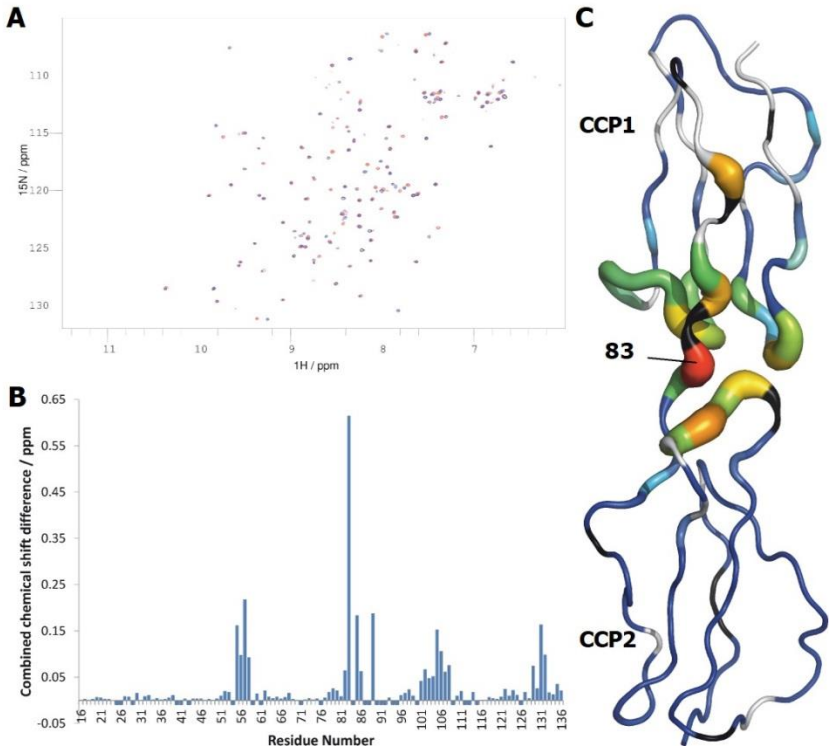

Figure 70 Analysis of HSQC spectra obtained from NMR spectroscopy of WT and R83S FH SCR 1-2

Taken from (Wong *et al.*, 2014). ^{1}H-^{15}N-heteronuclear single quantum coherence spectra of WT and R83S FH SCR1-2 were acquired at 37°C, and resonances were assigned where possible by comparison with previously assigned WT FH SCR1-2 spectra (Hocking *et al.*, 2008). (A) Overlay of ^{1}H-^{15}N-heteronuclear single quantum coherence spectra of FH SCR1–2 WT (blue) and R83S (red) show good chemical shift dispersion consistent with a well-structured protein, implying that R83S does not result in local unfolding of the protein. (B) The combined ^{1}H and ^{15}N chemical shift differences of R83S with respect to WT chemical shifts are represented graphically. Residues for which no chemical shift difference could be ascribed have been given a value of -0.01. Most residues exhibits only minor chemical shift differences (only 18 amino acids with combined chemical shift difference greater than 0.05 ppm), indicating that the overall fold of the protein should remain largely unchanged as a result of this mutation. (C) Chemical shift differences are represented on a cartoon: line thickness and colour (blue to red with increasing chemical shift difference) indicate the degree of chemical shift difference. The positions of proline residues (for which it is not possible to assign chemical shifts) are displayed in black, and residues with chemical shift that could not be confidently assigned are displayed in white. It is clear from this representation that the mutation R83 results in only localized changes in the structure of the protein; however, these changes are located at the intermodular interface between SCRs 1 and 2.

4.4.3 Structural Modelling of R83S Mutant Protein

The R83S variant was then modelled on the co-crystal structure of FH1-4:C3b and displayed using Pymol (Figure 71). It was found to be in direct opposition to C3b. NMR spectroscopy revealed only small localised changes in structure consistent with a secreted protein and normal FH levels. However, the amino acid change (R to S) results in the loss of electrical change, which I predicted would weaken the interaction of FH with C3b and impair the regulatory activity of FH in keeping with *in-silico* analysis. I therefore studied the functional consequences of the R83S variant.

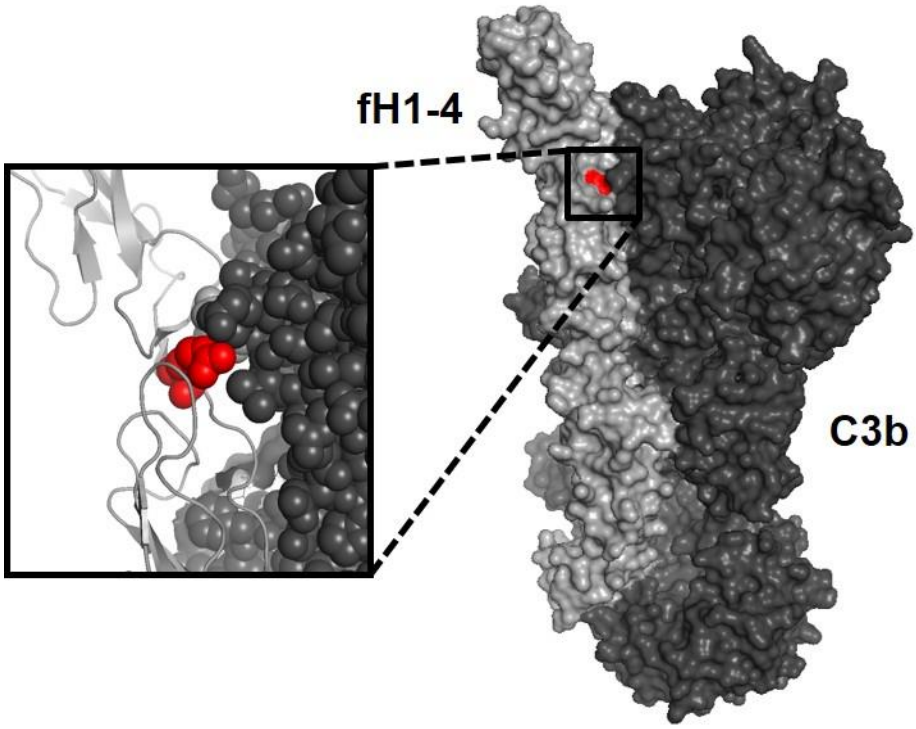

Figure 71 Structural modelling of R83S variant on co-crystal structure of FH SCR1-4 and C3b

Taken from (Wong et al., 2014). The mutated amino acid R83S is modelled on the co-crystal structure of FH:C3b using Pymol. R83S is on the surface of FH and is at the interface of FH SCR1-4 (light grey) and C3b (dark grey).

4.5 Functional Consequences of the R83S Mutant Protein

4.5.1 Expression and Purification of WT and R83S FH SCR1-4

In order to study the functional consequences of the R83S variant, recombinant wildtype (WT) and R83S sequence variants were generated in *Pichia pastoris* in the setting of SCR1-4 FH. Site-directed mutagenesis was used to introduce the R83S variant into pPICZαB containing cDNA encoding SCR1-4 of FH (residues 19-263). The mutation was confirmed by Sanger sequencing.

Expressed protein was purified by nickel-affinity chromatography and size exclusion and were free of aggregates and degradation products as shown in Figure 72.

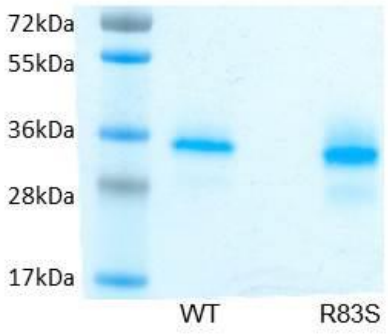

Figure 72 Purified FH SCR1-4 protein on SDS-PAGE – R83S

WT and R83S SCR1-4 fragments of FH expressed in Pichia pastoris are free of aggregates or degradation products following Nickel-affinity chromatography and size exclusion. Following 4-15% SDS-PAGE and under reducing conditions, bands are visualised at ~30kDa.

4.5.2 Determination of Affinity of FH SCR1-4 to C3b

The affinity of FH SCR1-4 to C3b was determined using SPR. The steady-state binding response of FH SCR1-4 fragments at different concentrations were plotted to generate an affinity curve. The affinity constant (K_D) was then determined using Biacore T200 Evaluation software in a steady state affinity model. The K_D was 10.7μM for the WT FH SCR1-4 interaction with C3b. There was minimal interaction of R83S FH SCR1-4 to C3b observed using the concentrations of protein available (up to 40μM). An estimated K_D for the R83S interaction with C3b was 75μM in the same conditions (Figure 73).

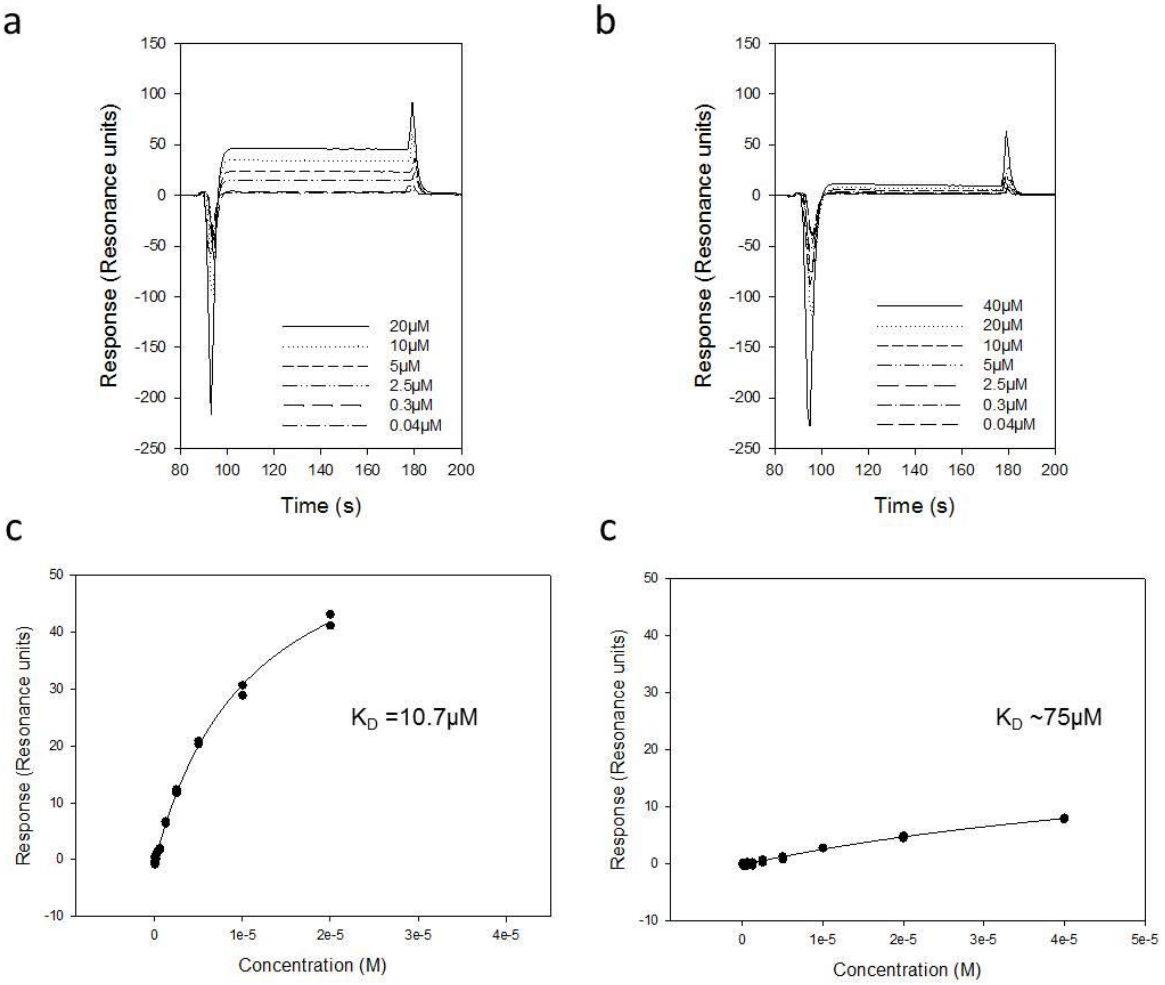

Figure 73 Binding of FH SCR1-4 to C3b using SPR – R83S

Taken from (Wong et al., 2014). C3b was immobilized to a CM5 chip using standard amine coupling. Injections of WT and R83S FH SCR1-4 were performed in duplicate in 10mM HEPES-buffered saline with 3mM EDTA and 0.05% surfactant P20. Overlaid sensograms show steady-state binding response (resonance units) of (a) WT (0.04–20 μM) and (b) R83S (0.04–40 μM) to C3b. An affinity curve was plotted from steady-state response (resonance units) versus concentration and used to estimate K_D and shown for (c) WT and (d) R83S.

4.5.3 Determining Decay Activity in Real Time using SPR

The functional consequences of impaired binding to C3b were then assessed. Decay acceleration was studied in an SPR-based decay acceleration assay. Figure 74 shows that R83S FH SCR1-4 exhibits minimal decay activity compared to WT FH SCR1-4.

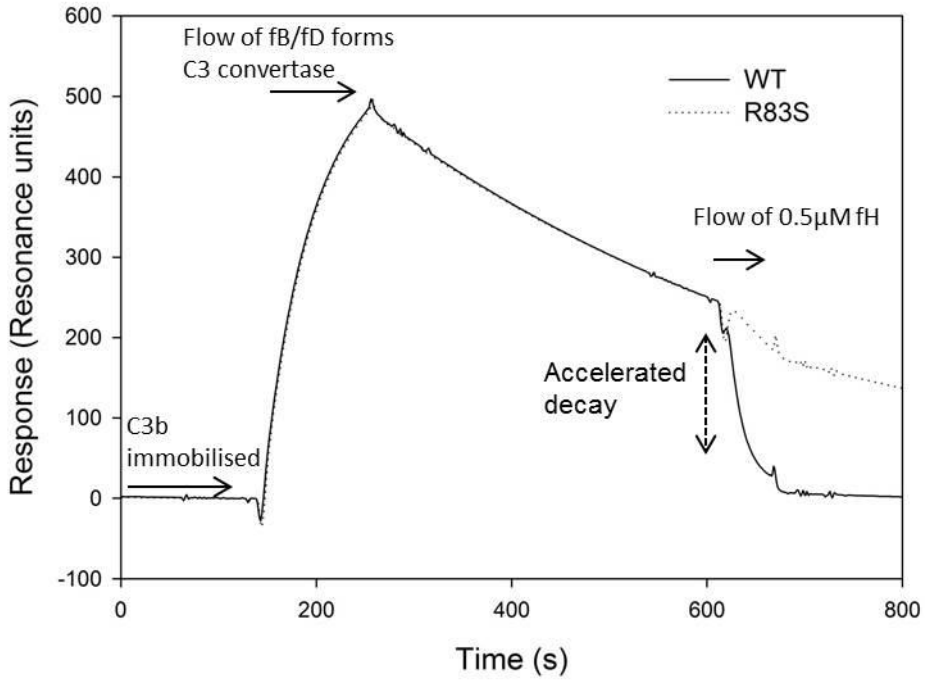

Figure 74 Decay activity in real time using SPR – R83S.

Adapted from (Wong et al., 2014). The flow of FB and FD on the surface of C3b immobilised on a CM5 biosensor chip forms an on-chip C3bBb as shown on overlyaing sensograms. C3bBb is allowed to naturally decay before the injection of 0.5µM FH SCR1-4 over the chip surface. WT FH SCR1-4 accelerates the natural decay of the on-chip C3bBb. R83S FH SCR1-4 has minimal effect on the natural decay of the on-chip C3bBb.

4.5.4 Determining Decay Activity in Sheep Erythrocyte Haemolysis Assays

Decay acceleration was also studied on the surface of sheep erythrocytes in a haemolytic assay. WT FH SCR1-4 was 9.1-times more effective than R83S FH SCR1-4 at protecting cells from lysis (IC_{50} for WT was 97.5 nM compared to IC_{50} for R83S 870 nM) (Figure 75).

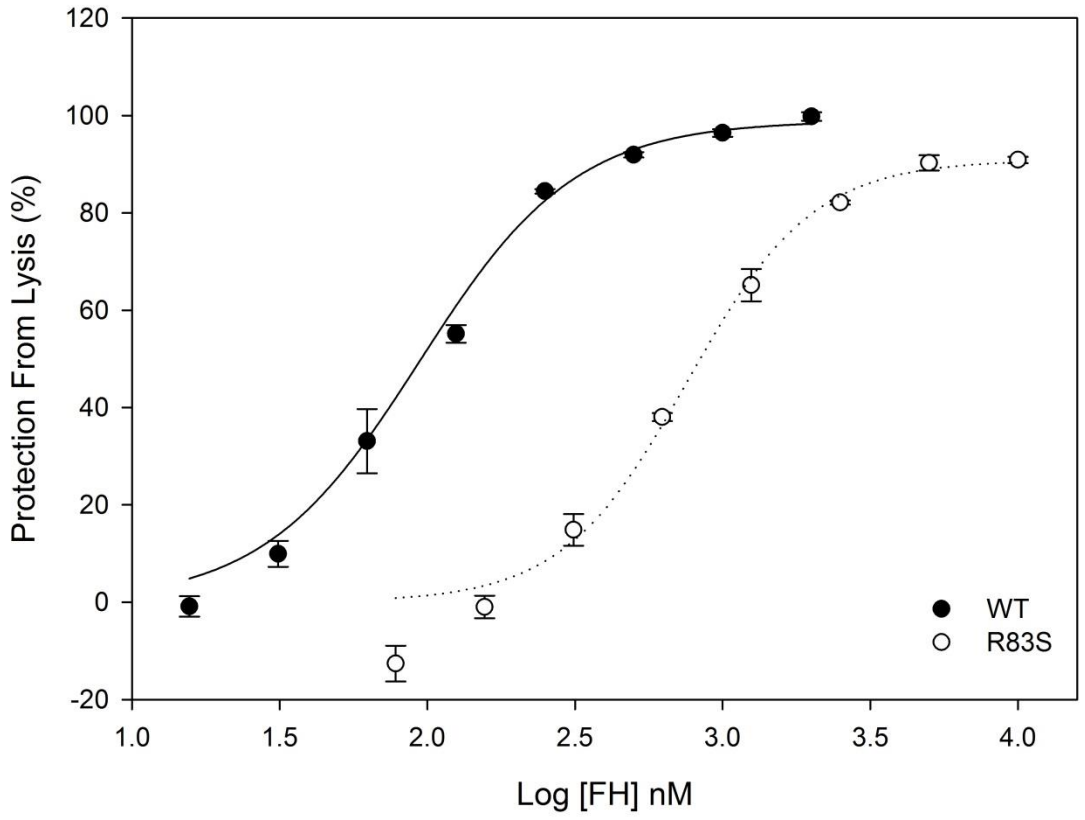

Figure 75 Decay activity on sheep erythrocytes – R83S

Adapted from (Wong et al., 2014). FH SCR1-4 was incubated with C3bBb formed on the surface of sheep erythrocytes. FH decays C3bBb and prevents lysis instigated by the addition of serum depleted of FB and FH. WT FH SCR1-4 is 9.1-times more effective than R83S FH SCR1-4 at protecting cells from lysis (IC_{50} WT95.5nM vs R83S 870 nM). Error bars represent 1 standard deviation from experiments performed in triplicate.

4.5.5 Determining Co-factor Activity in a fluid-phase assay

The effect of the R83S variant on co-factor activity was then studied in a fluid phase co-factor assay. WT FH SCR1-4 cleaved the α' chain of C3b to generate α chain fragments in a time course assay. R83S FH SCR1-4 had minimal activity compared to WT (Figure 76).

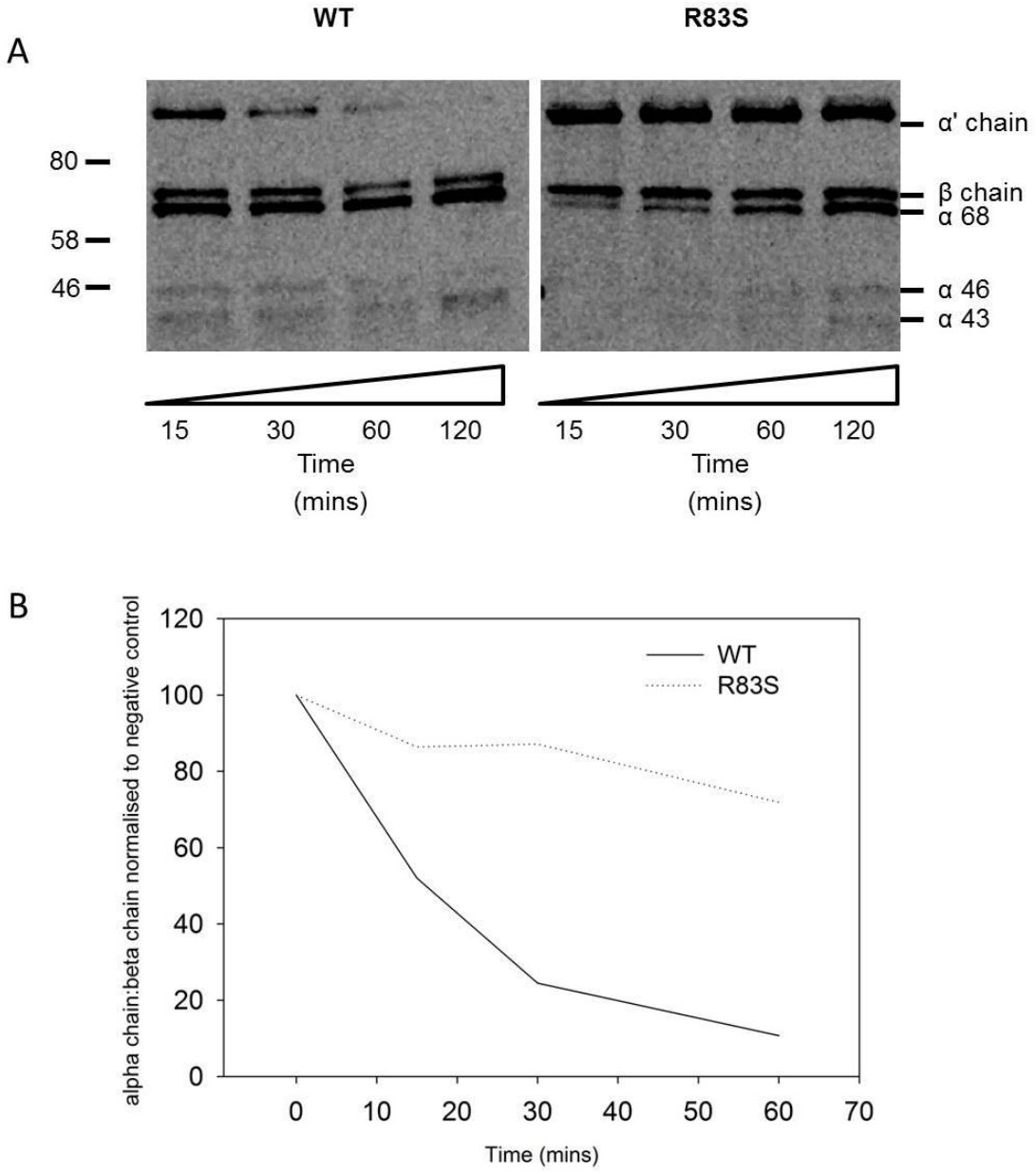

Figure 76 Co-factor activity in fluid-phase – R83S

Taken from (Wong et al., 2014). Limiting concentrations of WT and R83S FH SCR1-4 were incubated with FI and C3b for increasing time points. (A) The C3b-cleavage products were separated on 10% SDS-PAGE/Western blot under reducing conditions and scanned using densitometry and analysed by VisionWorks®LS Analysis Software. *(B) The time-course analysis shows the loss of C3b cleavage activity (ratio of α'-chain to β-chain normalized to percentage of negative control) of R83S compared with WT.*

4.5.6 Determining Co-factor Activity in Sheep Erythrocyte Haemolysis Assays

The loss of co-factor activity due to the R83S variant compared to the WT FH SCR1-4 was also confirmed on sheep erythrocyte cell surface assays (Figure 77). The IC_{50} for WT was 4.3µM. At this same concentration, the mutant R83S protein exhibited no co-factor activity.

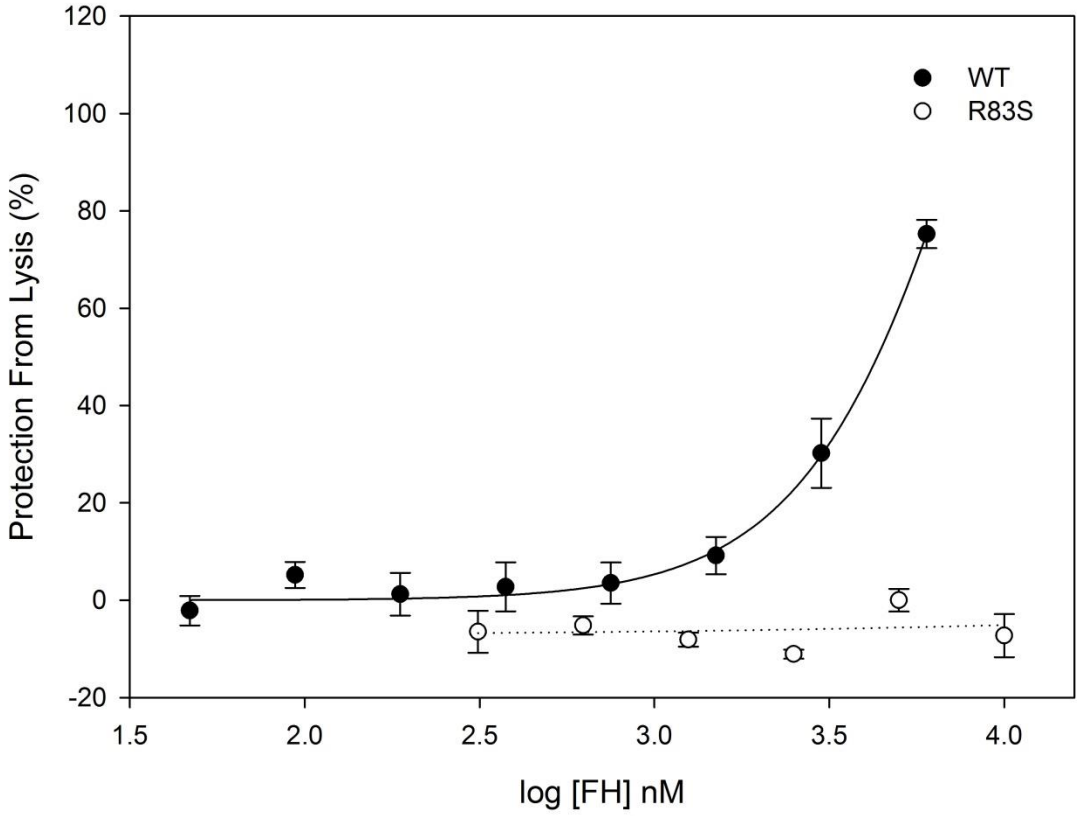

Figure 77 Co-factor activity on sheep erythrocytes – R83S

Adapted from (Wong et al., 2014). FH SCR1-4 was incubated with sheep erythrocytes coated with C3b and factor I for 8 minutes. C3bBb was formed on intact C3b and lysis instigated by the addition of serum depleted of FB and FH. C3b is inactivated in the presence of FI and its cofactor, FH. This prevents subsequent C3bBb formation and protects cells from lysis. WT FH SCR1-4 is more effective than than R83S FH SCR1-4 at protecting cells from lysis. Error bars represent 1 standard deviation from experiments performed in triplicate.

4.6 Discussion

4.6.1 Abnormalities of the Alternative Pathway in a Case of Familial MPGN

In this chapter I have described a rare genetic variant that was identified through screening for rare genetic variants in a case of familial MPGN. The variant, c.249G>T results in the amino acid substitution R83S and segregates completely with affected members. Structural studies suggested that the mutant protein results in only minor perturbations in structure localised to the intermodular interface of SCR1 and SCR2 of FH. This finding ruled out the possibility that R83S causes significant disruption to structure that might otherwise have led to unfolding and non-secretion of protein. FH levels in affected patients were not low and in keeping with a secreted mutant protein.

Structural modelling demonstrated that the affected amino was on the surface of FH directly interacting with C3b. *In-silico* analysis predicted the variant would have an effect on function, and functional studies confirmed that the affinity of the mutant R83S FH is markedly reduced compared to wildtype resulting in loss of decay and co-factor activity. These findings are similar to those of the R78G variant that was previously studied (Pechtl *et al.*, 2011).

The rare variant R83S was not the only complement abnormality identified in this family. Affected members also inherited the functionally significant risk polymorphisms in *C3* (Finn and Mathieson, 1993; Abrera-Abeleda *et al*., 2011) and risk haplotype in *CFH* (Abrera-Abeleda *et al*., 2006; Pickering *et al*., 2007) that associate with increase in AP activity and DDD. These common polymorphisms are likely to contribute to disease but cannot individually cause disease.

In addition to the genetic abnormalities, two acquired abnormalities were described in this family, C3 nephritic factor and autoantibodies to FH. C3 nephritic factor has been described in ~50-80% of cases of MPGN/C3G (Servais *et al.*, 2012). It is possible that the C3 nephritic factor is partly responsible in this family for the development of renal disease, in concert with the R83S variant leading to complement overactivation. The possibility that patient 2:2 had a C3 nephritic factor earlier in the clinical course cannot be excluded. C3 nephritic factor is observed in patient 2:4 who does not have renal disease. This suggests that in this family, the finding of C3 nephritic factor alone was insufficient for the onset of MPGN.

Patient 2:4 had an autoantibody to FH. The prevalence of autoantibody to FH in two cohorts of MPGN and C3G described in chapter 3 was 14.5 and 16.7%, a little higher than the 11%

reported in a French cohort (Blanc *et al.*, 2015). In most cases, autoantibodies to FH in these cohorts bound predominantly to an N-terminal fragment of FH. In this patient, the autoantibody bound well to SCR1-4, SCR6-8 and SCR15-18 suggesting polyclonal reactivity. Autoantibodies to FH have previously also been described in 11.5% of a cohort of rheumatoid arthritis (RA) and 6.7% of a cohort of systemic lupus erythematosus (SLE) (Foltyn Zadura *et al.*, 2012). Unlike in MPGN and C3G, where predominant binding to the N-terminal domain of FH is observed, autoantibodies to FH in RA and SLE were polyclonal with respect to epitope binding. Given the clinical features of patient 2.4 in addition to autoantibodies against dsDNA and anti-nuclear antibodies and C3 nephritic factor, the autoantibodies to FH in this patient are more consistent with underlying predisposition to autoimmunity rather than MPGN.

The familial nature of APL as first highlighted in this chapter was also a conundrum (Power *et al.*, 1990). The R83S variant in *CFH* did not segregate with APL. Whole exome sequencing was used by Dr. Rachel Challis to identify a rare genetic variant in lipodystrophy-associated genes (Appendix 7, Table 61). No variants were found in this analysis. All individuals with APL did however, have C3 nephritic factor and carried the C3 R102G polymorphism previously associated with C3 nephritic factor (Finn and Mathieson, 1993). The segregation of C3 nephritic factor with APL is consistent with previous studies (Finn and Mathieson, 1993; Misra *et al.*, 2004). A role for C3 nephritic factor in the pathogenesis of APL has been previously described (Mathieson *et al.*, 1993; Williams, 1997) and would be in keeping in this family in which C3 nephritic factor segregates with APL.

4.6.2 A Familial Case of MPGN and C3 Glomerulopathy?

The term C3G was developed to alert the clinician to the possibility of an underlying complement abnormality (Pickering *et al.*, 2013). The earliest classifications of C3G precluded the presence of any Ig staining within the glomerulus (Fakhouri *et al.*, 2010a). A diagnosis of C3G is currently considered if there is dominant C3 deposition, where dominant is defined as C3 intensity ≥2 orders of magnitude more than any other immune reactant on a scale of 0 to 3 (including 0, trace, 1+, 2+, 3+) (Pickering *et al.*, 2013). The presence of Ig staining on biopsy as observed in patient 1.2 does not exclude C3G and a diagnosis of C3G based upon the current consensus guidelines cannot be ruled out. Whilst this familial case has features in keeping with C3G a diagnosis of MPGN is certain. This case is a reminder that

abnormalities in AP can be found in cases of MPGN. The inability to positively identify a case of C3G on renal biopsy should not dissuade the clinician or researcher from similarly investigating the AP.

4.6.3 Limitations of study

Within the complexities of this familial case of MPGN, the finding and study of the R83S variant in *CFH* appears to elucidate a role of rare genetic variants in *CFH* in the pathogenesis of MPGN. Correlation of functional studies in this case and others in a cohort of patients is still required to understand their importance in defining disease phenotype and outcomes. Interpretation of the functional loss due to R83S variant assumes that these studies in the setting of FH SCR1-4 can be extrapolated to the full length protein.

4.7 Summary

Figure 78 summarises the genetic and acquired complement abnormalities described in this pedigree. Patients 1:2, 2:1 and 2:2 all have the renal phenotype and carry the rare genetic variant, R83S in heterozygosity. These also carry risk SNPs in *C3* and *CFH* that associate with increased AP activity. Acquired abnormalities in AP were also found in this family, these were also found in cases without the renal phenotype.

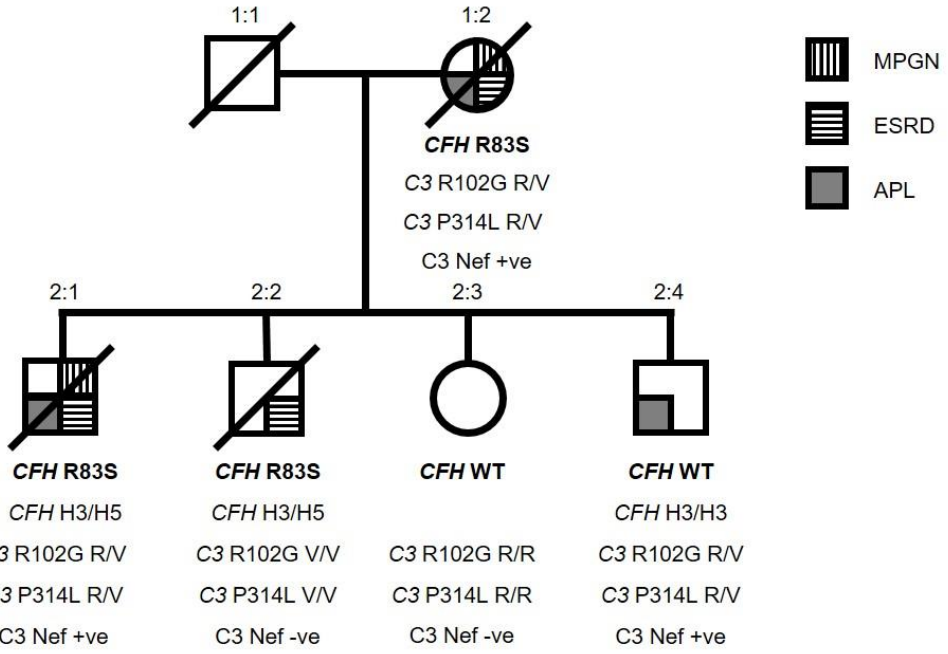

Figure 78 Updated pedigree and complement abnormalities in a case of familial MPGN

The results of CFH screening, CFH haplotype, C3 SNPs and C3 nephritic factor found in this pedigree are shown where available.

In summary, I have demonstrated that the rare genetic variant, R83S, in the N-terminal region of *CFH* in a case of familial MPGN was functionally significant and resulted in loss of regulatory function and solved a long standing conundrum in the field of complement-mediated renal disease. Further studies of other rare genetic variant in the N-terminal region of *CFH* are warranted.

Chapter 5 - Functional Study of rare N-terminal genetic variants in Complement Factor H

5.1 Introduction

Acquired and genetic abnormalities of the AP result in excess complement activation and associate with MPGN and C3G. Screening for such abnormalities in cohorts of MPGN/C3G has identified rare genetic variants in *CFH* in 4-16.2% (Servais *et al.*, 2012; Bu *et al.*, 2015; Iatropoulos *et al.*, 2016) of cases.

FH is the major fluid phase regulator of AP. Pathogenic variants may lead to quantitative FH deficiency (type 1 mutations) or normal levels of functionally impaired FH (type 2 mutation). The gold standard for confirming the pathogenic significance of type 2 mutations requires functional studies. If data from functional tests is not available, then the pathogenicity of a variant should be interpreted with caution and should be called variants of uncertain significance (VUS) (Kavanagh and Anderson, 2012).

Functional studies of rare genetic variants in *CFH* have generally been limited to those found within domains of known function. In aHUS, rare genetic variants in *CFH* cluster in the C-terminal recognition domain and have been extensively studied (Sanchez-Corral *et al.*, 2002; Ferreira *et al.*, 2009). Functional studies generally show that mutant FH proteins fail to regulate complement on host surfaces.

A smaller number of studies have described the functional consequences of variants in the N-terminal regulatory domain of *CFH* using either recombinant short fragments of FH or FH from serum, both purified and non-purified (Table 38).

Studies using non-purified FH from serum include a recent study in which the variant, W198R described in aHUS was tested in haemolytic assays in which patient serum was used to lyse sensitised sheep erythrocytes (Szarvas *et al.*, 2016). These assays do not differentiate between decay and co-factor activity and are dependent upon the lytic properties in the patient serum (Sanchez-Corral *et al.*, 2002).

A different approach to investigate the effects of variants within the N-terminal domain of *CFH* is to use purified FH from serum. Purified mutant FH protein from patient serum can be studied to determine the effects of a rare genetic variant on C3b binding, decay and co-factor activity (Licht *et al.*, 2006; Tortajada *et al.*, 2009). C-terminal function can also be tested in heparin and endothelial cell binding assays (Licht *et al.*, 2006).

A final approach has been to recombinantly express mutant proteins in a fragment of FH comprising the N-terminal regulatory domain (FH SCR1-4). Effects on individual regulatory functions can be determined and study of these mutants is not reliant upon patient sampling to obtain serum for functional tests (Pechtl *et al.*, 2011; Wong *et al.*, 2014).

Functional test	Recombinant protein[1]	Serum	Purified FH
C3b binding	SPR (Pechtl *et al.*, 2011)		SPR and peptide spot assay (Licht *et al.*, 2006)
Decay	SPR and haemolytic assay (Pechtl *et al.*, 2011)	Haemolytic assay[2] (Licht *et al.*, 2006)	
Co-factor fluid phase	Fluid phase and haemolytic assay (Pechtl *et al.*, 2011)		Fluid phase assay (Licht *et al.*, 2006)
Regulatory activity		Haemolytic assay[3] (Szarvas *et al.*, 2016)	
C-terminal function			C3d, heparin and endothelial cell binding assays (Licht *et al.*, 2006)

Table 38 Functional studies of rare disease-associated N-terminal mutations

[1]Recombinant protein was in the setting of SCR1-4 only. Assays used in the studies above (Pechtl et al., 2011) have also been described in full length FH protein purified from patients for the study of the common polymorphism, I62V (Tortajada et al., 2009), [2]Patient serum containing mutant FH is used to initiate lysis after C3bBb has formed on the surface of sheep erythrocytes in a decay assay, [3]Patient serum containing mutant FH is used to initiate lysis of sensitised sheep erythrocytes.

5.1.1 Chapter Aims

In this chapter I will

1. Review the rare genetic variants in *CFH* that have been described in cases MPGN/C3G.
2. Determine the functional significance of rare genetic variants in the N-terminal domain of *CFH* found in MPGN/C3G using recombinant FH SCR1-4 proteins in assays to test C3b binding, decay and co-factor activity.
3. Compare the functional effects of variants N-terminal domain of *CFH* in MPGN/C3G to those found in other diseases including aHUS and AMD.

5.2 Selection of Rare Genetic Variants in MPGN and C3G for Functional Study

5.2.1 Identification of Rare Genetic Variants in MPGN/C3G from the Literature

Rare genetic variants in *CFH* were identified from the literature in cases of MPGN or C3G. The published rare genetic variants and those identified in the cohort studies described in chapter 3 are summarised (Figure 79 and Appendix 8, Table 62). In total, 43 different rare variants in *CFH* were identified in 51 patients or pedigrees with MPGN or C3G. Two patients had 2 different rare variants. Seventeen variants (39.5%) were type 1 mutations that result in either complete or partial FH deficiency depending on whether they were inherited in homozygosity or heterozygosity respectively (Table 39). Functional studies have been performed for an additional 6 variants. Three of these, R78G (Pechtl *et al.*, 2011), delK224 (Licht *et al.*, 2006) and R83S (Wong *et al.*, 2014), (a variant identified in familial MPGN studied in chapter 4) are variants within the N-terminal domain of *CFH* and were shown to result in a mutant protein with complete loss of regulatory function. A fourth functionally significant variant was R1210C in the C-terminal domain (Recalde *et al.*, 2015). Functional studies have also been performed to determine the effect of the variants, Q950H and T956M. In these studies, a small loss of regulatory function was reported in the Q950H variant only (Szarvas *et al.*, 2016). Further data including FH levels and/or functional studies are required in the remaining variants to determine functional significance.

Five variants (P26S, R53C, DelE122-G128, D130N, A161S) identified in the functional N-terminal regulatory domain of *CFH* associate with normal FH levels and are the logical first choice variants for functional studies (Figure 79, Table 40).

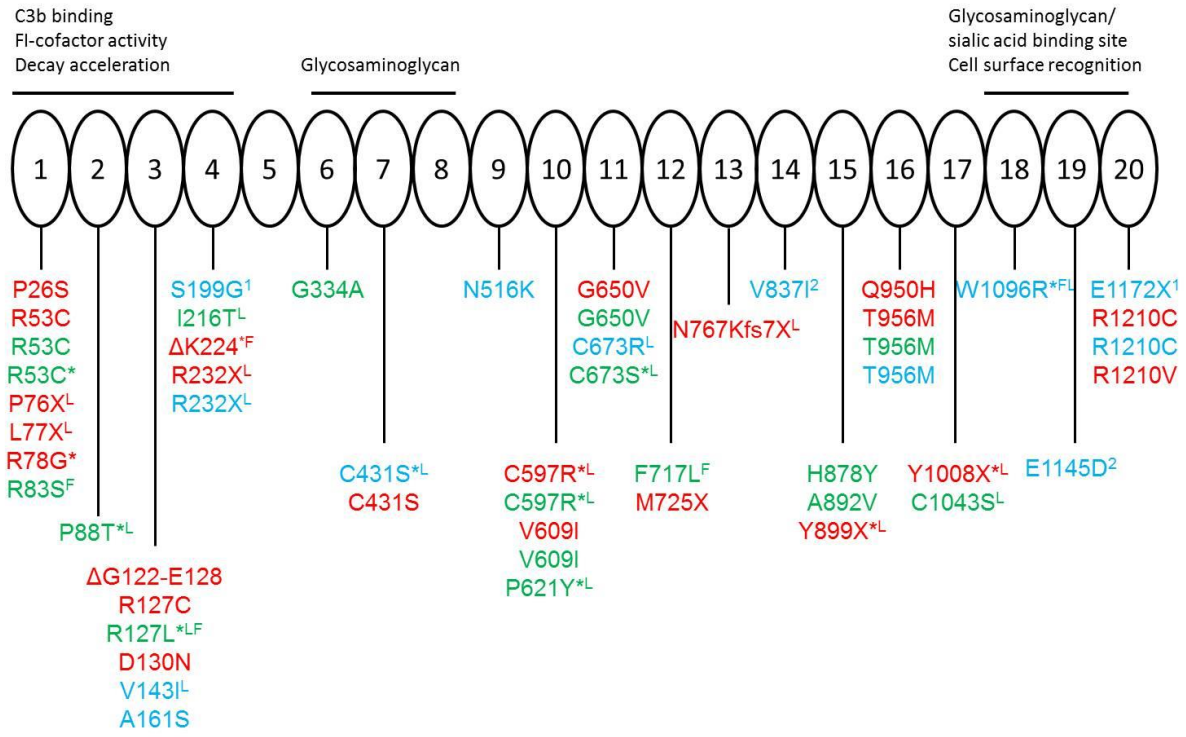

Figure 79 Rare genetic variants identified in CFH in MPGN and C3G

*43 rare variants identified in 51 patients or pedigrees have been annotated on a representation of the 20 short consensus repeats of FH and the functional domains. Disease types have been labelled in colour - C3GN (red), MPGN (green) and DDD (blue). * homozygous L low FH levels / Type 1 mutations F = familial members affected. Two patients carried 2 rare variants each, S199G/E1172X[1] and V837L/E1145D[2]. IVS11+5 not shown. Original sources are referenced in Appendix 8.8, Table 62.*

	Type 1 mutations – low FH levels		Functionally significant type 2 mutations
Homozygous	P88T[1]	C673S	R78G
	R127L[1]	Y899X	DelK224[1]
	C431S	Y1008X	
	C597R (2)	W1096R[1]	
	P621Y		
Heterozygous	P76X	R232X (2)	R83S[1]
	L77X	C673R	R1210C (2)
	V143I	K768X	
	I216T	C1043X	

Table 39 Rare genetic variants in CFH in MPGN and C3G leading to FH deficiency

[1]indicates familial disease () = number of patients with variant.

Normal FH levels	FH levels not known
P26S	R127C
R53C (3)	S199G/E1172X
DelG122-E128	N516K
D130N	V609I (2)
A161S	M725X
G334A	V837I/E1145D
C431S	Q950H[1]
IVS11+5	T956M (3)[1]
G650V (2)	
F717L (2)	
H878Y	
A892V	
R1210V	

Table 40 Rare genetic variants in CFH in MPGN and C3G of uncertain significance

() = Indicates number of patients with a particular rare variant.[1]Q950H and T956M had normal levels in published cases of aHUS in which functional studies were performed. Their levels in MPGN/C3G patients are not known.

5.2.2 N-terminal Rare Variants in *CFH* in other Renal Diseases

A total of 26 rare genetic variants in the N-terminal domain of *CFH* were identified in 28 patients with aHUS from the literature and includes 3 unpublished variants from the Newcastle aHUS registry (Table 41 and Appendix 9, Table 63). All were inherited in heterozygosity. Of these, 11 were type 1 mutations and 2 were functionally significant type 2 mutations. Of the remaining 13 variants, 6 associated with normal FH levels. FH levels in the remaining 7 cases could not be determined from the available literature. Two variants, R53C and A161S, have also been described in cases of MPGN/C3G.

Three rare genetic variants, also in the N-terminal domain of *CFH*, were identified in other renal phenotypes (Table 42). These all associated with low levels of FH.

Type 1 mutations	Functionally Significant	VUS (Normal FH levels)	VUS (Unknown FH levels)
R28Ifs5X	R53H	R53C	R53S
T30Nfs10X	R78G	W71R	S58A
I32X		Q81P	V111E
Y118Ifs4X		A161S (3)	W134R
I124Mfs12X		M162V	R166L
R127L		W198R	Y235C
P139S[1]			C247G
S159N[2]			
G218E			
R232X			
P258L			

Table 41 N-terminal rare genetic variants in CFH in aHUS

() = indicates number of patients with a particular rare variant. All were inherited in heterozygosity.[1]P139S associated with low FH levels in (Schejbel et al.*, 2011), [2]S159N was initially identified in AMD in Newcastle Complement Genetics Service with associated normal FH levels and selected for study.*

	Variant	Disease	Reference
Complete FH deficiency[1]	P139S E189X[2]	Endocapillary GN Lupus Nephritis	(Schejbel *et al.*, 2011) (Brai *et al.*, 1988) (Sanchez-Corral *et al.*, 2000)
Partial FH deficiency[3]	A48S	IgA Nephropathy	(Schmitt *et al.*, 2011)

Table 42 N-terminal rare genetic variants in CFH in other renal phenotypes

[1]inherited in homozygosity, [2]familial disease, [3]inherited in heterozygosity.

5.2.3 N-terminal Rare Variants in *CFH* in AMD

N-terminal variants in *CFH* have also been described in AMD, a disease associated with DDD. Two variants, G69E (Raychaudhuri *et al.*, 2011) and D90G (Yu *et al.*, 2014) had been described during the research period and were studied alongside those identified in renal disease. A third variant, S159N was identified in a case of AMD in the Newcastle Complement Genetics Service with normal FH levels. This was also selected for study. It was subsequently identified in a case of aHUS in the Newcastle Complement Genetics Service and associated with low FH levels (Table 40). A larger number of variants in AMD that were recently identified will be discussed later in this chapter.

5.2.4 Structural Modelling of N-terminal Variants Selected for Functional Study

The variants of uncertain significance in the N-terminal domain of *CFH* in MPGN, DDD, C3GN, aHUS and AMD were considered for functional studies. The variants P26S, W71R, W198R and S199G were not known to me at the time of study. The variants selected for study in this chapter were R53C, G69E, Q81P, D90G, delG122-E128, D130N, S159N, A161S and M162V.

The variants in the N-terminal domain of *CFH* described in renal diseases result in an amino acid substitution in SCR 1 (R53C, Q81P), in SCR 2 (D130N) and SCR 3 (S159N, A161S and M162V). One large deletion results in the loss of 7 amino acids in exon 2 (delG122-E128). The secreted variants in the N-terminal domain of *CFH* described in AMD only, result in an amino acid substitution in SCR 1 (G69E) and SCR2 (D90G) (Figure 80). Five variants (Q81P, D90G, S159N, A161S and M162V) affect amino acids that are on the surface of FH and interface with C3b. Two variants (R53C and D130N) are on the surface of FH and face away from C3b and on a putative surface that interacts with FI. The deletion G122-E128 results in the loss of 7 amino acids that are mostly on a surface that faces away from C3b. G69E faces away from both C3b and FI binding sites.

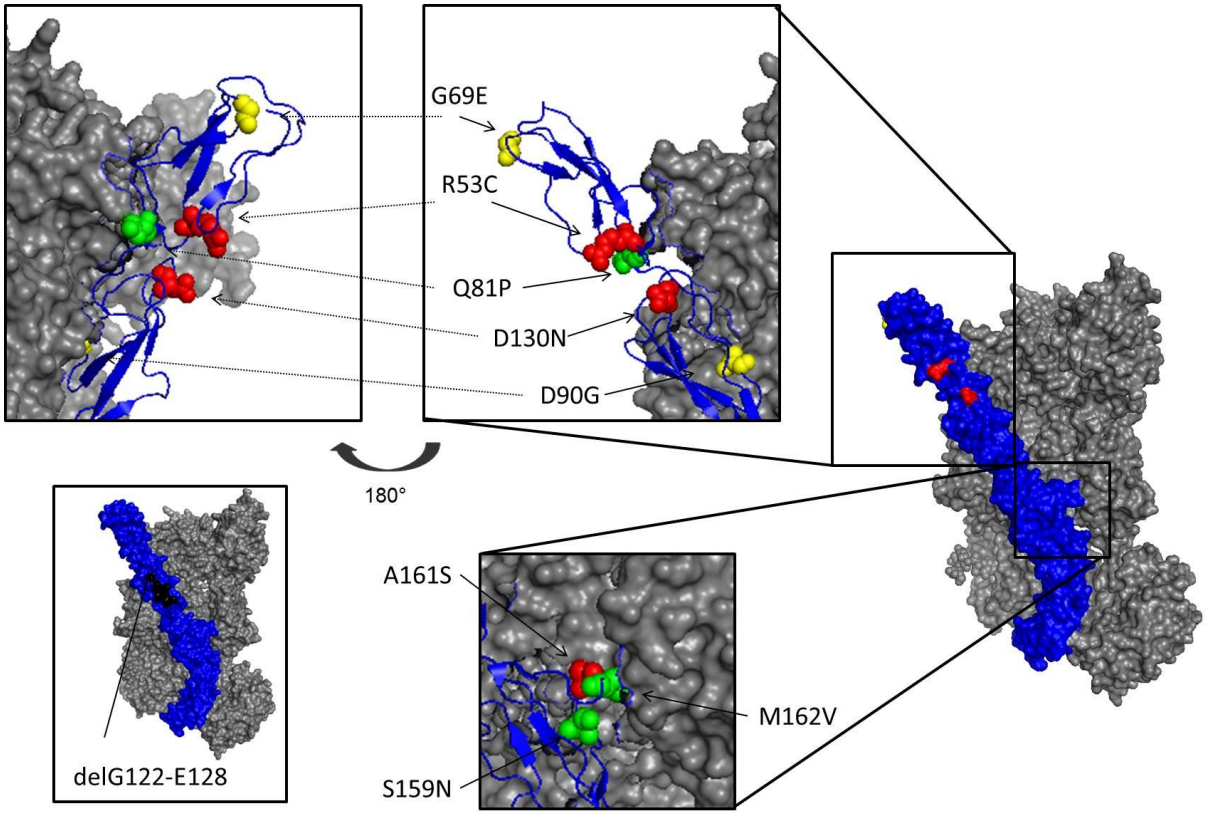

Figure 80 Co-crystal structure of FH SCR1-4:C3b showing rare genetic variants selected for functional study

Genetic variants in the N-terminal domain of CFH that had normal serum FH levels are modelled on a co-crystal structure of FH SCR1-4 (blue) and C3b (grey). The variants are all found on the surface of FH SCR1-4. Variants are coloured red if been described in MPGN/DDD/C3GN. The remaining variants are coloured green if described in aHUS (green) and lastly blue, if only described in AMD. The residues involved in the deletion G122-E128 (described in DDD) are shown in black to distinguish from other variants.

5.3 Functional Study of Rare Genetic Variants in the N-terminal Domain of *CFH*

5.3.1 Small Scale Expression of Mutant Protein

In order to study the functional consequences of the variants described in 5.2.4, recombinant WT and mutant sequence variants in the pPICZαB vector were generated in *Pichia pastoris* in the setting of FH SCR1-4. Figure 81 confirms successful introduction of each mutation by site-directed mutagenesis into the FH SCR1-4 clone in pPICZαB. A clone of the S159N variant was a gift from Professor Alan Wright, University of Edinburgh. Confirmation of introduction of this mutation is also shown. The 21bp deletion in the delG122-E128 variant was also successfully introduced and shown in Figure 82.

Following transformation of KM71H clones with the mutated FH SCR1-4 clones in pPICZαB, small scale expression of protein was performed. Figure 83 shows the successful expression of several mutant proteins in one experiment as demonstrated by the detection of bands on Western blotting. Bands migrating at the expected 25kDa under non-reducing conditions, consistent in size with a FH SCR1-4 protein were visualised. A band at this size was not present in the empty vector control and not in the delG122-E128 clone, suggestive of impaired secretion of FH of the delG122-E128 clone.

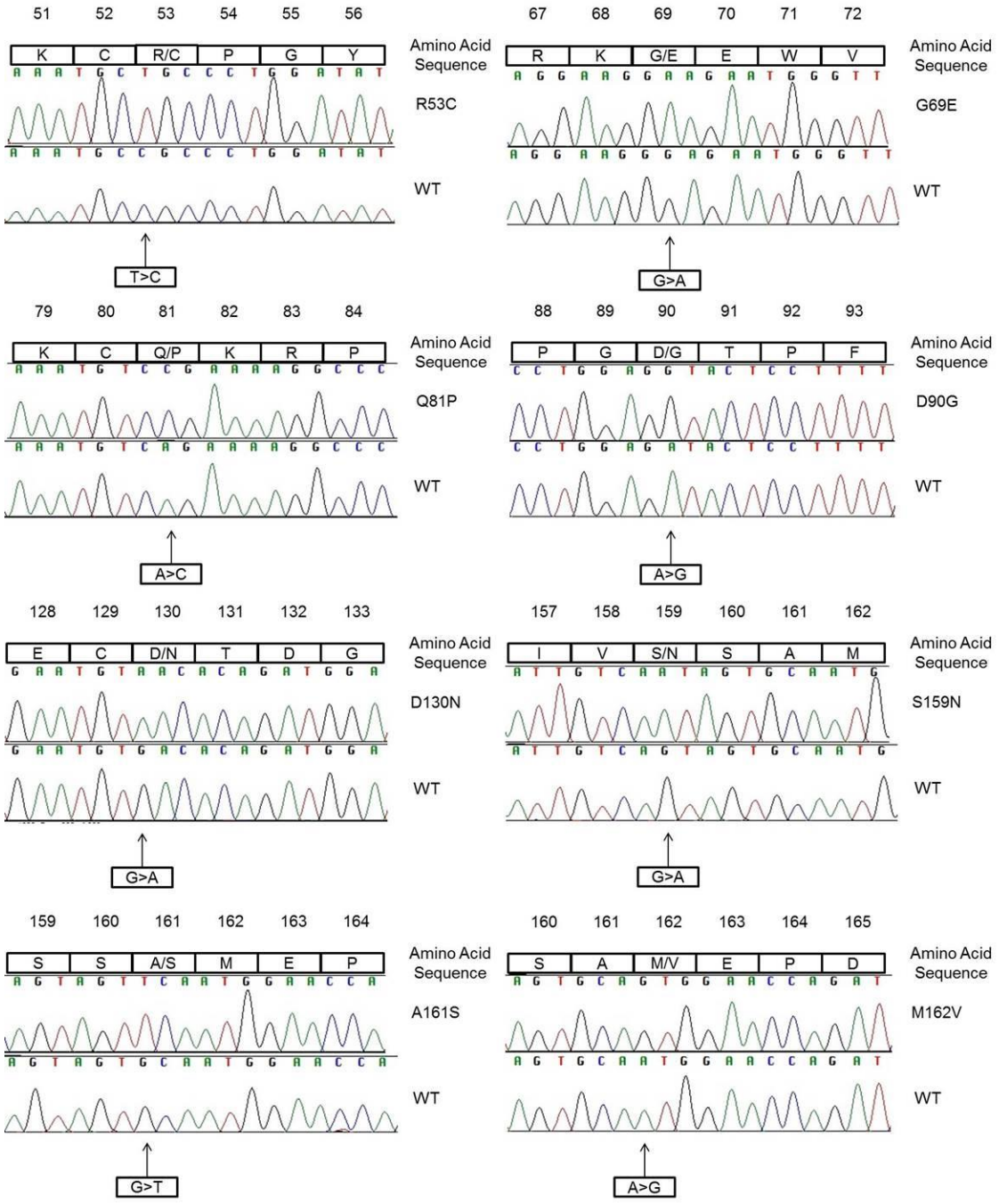

Figure 81 Chromatograms following Sanger sequencing of maxiprep DNA to confirm introduction of point mutations

Point mutations were introduced in order to express the mutant proteins R53C, G69E, Q81P, D90G, D130N, S159N, A161S and M162V in the setting of the FH SCR1-4 clone in pPICZαB. Amino acids and the positions flanking the mutated amino acid are shown above chromatograms for mutant and wildtype sequence.

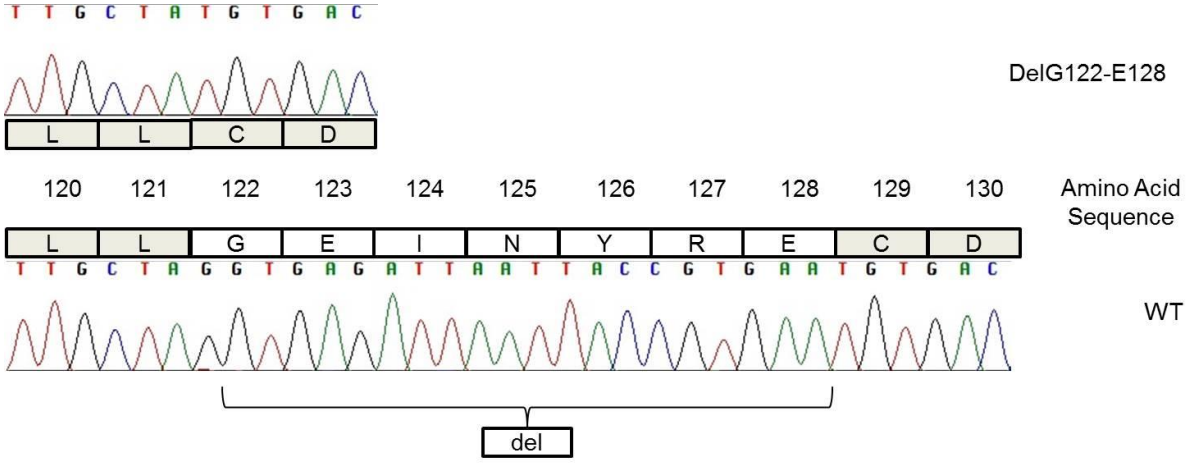

Figure 82 Chromatograms following Sanger sequencing of maxiprep DNA to confirm a 21bp deletion

The sequence of 21bp encoding the amino acids from G122-E128 were deleted from the FH SCR1-4 clone in pPICZαB. The 6bp flanking either side of the deleted sequences are shown encoding L120-L121 and C129-D130 (shaded boxes). These four amino acid are adjacent and in-frame in the mutated sequence.

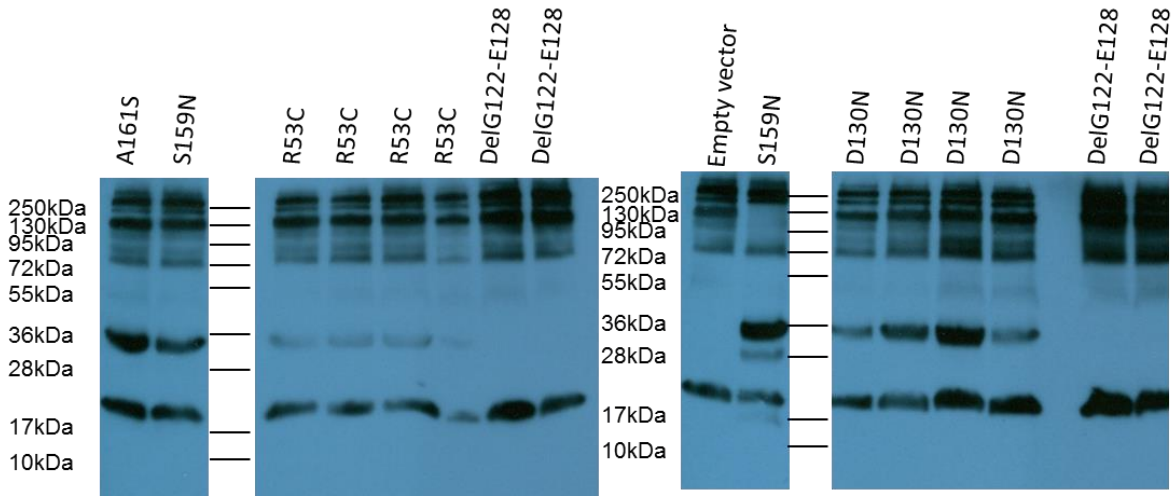

Figure 83 Expression of FH SCR1-4 variants on Western blot

Small scale cultures were centrifuged and the remaining supernatant was collected. Samples were separated on 4-15% gradient SDS-PAGE under reducing conditions and then transferred onto nitrocellulose membrane for Western blotting using a goat anti-FH antibody (1:14000) and rabbit anti-goat-HRP secondary antibody (1:3000). The Western blots show the presence of a band migrating to ~30kDa consistent in size with FH SCR1-4 in R53C, D130N, S159N and A161S. these bands were not visible in the empty vector control (that does not contain cDNA for FH SCR1-4) or DelG122-E128.

5.3.2 Confirmation of Purity of Purified Protein

Expressed protein was purified by nickel-affinity chromatography and size exclusion and was free of aggregates and degradation products. Figure 84 shows purified wildtype and mutant proteins.

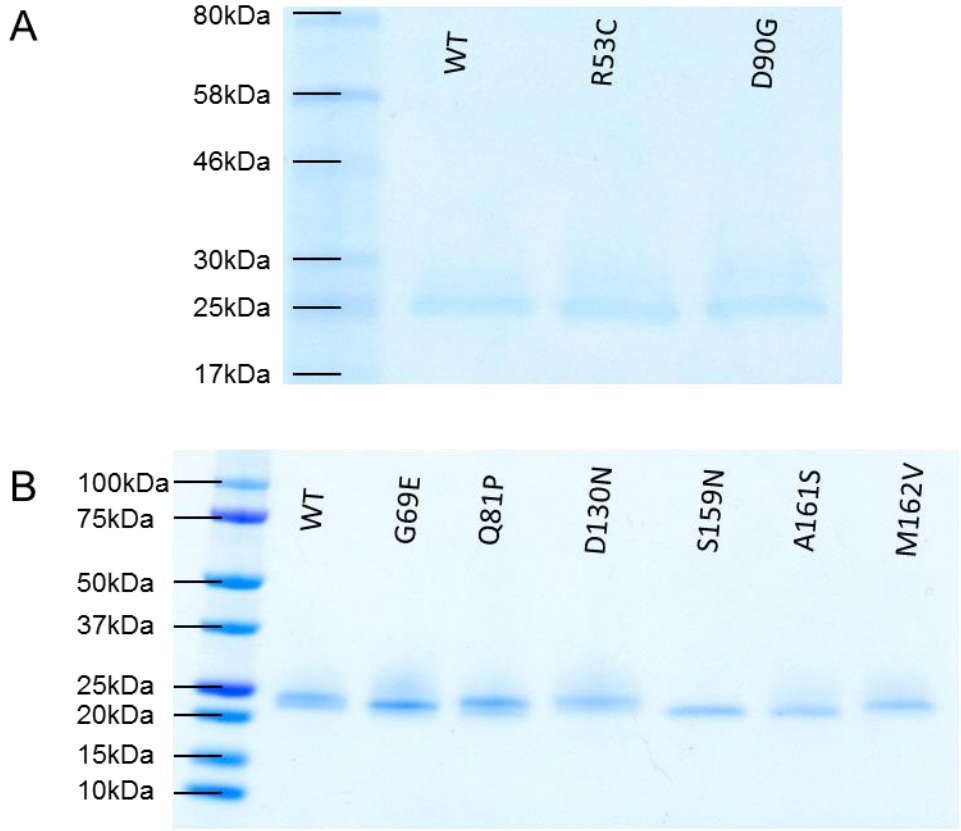

Figure 84 Purified FH SCR1-4 protein on SDS-PAGE

Samples of pooled purified protein (20µL, 1.5µM) were separated by 4-15% gradient SDS-PAGE under non-reducing conditions. Bands at ~25kDa are consistent with FH SCR1-4 and free from aggregates or degradation products. A) and B) show proteins expressed in different batches.

5.3.3 Reporting of Functional Effects of Rare Genetic Variant

Functional testing of the variants R53C and D90G was performed in comparison with wildtype in one batch of testing. Functional testing of the variants G69E, Q81P, D130N, S159N, A161S and M162V was performed during another batch of testing. Small changes to the testing protocol were made as part of ongoing optimisation of assays and are described in each individual section. For each variant, experiments were always performed in direct comparison with wildtype protein. The effect of a variant on K_D or IC_{50} was reported relative to the value determined for wildtype for each experiment. Representative data for experiments testing R53C and D90G are shown in separate figures to experiments testing the other variants.

5.3.4 Determination of Affinity of FH SCR1-4 to C3b

The affinity of FH SCR1-4 to C3b was determined using SPR. The steady-state binding response of FH SCR1-4 fragments at different concentrations were plotted to generate an affinity curve. The affinity constant (K_D) was then determined using Biacore T200 Evaluation software in a steady state affinity model.

The interaction between Q81P and C3b was the weakest of all of the variants tested. A K_D for this interaction could not be calculated using the concentration of protein available. The remaining variants bound well, with small differences ranging between 0.92-fold to 1.47-fold the K_D calculated for the interaction of WT FH SCR1-4:C3b (Figure 85 and Figure 86), suggestive that there was slight impairment of affinity for some variants.

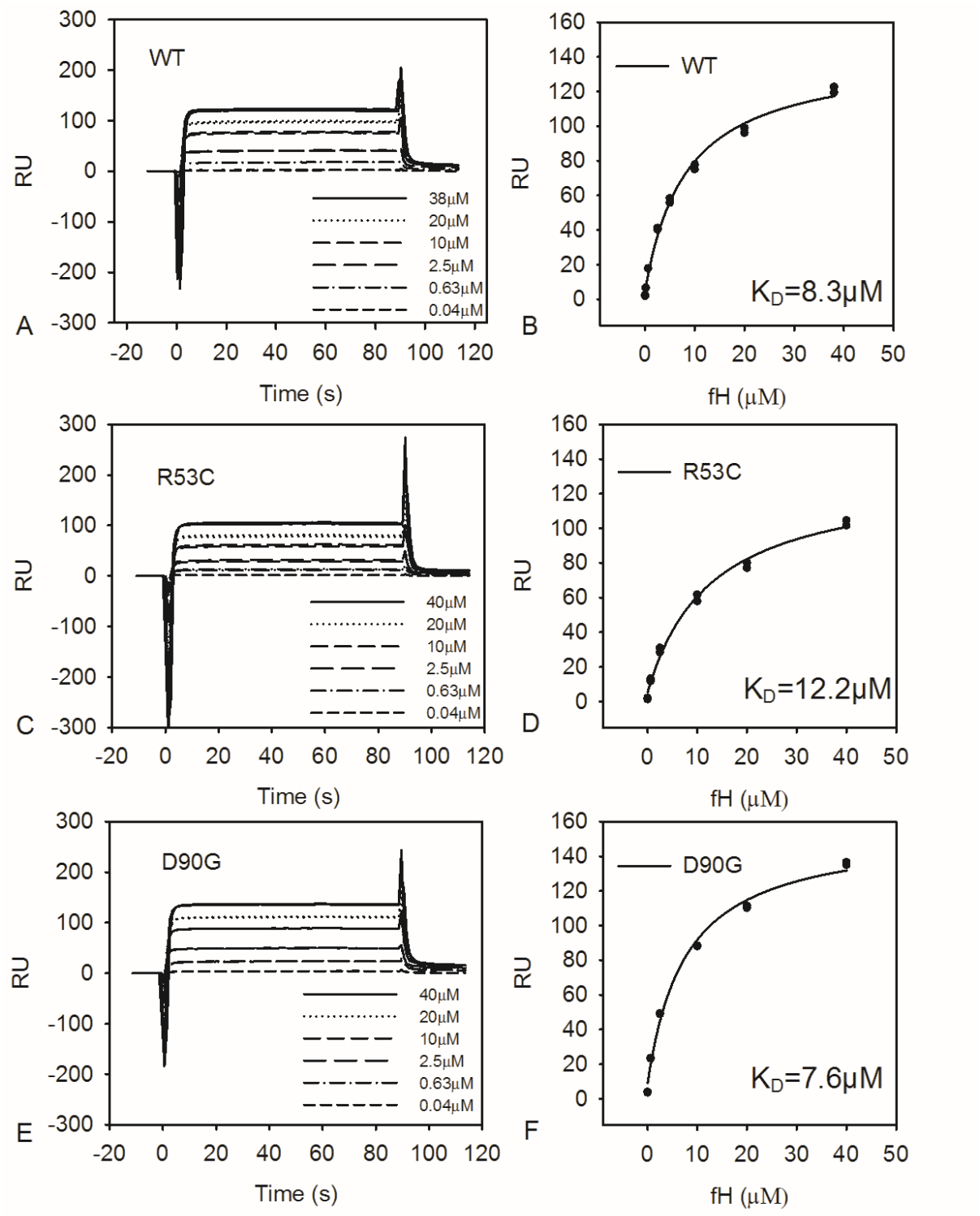

Figure 85 Binding of FH SCR1-4 to C3b using SPR - R53C and D90G

860 Resonance units (RU) of C3b was immobilised on the surface of a CM5 chip. FH SCR1-4 (WT, R53C and D90G) was flowed over the surface at a range of concentrations (0.4-40μM). Overlaying sensograms demonstrate binding of FH SCR1-4 to C3b for WT, R53C and D90G (A, C, E). The steady state response was plotted against the concentration of FH SCR1-4 to generate an affinity curve. A K_D was calculated for the interaction between mutant FH SCR1-4 and C3b. The ratio of K_D for the mutant FH SCR1-4 interaction compared to the WT FH SCR1-4 interaction with C3b was 1.47-fold for R53C and 0.92-fold for D90G.

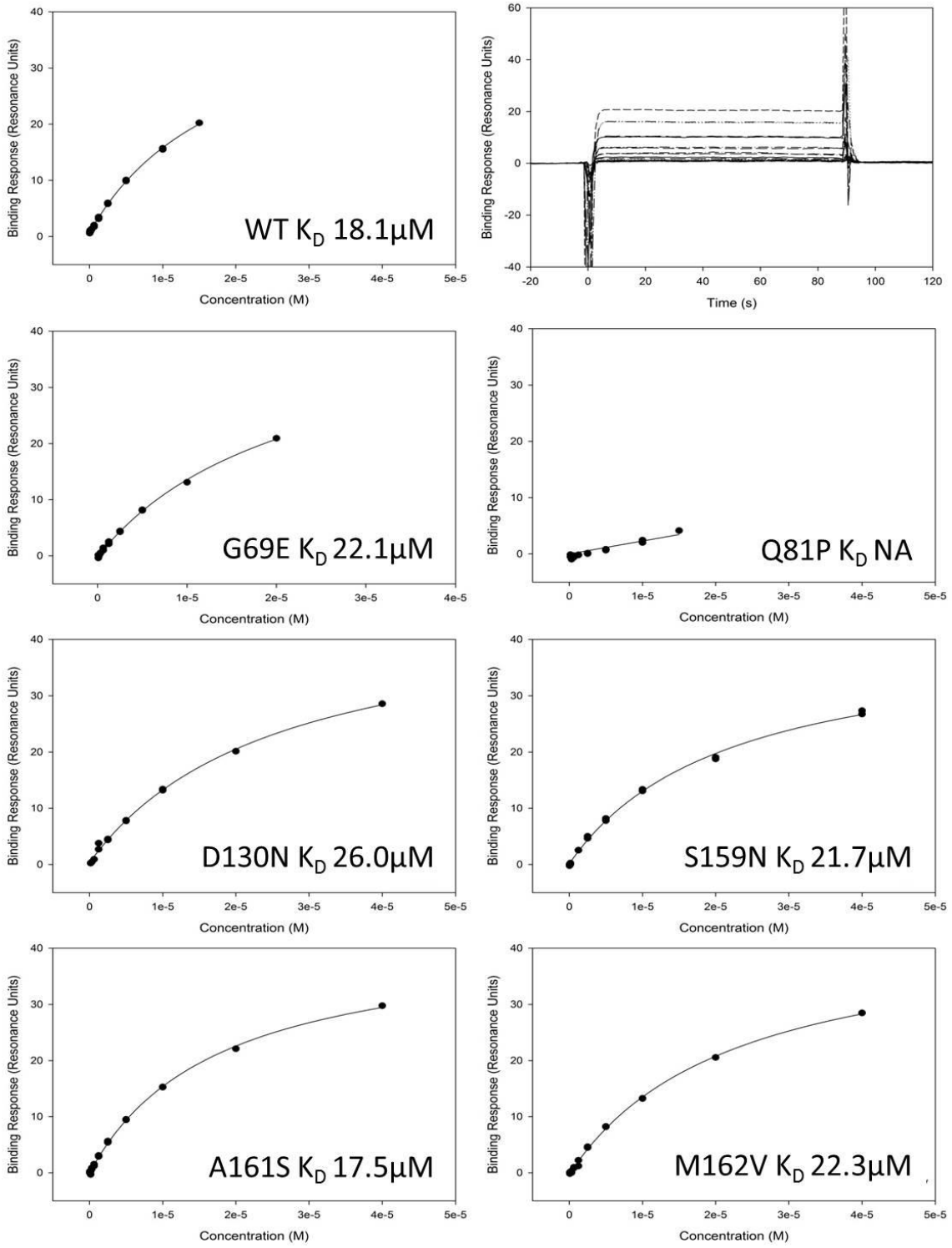

Figure 86 Binding of FH SCR1-4 to C3b using SPR - G69E, Q81P, D130N, S159N, A161S and M162V

480 RU C3b was immobilised on the surface of a CM5 chip. FH SCR1-4 (WT, G69E, Q81P, D130N, S159N, A161S and M162V) was flowed over the surface at a range of concentrations (0.4-40µM). Representative overlaying sensograms for WT (top right) shows that steady state binding was achieved over a range of concentrations. The steady state response was plotted against the concentration of FH SCR1-4 to generate an affinity curve and a K_D was calculated for the interaction between the mutant FH SCR1-4 and C3b. The difference in K_D compared to the WT FH SCR1-4 interaction with C3b was 1.22-fold for G69E, 1.44-fold for D130N, 1.20-fold for S159N, 0.97-fold for A161S and 1.23-fold M162V. A K_D could not be determined for the interaction between Q81P and C3b.

5.3.5 Determining Decay Activity in Real-time Using SPR

The functional consequences of impaired binding to C3b were then assessed. Decay acceleration was studied in an SPR-based decay acceleration assay. C3bBb was generated on immobilised C3b captured on the surface of a CM5 biosensor chip. In the experiments testing R53C and D90G, C3bBb was generated using 500nM FB and 60nM FD. The subsequent batch of experiments testing G69E, Q81P, D130N, S159N, A161S and M162V were performed using 250nM FB and 30nM FD during the formation of C3bBb as described in the methods. All convertases were allowed 210 seconds of natural dissociation before the injection of wildtype and mutant FH SCR1-4 protein to determine its effect on decay of the remaining convertase visualised in real-time. Sensograms (Figure 87 and Figure 88) are overlaying showing C3bBb formation and decay using wildtype and mutant FH SCR1-4 at equimolar concentrations. Sensograms have been normalised from the baseline prior to the injection of FB and FD (0%) to the maximal binding at the end of the injection (100%).

At 500nM, R53C had minimal convertase decay activity compared to wildtype whereas D90G had comparable decay activity to wildtype (Figure 87).

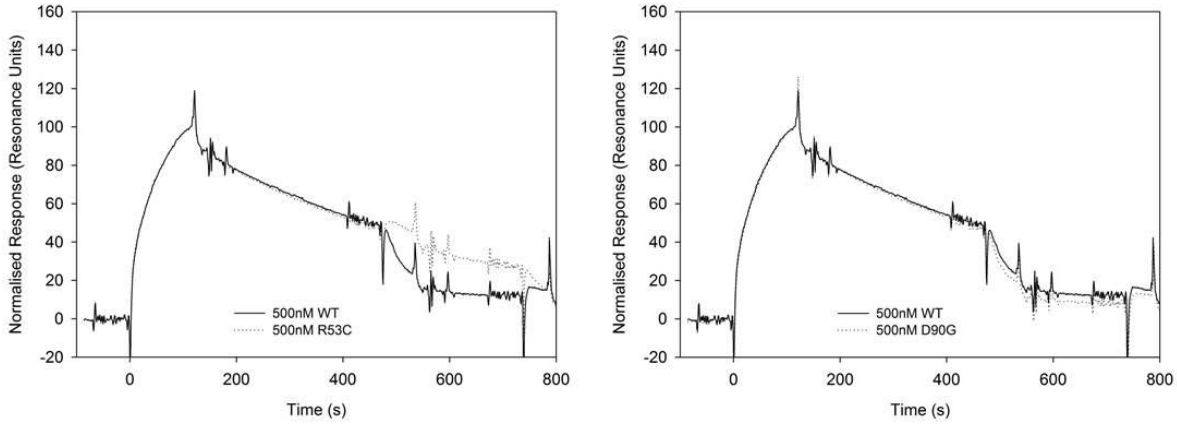

Figure 87 Decay activity in real time using SPR - R53C and D90G

Adapted from (Yu et al., 2014) The flow of FB and FD on the surface of C3b immobilised on a CM5 biosensor chip forms an on-chip C3bBb and is shown in overlaying sensograms. C3bBb is allowed to naturally decay before the injection of 0.5μM FH SCR1-4 over the chip surface. WT FH SCR1-4 and D90G FH SCR1-4 accelerate the natural decay of the on-chip C3bBb. R53C FH SCR1-4 has minimal effect on the natural decay of the on-chip C3bBb.

In the second batch of experiments, 166nM of WT achieved complete C3bBb decay. At this concentration, Q81P had only a minimal effect on C3bBb decay. All other mutants demonstrated ability to decay C3bBb though small losses in decay activity compared to WT were observed and were greatest for G69E and D130N (Figure 88).

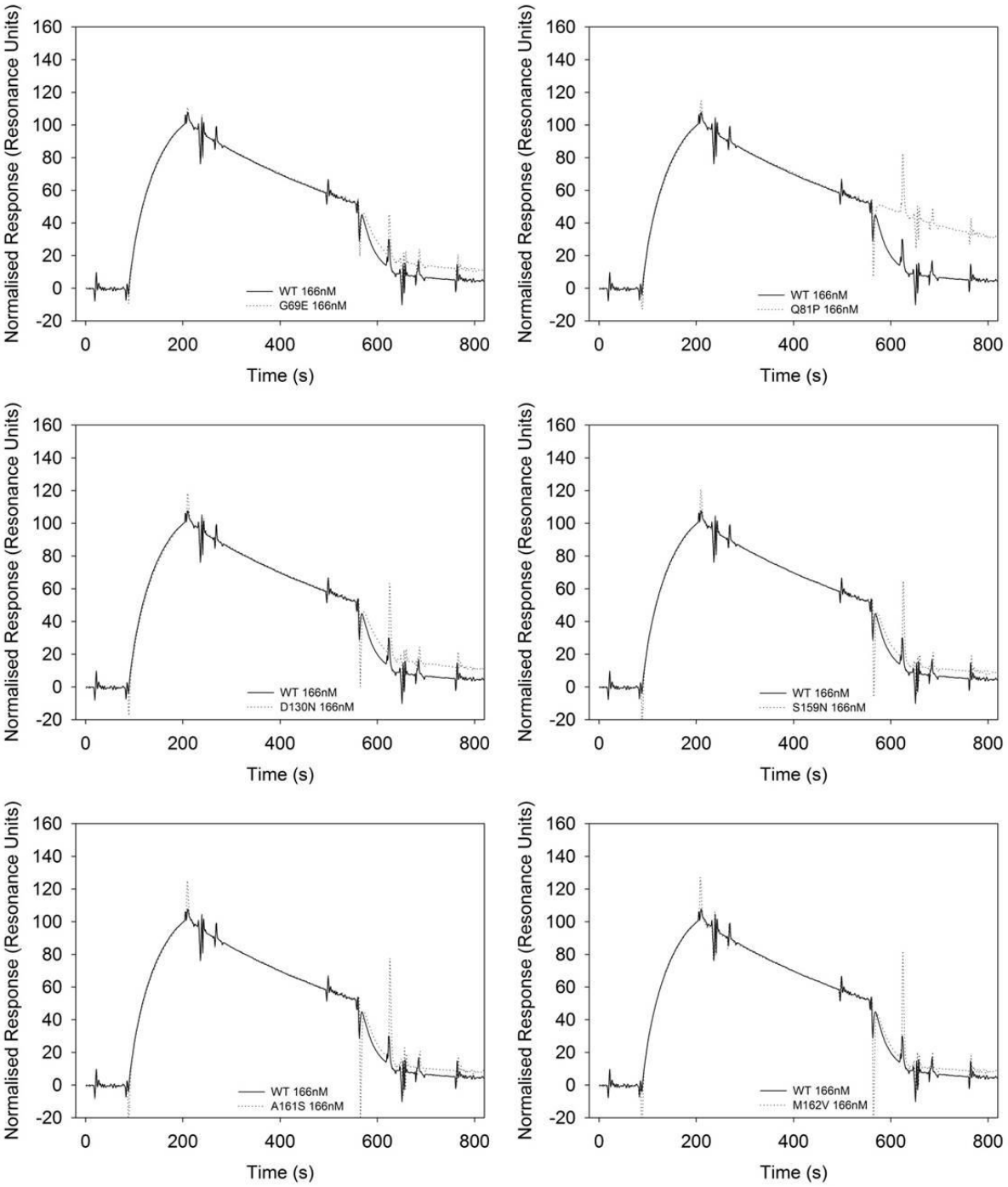

Figure 88 Decay activity in real time using SPR - G69E, Q81P, D130N, S159N, A161S and M162V

The flow of FB and FD on the surface of C3b immobilised on a CM5 biosensor chip forms an on-chip C3bBb and is shown in overlaying sensograms. C3bBb is allowed to naturally decay before the injection of 0.17μM FH SCR1-4 over the chip surface. WT FH SCR1-4 and mutant FH SCR1-4 (except Q81P) accelerate the natural decay of the on-chip C3bBb though G69E and D130N appear silghtly less active. Q81P FH SCR1-4 has minimal effect on the natural decay of the on-chip C3bBb.

5.3.6 Determining Decay Activity in Sheep Erythrocyte Haemolysis Assays

The effect of each rare variant on decay acceleration activity was then tested on C3bBb formed on the surface of sheep erythrocytes. R53C had a loss of decay activity on C3bBb formed on the surface of sheep erythrocytes. At the equivalent concentration of IC_{50} for WT, R53C had no decay activity (Figure 89). Q81P also had a loss of decay activity on C3bBb formed on the surface of sheep erythrocytes. As in the case for R53C, at the equivalent concentration of IC_{50} for WT, Q81P had no decay activity (Figure 90). In these experiments, R53C and Q81P required 22.4-fold and 25.1-fold higher concentration of FH than wildtype respectively to achieve 50% protection from lysis. D130N, G69E and A161S had slight impairment of decay activity requiring 2-fold, 1.3-fold and 1.3-fold more FH respectively to achieve 50% protection from lysis. There was no change in decay acceleration regulatory activity for the remaining variants.

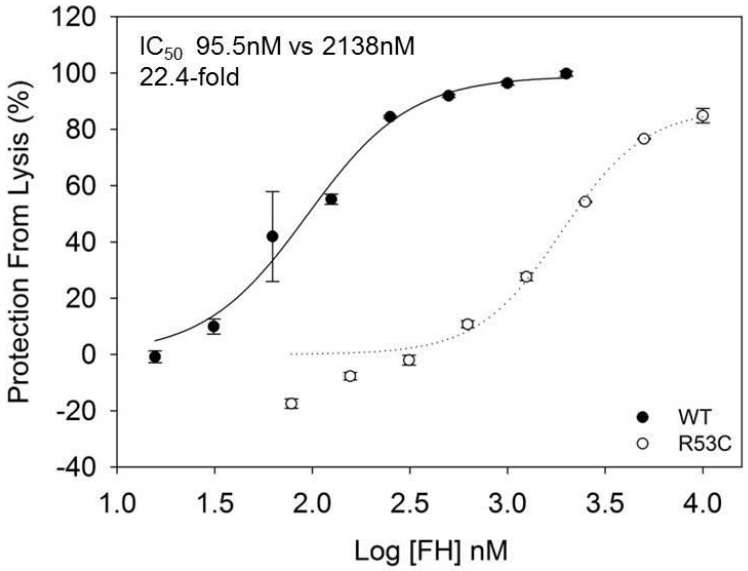

Figure 89 Decay activity on sheep erythrocytes - R53C

FH SCR1-4 was incubated with C3bBb formed on the surface of sheep erythrocytes. FH decays C3bBb and prevents lysis instigated by the addition of serum depleted of FB and FH. WT FH SCR1-4 is 22.4-times more effective than R53C FH SCR1-4 at protecting cells from lysis (IC_{50} WT 95.5nM vs R53C 2138 nM). Error bars represent 1 standard deviation from experiments performed in triplicate.

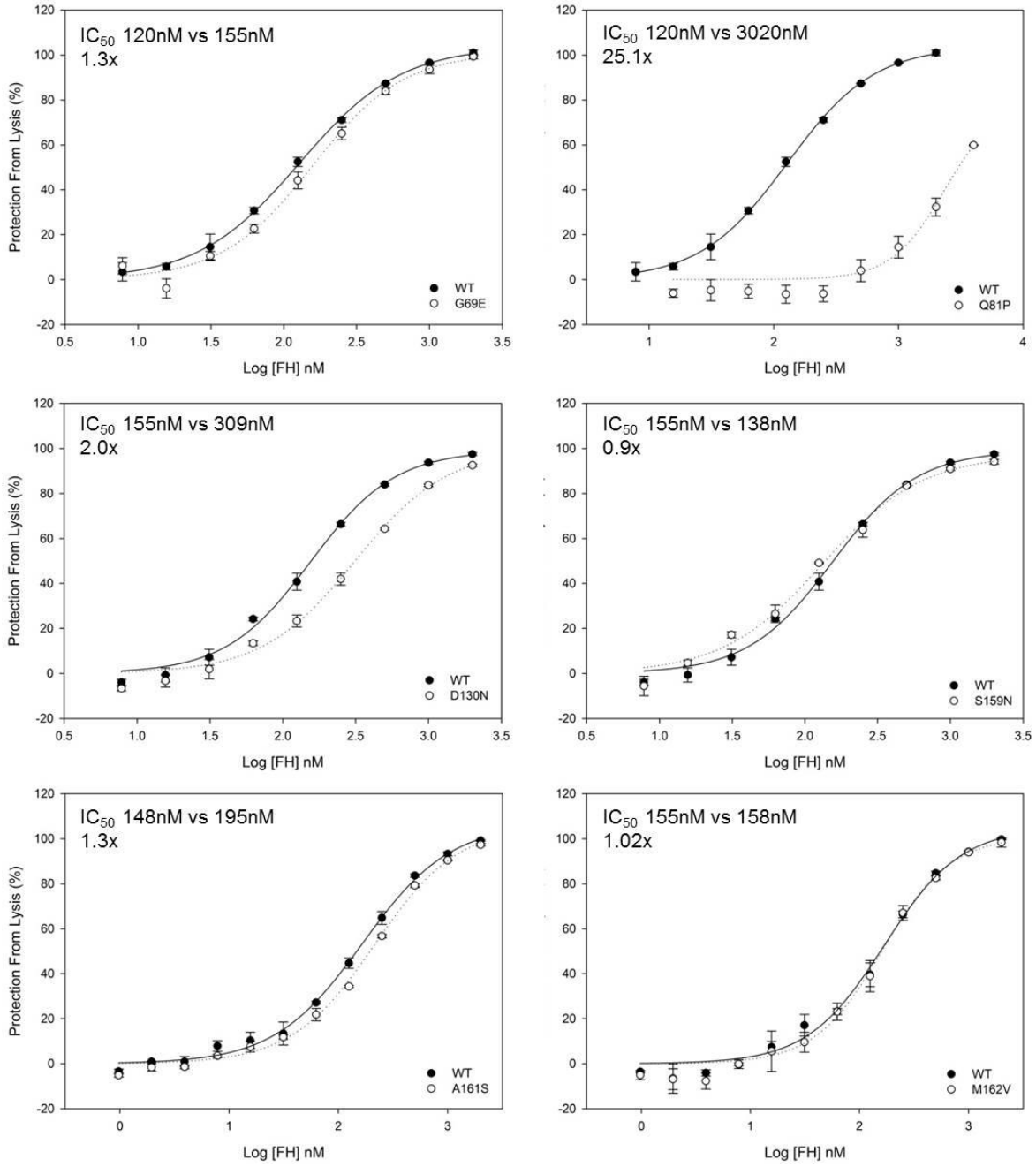

Figure 90 Decay activity on sheep erythrocytes - G69E, Q81P, D130N, S159N, A161S and M162V

FH SCR1-4 was incubated with C3bBb formed on the surface of sheep erythrocytes. FH decays the C3bBb and prevents lysis instigated by the addition of serum depleted of FB and FH. Q81P FH SCR1-4 is 25.1-times less effective than WT FH SCR1-4 at protecting cells from lysis. The difference in IC_{50} for mutants compared to WT FH SCR1-4 was 1.3-fold for G69E, 2.0-fold for D130N, 0.9-fold for S159N, 1.3-fold for A161S and 1.0-fold M162V. Error bars represent 1 standard deviation from experiments performed in triplicate.

5.3.7 Determining Co-factor Activity in a Fluid-phase Assay

The ability of FH SCR1-4 to act as a co-factor in the FI-mediated proteolytic cleavage of C3b into iC3b was assessed in the fluid phase. In Figure 91, co-factor activity as determined by the loss of the C3b α' chain and appearance of the α_1 chain appears to be slightly impaired. These assays were repeated independently using WT, R53C and D90G FH SCR1-4 expressed in mammalian cell lines by Dr. Elizabeth Schramm, Washington University School of Medicine, St. Louis. In these experiments, the loss of the α'-chain was quantified using densitometry and normalised to the intensity of the β-chain and plotted over a time-course. A small difference in activity could be detected for both R53C and D90G compared to WT (Figure 92).

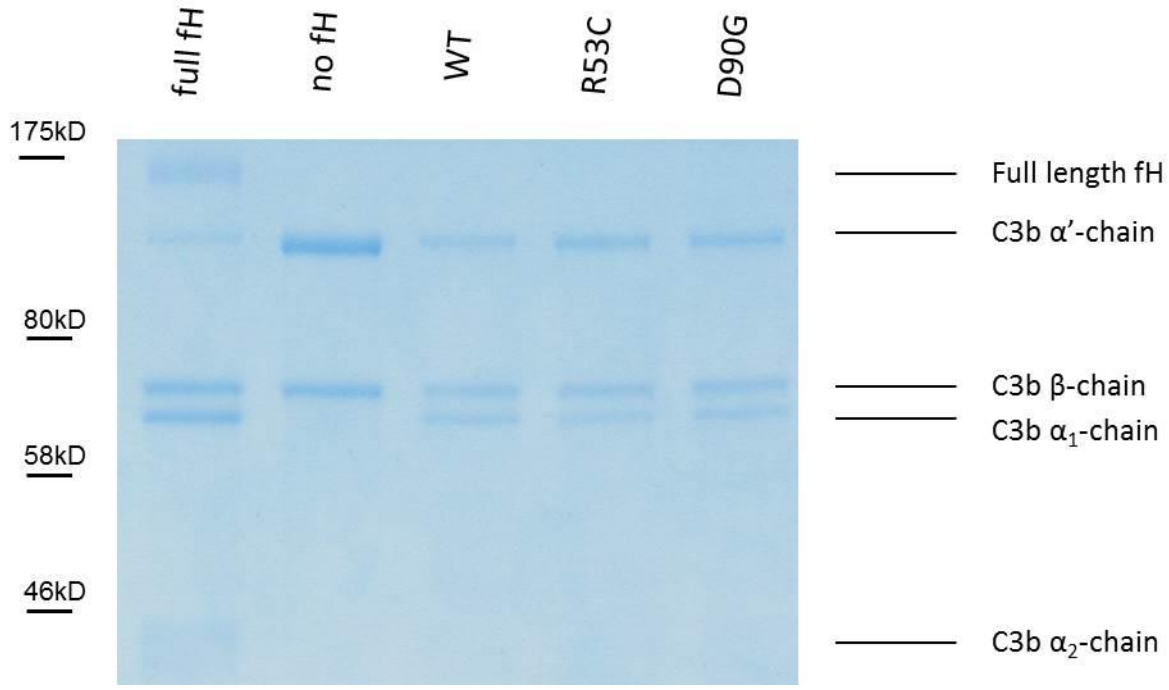

Figure 91 Co-factor activity in fluid phase - R53C and D90G

WT and mutant FH SCR1-4 were incubated with FI and C3b for 30 minutes. The C3b-cleavage products were separated on 10% SDS-PAGE under reducing conditions Co-factor activity is observed by the loss of α'-chain and the appearance of the β-chain for all mutants.

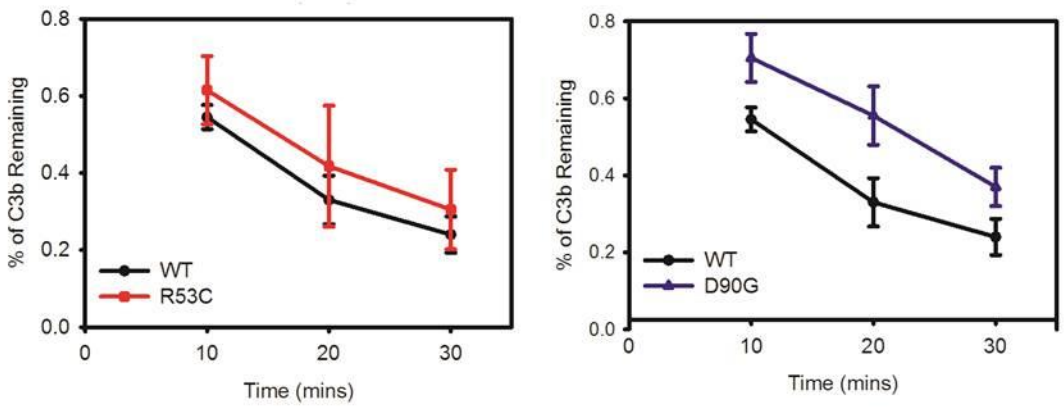

Figure 92 Co-factor activity in fluid phase – time course - R53C and D90G

Adapted from (Yu et al., 2014). Experiments performed by Dr. Elizabeth Schramm using FH SCR1-4 expressed in HEK293 cells as described in (Yu et al., 2014). Percentage (%) of C3b remaining was determined by quantitation of the a′ produced over time relative to the amount of a′ present originally are the average of four independent experiments; error bars represent one standard deviation. Quantification was done by densitometrically scanning the a′ bands and normalizing to the amount of C3b in that lane (β- chain). R53C has lower CA in the fluid phase assay ($P<0.05$ at all time points, two-tailed t-test.) D90G has a significant defect in co-factor activity (CA) ($P<0.01$ at all time points, two-tailed t-test.

In a second batch of fluid phase co-factor assays, the Q81P variant clearly had very little co-factor activity, as seen by the clear intact α' band and virtually no formation of the α_1 cleavage products (Figure 93). All other variants exhibited co-factor activity.

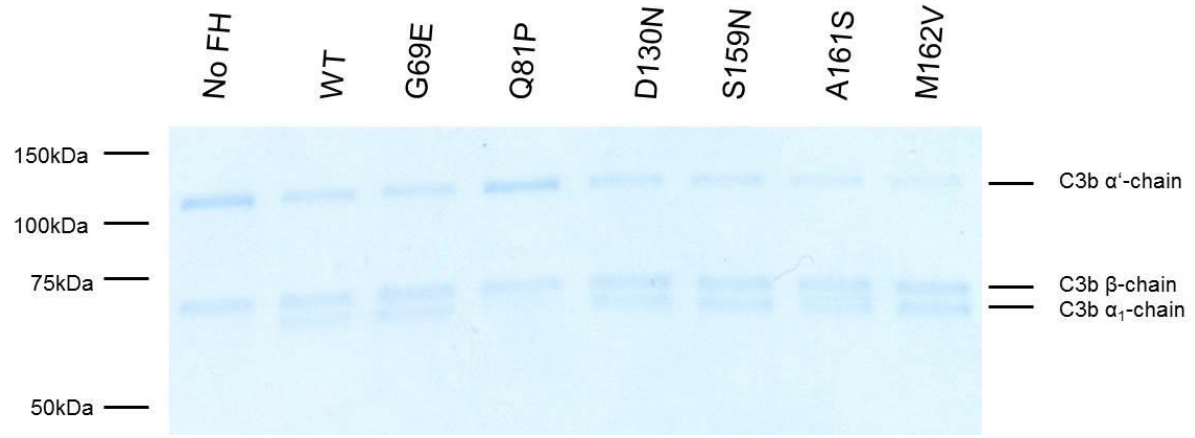

Figure 93 Co-factor activity in fluid phase - G69E, Q81P, D130N, S159N, A161S and M162V

WT and mutant FH SCR1-4 were incubated with FI and C3b for 30 minutes. The C3b-cleavage products were separated on 10% SDS-PAGE/Western blot under reducing conditions Co-factor activity is observed by the loss of α'-chain and the appearance of the β-chain. Co-factor activity was evident for all mutants except Q81P.

5.3.8 Determining Co-factor Activity in Sheep Erythrocyte Haemolysis Assays

The effect of each rare variant to act as a co-factor in the FI-mediated proteolytic cleavage of C3b into iC3b was then assessed on the surface of sheep erythrocytes. R53C had evidence of co-factor activity when compared to wildtype. Approximately 1.3-fold higher concentration of R53C was required to achieve 50% protection from lysis compared to wildtype (Figure 94).

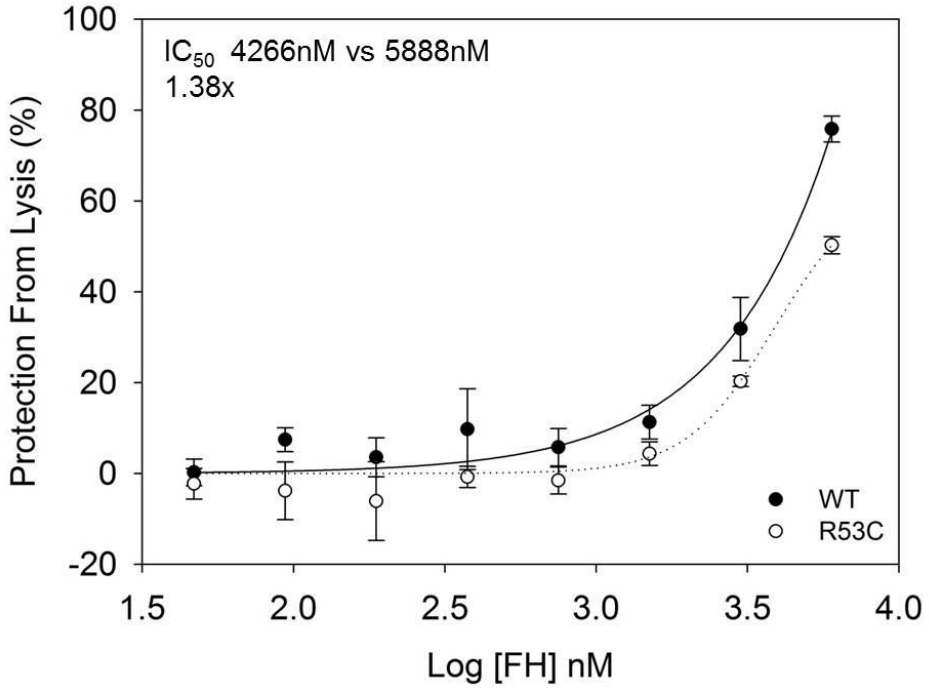

Figure 94 Co-factor activity on sheep erythrocytes - R53C

Adapted from (Yu et al., 2014). FH SCR1-4 was incubated with sheep erythrocytes coated with C3b and factor I for 8 minutes. C3bBb was formed on intact C3b and lysis instigated by the addition of serum depleted of FB and FH. FH acts as a co-factor and C3b is inactivated in the presence of factor I, This prevents subsequent C3bBb formation and protects cells from lysis. WT FH SCR1-4 is slightly more effective than R53C FH SCR1-4 at protecting cells from lysis (IC_{50} WT 4266nM vs R53C 5888 nM). Error bars represent 1 standard deviation from experiments performed in triplicate.

Q81P had a loss of co-factor activity on the surface of sheep erythrocytes. At the equivalent concentration of IC_{50} for WT, Q81P had no co-factor activity. Q81P required 11- fold and higher concentration of FH than wildtype respectively to achieve 50% protection from lysis (Figure 95). The other variants had differences in co-factor activity requiring 1.1-1.66-fold more FH to give sheep erythrocytes 50% protection from lysis.

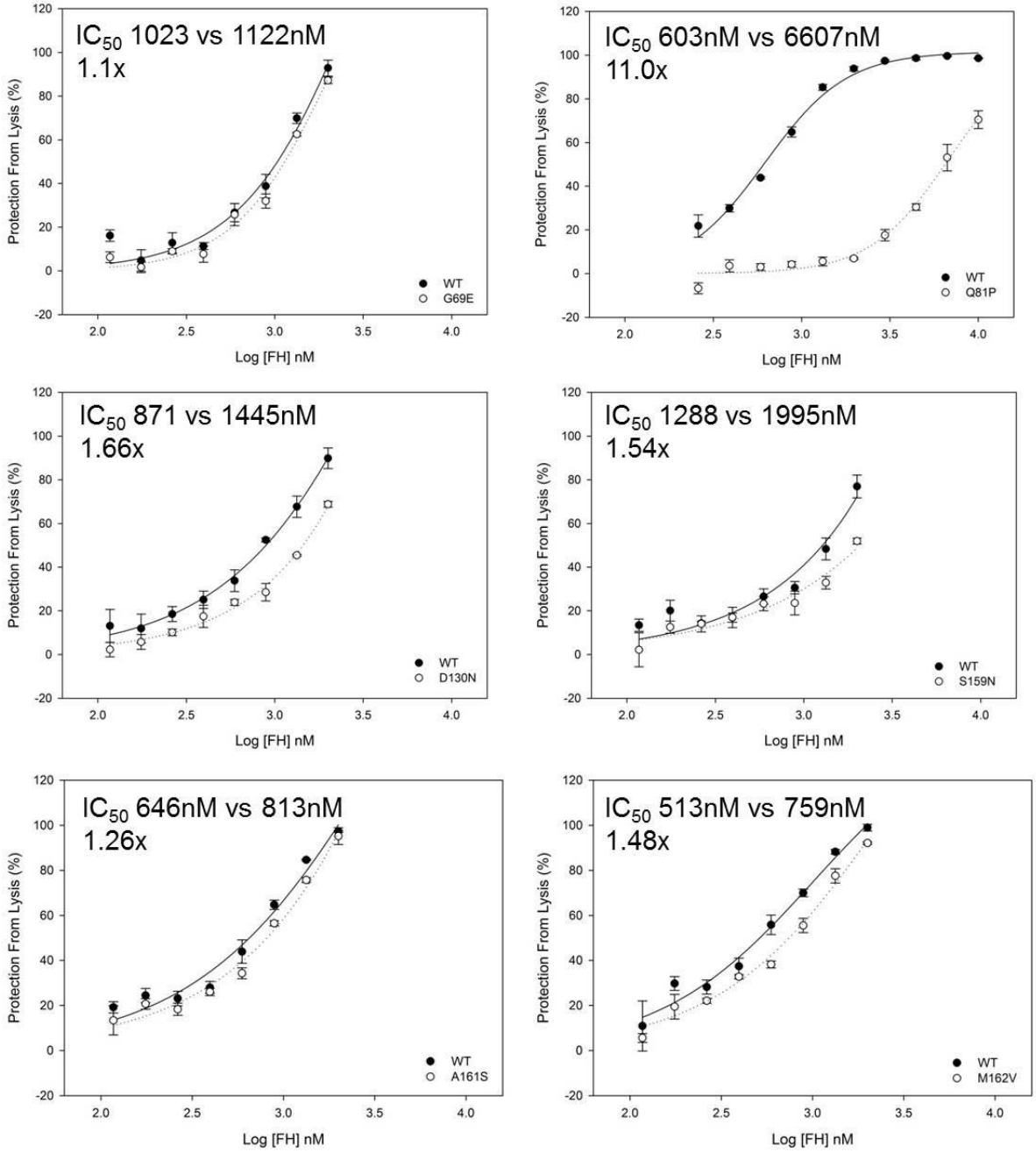

Figure 95 Co-factor activity on sheep erythrocytes - G69E, Q81P, D130N, S159N, A161S and M162V

FH SCR1-4 was incubated with sheep erythrocytes coated with C3b and factor I for 15 minutes. C3bBb was formed on intact C3b and lysis instigated by the addition of serum depleted of FB and FH. FH acts as a co-factor and C3b is inactivated in the presence of factor I, This prevents subsequent C3bBb formation and protects cells from lysis. WT FH SCR1-4 is 11-fold more effective than Q81P (IC50 WT 603nM vs Q81P 6607nM). The difference in IC_{50} for the other mutants compared to WT FH SCR1-4 was 1.1-fold for G69E, 1.66-fold for D130N, 1.54-fold for S159N, 1.26-fold for A161S and 1.48-fold forM162V. Error bars represent 1 standard deviation from experiments performed in triplicate.

5.4 Discussion

In this chapter, I have characterised 8 rare disease-associated genetic variants in the setting of a recombinant FH SCR1-4 protein. The functional characteristics of these, and those published previously in the setting of FH SCR1-4 are summarised in Table 43. The functional characteristics of the common DDD-associated risk polymorphism V62 compared to I62 are also shown. A 9th variant (delG122-E128) was transformed into *Pichia pastoris* but there was no evidence of any protein expression.

	Effect of Variant on				
Variant	Affinity to C3b	Decay on SPR Assay	Decay on Sheep E	Co-factor Fluid Phase	Co-factor on Sheep E
R53C	1.47	↓	22.4	↓[1]	1.38
R53H[2]	1.20	↓	40.0	↓	2.6
I62V[2]	1.4	↔	1.1	↓	2
G69E	1.2	↓	1.3	↔	1.1
R78G[2]	3.40	↓	25.1	↓	2.6
Q81P	↓	↓	25.1	↓	11.0
R83S	7.01	↓	9.1	↓	↓
D90G	0.92	↔	N/P	↓[1]	N/P
D130N	1.44	↓	2.0	↔	1.66
S159N	1.20	↔	0.9	↔	1.54
A161S	0.97	↔	1.3	↔	1.26
M162V	1.23	↔	1.02	↔	1.48

Table 43 Functional effects of variants studied in the setting of FH SCR1-4

Disease-associated variants described in this chapter are summarised alongside R83S described in Chapter 4 and the published variants R53H and R78G. The common polymorphism associated with risk of aHUS and DDD, I62V is also shown. SPR = surface plasmon resonance, E= erythrocytes, N/P = not performed. Numbers are ratio of K_D or IC_{50} of mutant:WT. Numbers >1 suggest less C3b binding or regulatory function. Where K_D or IC_{50} has not been determined, arrows indicate a decrease (↓) or no effect (↔) on affinity to C3b or regulatory activity [1]small differences in co-factor activity were confirmed using densitometry (Yu et al., 2014), [2]are data from previous studies (Pechtl et al., 2011).

I have determined the affinity of each variant to C3b using SPR, and tested decay and co-factor regulatory activity using SPR, fluid phase and sheep erythrocyte haemolytic assays.

The variant Q81P studied in this chapter exhibited a clear loss of decay and co-factor activity in functional assays, a pattern consistent with two previously studied variants in the setting of FH SCR1-4, R78G (Pechtl *et al.*, 2011) and R83S (Wong *et al.*, 2014). The variant R53C also exhibited a clear loss of decay activity with some loss of co-factor activity, a pattern consistent with the previously studied variant R53H (Pechtl *et al.*, 2011). The functional consequences of these 5 rare variants are of complete loss in at least one of the regulatory functions of FH. The magnitude of this loss of regulatory function is different to that demonstrated for the other 6 disease-associated rare variants studied in this chapter and that of the previously studied disease-associated common polymorphism I62V (Pechtl *et al.*, 2011). The differences in magnitude of effect on complement regulatory function suggests these variants may not have the same pathogenicity in disease.

5.4.1 Rare genetic variants in *CFH* resulting in complete loss of regulatory function

Rare genetic variants in *CFH* leading to FH deficiency, or type 1 mutations can be found in a number of renal phenotypes, including MPGN/C3G and aHUS (Dragon-Durey *et al.*, 2004). The regulatory function of FH is contained within the N-terminal domain. A variant in the N-terminal domain of *CFH* may result in a defective protein that fails to regulate complement by decay or co-factor activity. If the mutant protein is completely defective, then levels of functional FH could be expected to be similar to those with a type 1 mutation.

The Q81P variant studied in this chapter is in close proximity to two other disease-associated variants, R78G and R83S. All three of these variants are at the binding interface with C3b (Figure 96). In the case of R78G and R83S, the amino acid substitution results in the loss of a positive charge. In the case of Q81P, there is a loss of a polar side chain (Table 44). Taking into account *in-silico* prediction software tools, all three variants were predicted to be functionally significant. In studies of these three variants in a mutant FH SCR 1-4 protein, a clear loss of C3b binding was demonstrated resulting in loss of both decay and co-factor activity.

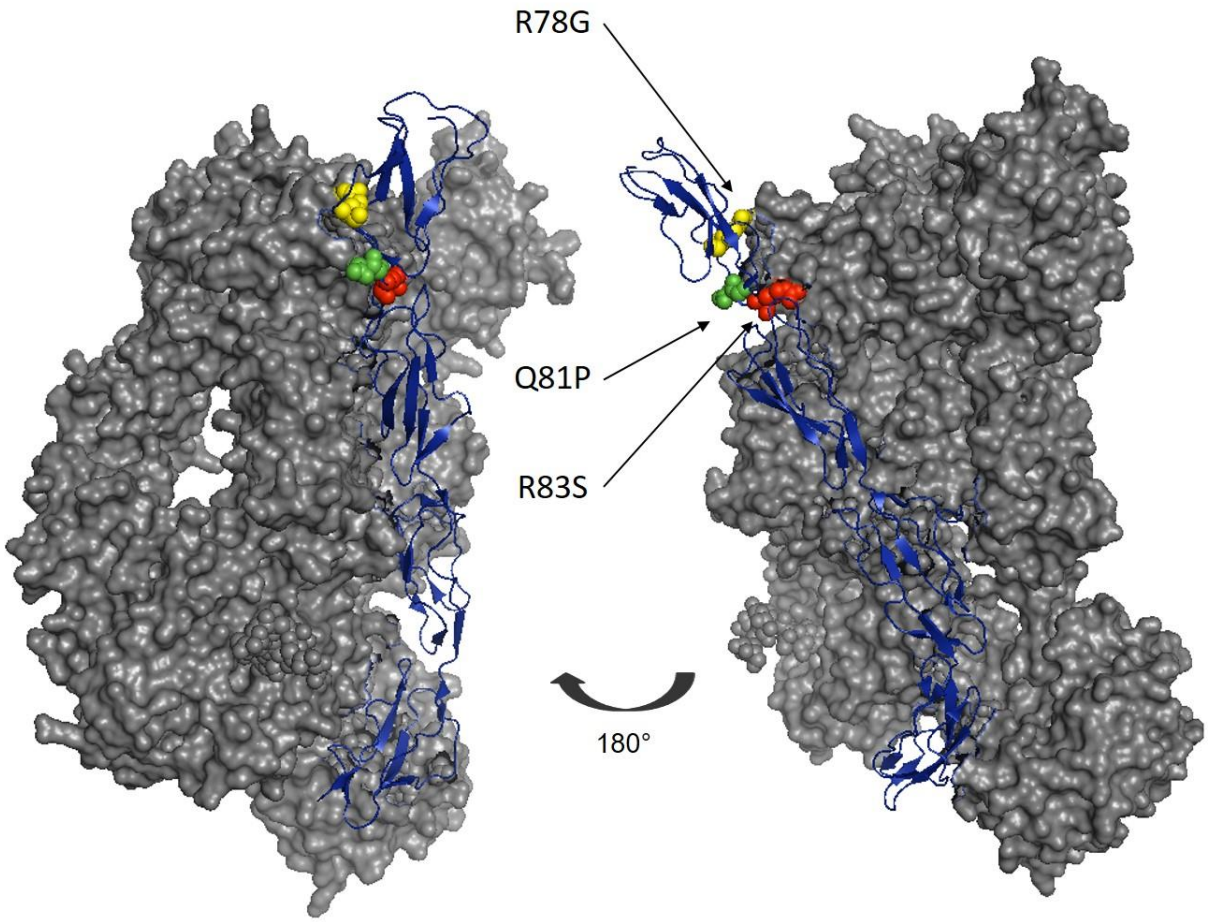

Figure 96 Structural modelling of R78G, Q81P and R83S associated with complete loss of regulatory function

The mutated amino acids R78G (yellow), Q81P (green) and R83S (red) are modelled on the co-crystal structure of FH/C3b using Pymol. All three are on the surface of FH and are at the interface of FH SCR1-4 (blue ribbon) and C3b (dark spheres).

Variant	Electrostatic property change		Polyphen Prediction	Provean Prediction
R78G	Electrically charged (+ve)	Non-polar side chains	Deleterious	Possibly damaging
Q81P	Polar side chains	Non-polar side chains	Deleterious	Probably damaging
R83S	Electrically charged (+ve)	Polar uncharged side chain	Deleterious	Possibly Damaging

Table 44 Electrostatic effects and in-silico prediction for effect of R78G, Q81P and R83S

The amino acid R53 is on the surface of FH and faces a putative binding site with FI (Figure 97). The current and previous functional study of two mutant proteins (R53C and R53H) in the setting of FH SCR1-4 demonstrated only a small reduction of C3b binding and co-factor

activity. However, there was a profound loss of decay acceleration activity. One amino acid change (R to C) results in the loss of positive charge but the other change (R to H) does not. Both arginine and histidine have basic side chains at neutral pH resulting in a positive charge. However, one distinction between these amino acids is that arginine holds onto the proton more strongly than histidine due to its higher pKa. When also taking into account *in-silico* prediction models, both variants were predicted to be functionally significant (Table 45). A large effect on C3b binding would not be expected based upon the structural modelling as shown. A larger effect on co-factor activity might have been expected based upon the putative binding site with FI. Instead, a large effect on decay activity was observed and suggests that the affected amino acid might play a more important role in decay rather than co-factor activity.

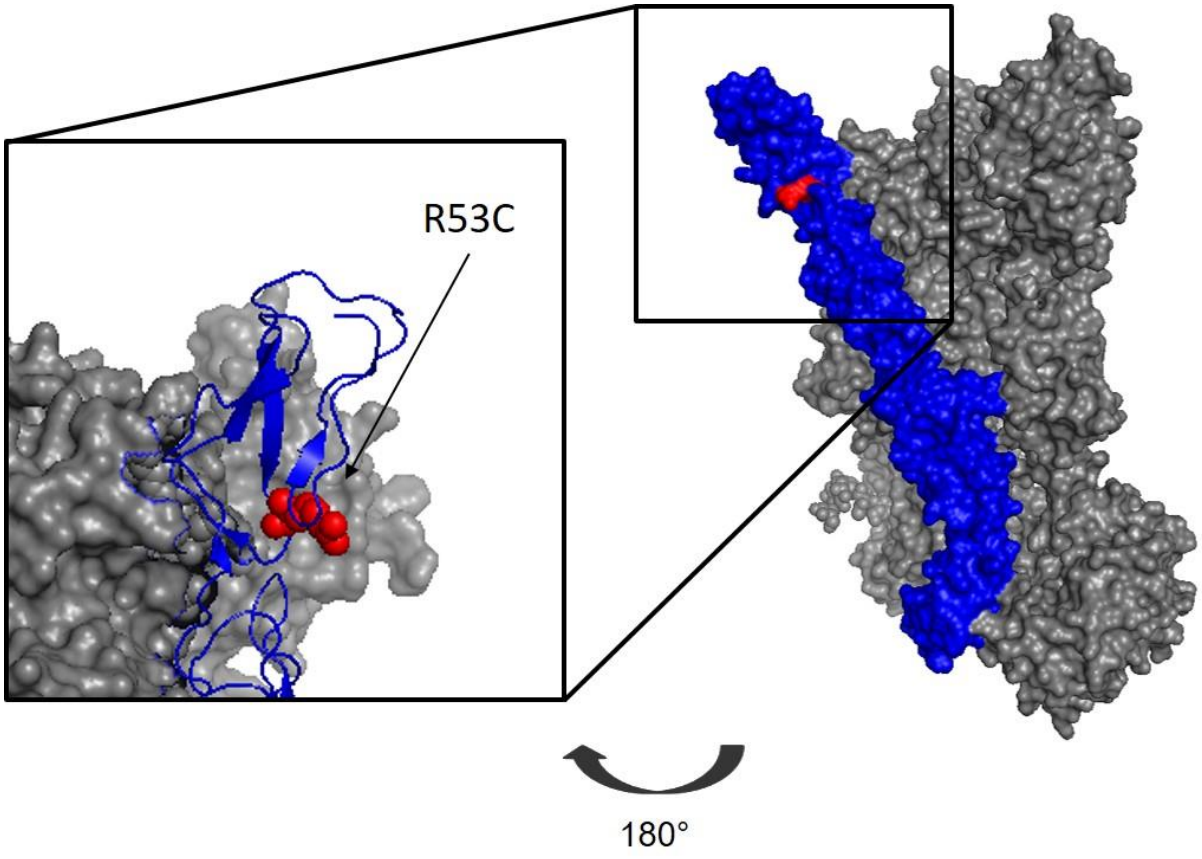

Figure 97 Structural modelling of R53C associated with complete loss of decay activity

The mutated amino acid R53 (red) representative of both the R53C and R53H mutant proteins, is modelled on the co-crystal structure of FH1-4:C3b using Pymol. It is on the on the surface of FH and at a putative interface of FH SCR1-4 (blue ribbon) and FI (not shown). C3b is also shown (dark spheres).

Variant	Electrostatic property change		Polyphen Prediction	Provean Prediction
R53C	Electrically charged (+ve)	Polar uncharged side chain	Deleterious	Probably Damaging
R53H	Electrically charged (+ve)	Electrically charged (+ve)	Deleterious	Possibly Damaging
R53S	Electrically charged (+ve)	Polar uncharged side chain	Deleterious	Probably Damaging

Table 45 Electrostatic effects and in-silico prediction for effect of R53C, R53H and R53S

Complement levels of patients with these rare variants resulting in loss of regulatory function and of patients with type 1 mutations are shown in Table 46. All C3 levels are low even in the absence of a C3 nephritic factor and regardless of the differences in FH levels between those with type 1 and type 2 mutations. They are lowest in patients who have a rare variant inherited in homozygosity.

Rare genetic variants in *CFH* that lead to complete loss of function should be considered functionally significant and pathogenic.

Variant	He Ho	Type 1 or 2	Disease	C3	C4	FH	FI	C3 NEF	Reference
C431S	Ho	1	DDD	215	112	<20	49	N	(Servais *et al.*, 2012)
C597R	Ho	1	C3GN	179	218	<20	75	N	(Servais *et al.*, 2012)
C597R	Ho	1	MPGN1	<40	133	<10	75	N	(Servais *et al.*, 2012)
R53C	Ho	2	MPGN1	144	234	643	65	N	(Servais *et al.*, 2012)
R53C	Ho	2	MPGN1	↓	↔	↔	↔	P/N[1]	(Janssen van Doorn *et al.*, 2013)
L77X	He	1	C3GN	616	271	260	74	N	(Servais *et al.*, 2012)
V132I	He	1	DDD	530	199	270	58	P	(Servais *et al.*, 2012)
R232X	He	1	DDD	538	160	275	52	P	(Servais *et al.*, 2012)
C673R	He	1	DDD	377	147	204	56	P	(Servais *et al.*, 2012)
C1043S	He	1	MPGN1	85	69	286	52	P	(Servais *et al.*, 2012)
R53C	He	2	C3GN	572	195	658	74	N	(Servais *et al.*, 2012)
R53C	He	2	aHUS[2]	509	160	689	65	ND	(Fakhouri *et al.*, 2010b)
Q81P	He	2	aHUS[2]	497	326	418	57	ND	(Fakhouri *et al.*, 2010b)
R83S[3]	He	2	MPGN1	↓	↔	↔	↔	P	(Wong *et al.*, 2014)
R83S[3]	He	2	MPGN1	↓	↔	↔	↔	P/N[1]	(Wong *et al.*, 2014)
R83S[3]	He	2	MPGN1	↓	↔	↔	↔	N	(Wong *et al.*, 2014)

Table 46 Complement levels associated with rare genetic variants in CFH leading to complete loss of function

Normal range of C3 (660-1250mg/L), and C4 (90-380mg/L), FH (338-682mg/L) and FI (42-78mg/L) (Fakhouri et al., 2010b; Servais et al., 2012), Complement levels in other studies adopt a different normal range and arrows denote low or normal levels. MPGN1= MPGN type 1, He = heterozygous, Ho = homozygous, C3 NEF = C3 nephritic factor, ↓ = below normal range, ↔ = within normal range, ND = not done, [1]Results from serial measurements of C3 nephritic factor were not the same at all time points, [2]pregnancy-related, [3]familial.

5.4.2 N-terminal Rare Genetic Variants in *CFH* that do not lead to Complete Loss of Regulation

Functional assays of 6 other rare genetic variants in the N-terminal domain of *CFH* (G69E, D90G, D130N, S159N, A161S and M162V) do not cause a complete loss of complement regulatory activity. In all assays, the mutant FH SCR1-4 proteins exhibited evidence of C3b binding, decay and co-factor activity at concentrations where the wildtype protein was functional.

The functional effects of these 6 rare variants should be considered in the context of the common polymorphism I62V. This polymorphism is associated with an increased risk of developing aHUS and DDD (Abrera-Abeleda *et al.*, 2006; Pickering *et al.*, 2007). The risk allele is carried by 80% of healthy individuals (evs.gs.washington.edu/). In functional studies, there was no effect on decay activity but it did have a small but significant effect on C3b binding and cofactor activity (Tortajada *et al.*, 2009; Pechtl *et al.*, 2011). In my studies of this group of variants, mutant FH was up to 1.66-fold less active than wildtype for co-factor activity and up to 2-fold less active than wildtype for decay activity. This compares with previous studies of the I62V variant that was 2-fold less active in co-factor activity (Pechtl *et al.*, 2011).

Of these 6 variants and including the common disease-associated polymorphism, I62V, five were found at the interface of FH and C3b (Figure 98). The amino acid substitutions did not affect electrical charge or polarity of side chains in the case of 4 of these, I62V, S159N, A161S or M162V. Furthermore, *in-silico* prediction did not predict a functional effect for these 4 variants (Table 47). The small effects on co-factor and decay activity in my studies for S159N, A161S and M162V are thus consistent with each other, and with the magnitude of the small but statistically significant effect of the common polymorphism I62V.

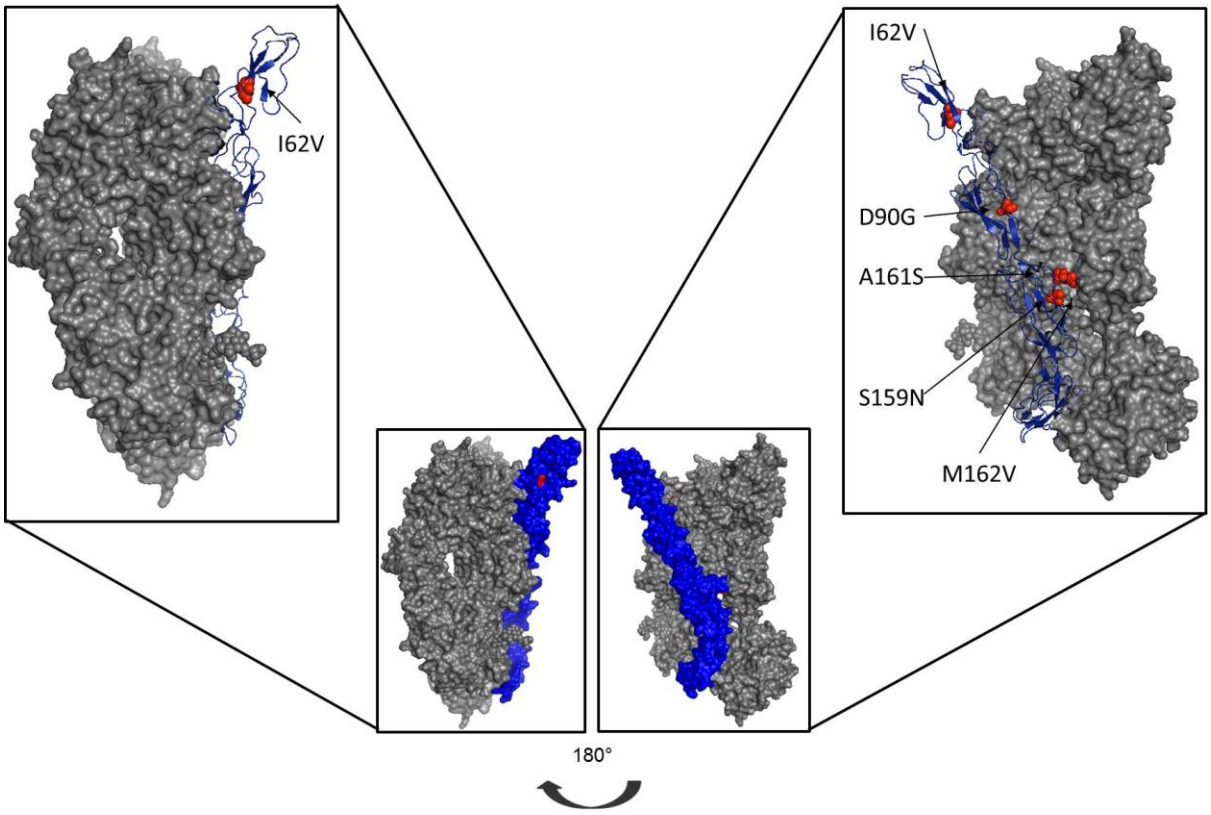

Figure 98 Structural modelling of I62V, D90G, S159N, A161S and M162V

The mutated amino acids (red spheres) are modelled on the co-crystal structure of FH1-4:C3b using Pymol. All are near or at the surface of FH and at a putative interface of FH SCR1-4 (blue ribbon) and C3b (dark spheres).

Of those variants at the interface of FH with C3b, the amino acid change and *in-silico* prediction for a 5th variant, D90G are comparable (Table 47) to those reported for the functionally significant variants R78G, Q81P and R83S. This suggests that D90G should result in a functional effect. Given the position of D90G at the C3b binding site (Figure 98), a functional effect resulting in complete loss of function would have been predicted. However, although statistically significant, the actual magnitude of effect on function was small and limited to co-factor activity.

Two variants studied were found at a putative FI binding site (Figure 99). Of all of the variants described in this sub-section, D130N consistently had the largest effect on decay and co-factor activity, being 2-fold and 1.66-fold less active than wildtype respectively. Nonetheless, the D130N mutant protein did demonstrate both decay and co-factor activity and did not result in complete loss of regulatory activity. From this perspective, the D130N mutant is consistent with the *in-silico* prediction that there would be no effect on function (Table 47).

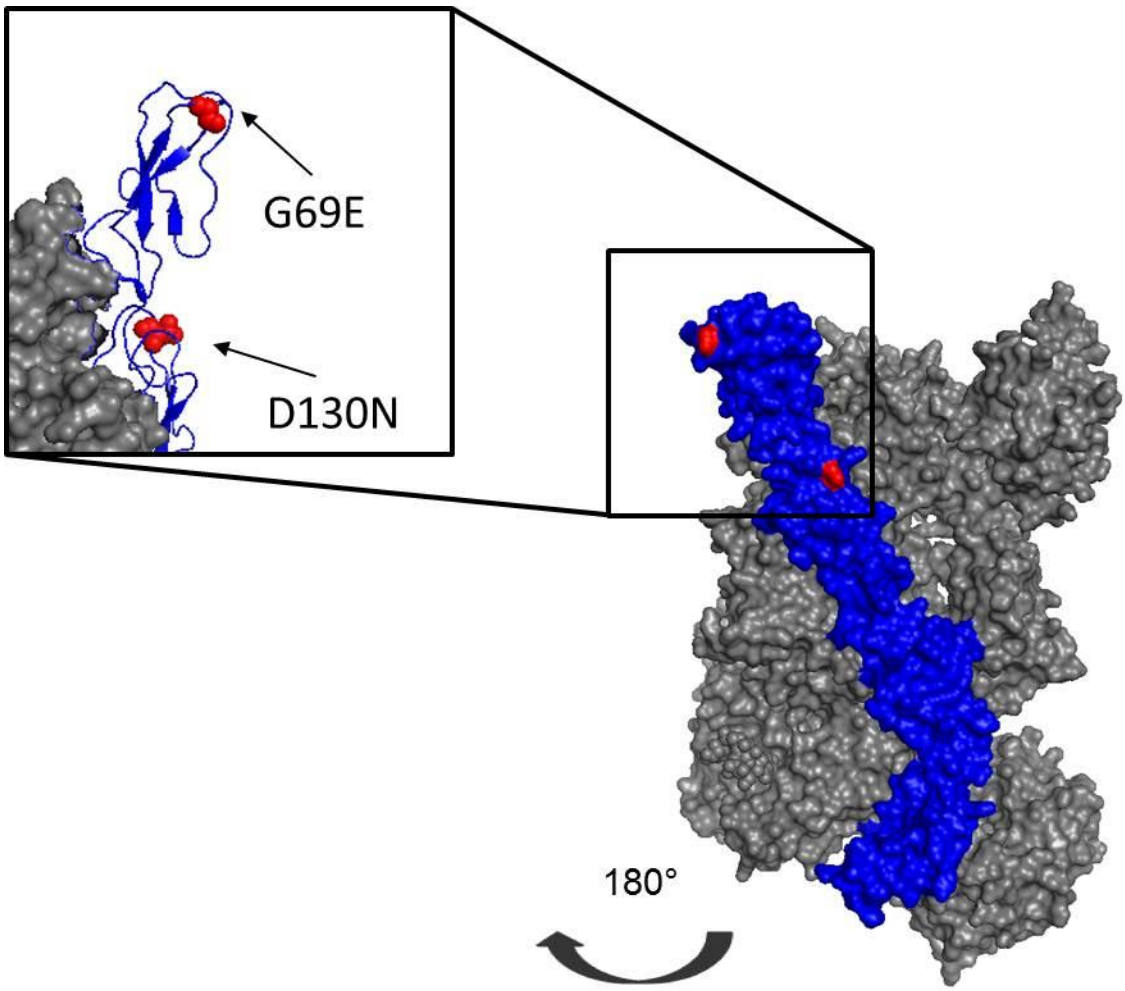

Figure 99 Structural modelling of G69E and D130N

The mutated amino acids are modelled on the co-crystal structure of FH/C3b using Pymol. They are on the on the surface of FH SCR1-4 (blue ribbon). C3b is also shown (dark spheres).

The final variant studied was G69E. *In-silico* analysis predicted a 'deleterious' and a 'probably damaging' effect. Both C3b binding and co-factor activity were only minimally affected and is consistent with structural modelling shows that this variant affects an amino acid that is not in proximity with C3b or a putative FI binding site. The effect of this variant on decay activity was also minor. It is possible that despite the predicted effects, that the position of the affected amino acid is not directly involved in the regulatory activity of FH.

Variant	Electrostatic property change		Polyphen Prediction	Provean Prediction
V62I	Non-polar side chains	Non-polar side chains	Neutral	Benign
D90G	Acidic side chain	Non-Polar uncharged side chain	Deleterious	Probably Damaging
S159N	Polar uncharged side chain -	Polar uncharged side chain -	Neutral	Probably Damaging
A161S	Non-Polar uncharged side chain	Non-Polar uncharged side chain	Neutral	Benign
M162V	Non-Polar uncharged side chain	Non-Polar uncharged side chain	Neutral	Benign
D130N	Acidic side chain	Polar uncharged side chain	Neutral	Benign
G69E	Non-Polar uncharged side chain	Acidic side chain	Deleterious	Probably Damaging

Table 47 Electrostatic effects and in-silico prediction for effect of variants with small changes in function

The variants discussed in this section did not exhibit a complete loss of function. The functional effects were small and comparable to the effects of the common polymorphism, I62V. The largest functional effect observed on decay and co-factor activity was a 2-fold and 1.66-fold reduction in activity respectively, compared to wildtype. The levels of functional FH in an individual who inherits such a variant in heterozygosity are unlikely to be as low as those with a type 1 mutation that associate with low C3 levels.

C3 levels in patients with these variants that do not cause complete loss of regulatory function are shown in Table 48. Functional studies of one variant, S159N identified in aHUS and AMD were performed on the basis that this protein was normally secreted, suggested by normal FH levels in a case of AMD. In functional studies it demonstrated only a small loss of co-factor activity. However, in a case of aHUS, low C3 levels were present and in association with low FH levels. It is possible that S159N is in fact a type 1 mutation.

C3 levels (where available) were within normal range for the remainder of the variants studied. This includes the variant, delG122-E128 that I had intended to study. This protein

did not express in small scale test expressions yet FH levels in a patient were within normal range. An explanation could be that normal FH levels are due to the wildtype allele alone. Study of FH directly from patient serum may be helpful to determine if a secreted truncated protein is present. If the patient was heterozygous for the common polymorphism, Y402H, relative levels of each variant could be measured (Hakobyan *et al.*, 2010).

In this section, functional testing distinguished these variants from those that led to complete loss. Based on the functional testing alone, these variants should not be regarded as functionally significant and pathogenic. Instead, as exemplified by the common polymorphism, I62V, they should be regarded as disease-associating and not individually pathogenic.

Variant	Disease	C3	C4	FH	FI	C3 NEF	Reference
DelE122-G128	DDD	883	254	449	73	P	(Servais *et al.*, 2012)
D130N	DDD	1240	328	663	55	N	(Servais *et al.*, 2012)
S159N[1]	aHUS	↓	↓	↓	↔	ND	Newcastle cohort, unpublished
A161S	aHUS[2]	733	248	530	76	ND	(Fakhouri *et al.*, 2010b)
A161S	aHUS	↔	↔	↔	↔	ND	Newcastle cohort, unpublished
A161S	DDD	802	345	602	68	P	(Servais *et al.*, 2012)
M162V	aHUS	NK	NK	↔	NK	ND	(Fremeaux-Bacchi *et al.*, 2013)

Table 48 Complement profile of patients with N-terminal rare variants in CFH that do not lead to complete loss of function

Normal range of C3 (660-1250mg/L), and C4 (90-380mg/L), FH (338-682mg/L) and FI (42-78mg/L) (Fakhouri et al., 2010b; Servais et al., 2012), Complement levels in Newcastle adopt a different normal range and arrows denote low or normal levels. All variants were heterozygous. C3 NEF = C3 nephritic factor, ↓ = below normal range, ↔ = within normal range, ND = not done in aHUS patients, NK = not known, [1] = initially selected for study in association with normal levels of FH in case of AMD in Newcastle Complement Genetics Service, [2] Pregnancy-associated.

5.4.3 Pleotropic Effects of Rare Genetic Variants in *CFH*

A recently studied example of a rare genetic variant that highlights the pleotropic effects of FH is R1210C (Recalde *et al.*, 2015). C-terminal variants were generally considered a cause of loss of complement regulation at the host cell surface leading to aHUS. In order to explain the association of the R1210C variant with MPGN/C3G, aHUS and AMD, studies using monoclonal antibodies confirmed that the covalently linked albumin on the free cysteine residue affects the functional domains in SCR1-4, SCR6-8 and SCR19-20. Thus, this was a variant of global FH dysfunction and not an isolated loss of C-terminal function. Patients with this variant in heterozygosity effectively have partial FH deficiency, like those with variants described in section 5.4.1.

In this chapter, rare genetic variants in the N-terminal domain of *CFH* have been described in MPGN/C3G, aHUS and AMD. Other rare genetic variants in the N-terminal domain of *CFH* have been identified in AMD cohorts and are summarised (Appendix 10, Table 64) (Duvvari *et al.*, 2015; Triebwasser *et al.*, 2015). A review of these variants identifies a total of 7 variants that associate with renal disease and AMD (Table 49). Most notably, the R53C and A161S variants have been described in MPGN/C3G, aHUS and AMD and is further evidence of the pleotropic effects of FH.

Protein	SCR	MPGN/C3G	aHUS
p.P26S	1	Y	
p.R53C	1	Y	Y
p.R53H	1		Y
p.S58A	1		Y
p.D130N	2	Y	
p.S159N	3		Y
p.A161S	3	Y	Y
p.I216T	4	Y	

Table 49 Pleotropic effects of Rare genetic variants in N-terminal domain of CFH found in AMD

Rare genetic variants identified in AMD have also been described in cases of MPGN/C3G and aHUS. R53C and A161S have been identified in all of these phenotypes.

5.4.4 Limitations of Study

The functional tests described in this chapter were performed in the setting of the short fragment, FH SCR1-4 only. None, except for the previously studied common polymorphism I62V (Tortajada *et al.*, 2009; Pechtl *et al.*, 2011) have been studied in both the setting of FH SCR1-4 and full length protein in which only small difference in co-factor activity was demonstrated. Therefore, extrapolation of the effects of the functionally significantly mutant proteins from studies in short fragments to full length protein is based on studies of this polymorphism alone. Confirmatory studies of functionally significant rare genetic variants in the N-terminal domain in the setting of full length protein should be considered.

In this study, functional studies of the S159N mutant protein that were initially based on the normal FH levels identified in a case of AMD, did not demonstrate a complete loss of function. However, analysis of FH levels from another case identified low FH levels suggestive of a type 1 mutation. Functional studies alone may have been misleading. Sampling of patient serum could allow additional evaluation of FH, to look for the possibility of degradation of protein by Western blot or the relative amounts of each variant, though this is reliant upon access to individual patients and in the latter, heterozygosity for the Y402H polymorphism and (Hakobyan *et al.*, 2010; Szarvas *et al.*, 2016).

Finally, the clinical need to understand the pathogenicity of individual variants in the MPGN/C3G cohort is an important factor driving this research. In aHUS, confirmation of the pathogenicity of variants in *CFH* allows stratification of patient prognosis and management (Wong *et al.*, 2015). Whilst functional studies have confirmed pathogenicity for a small number of variants, correlation with longitudinal data in these patient cohorts is yet to be done.

5.5 Summary

Rare genetic variants in the regulatory domain of *CFH* have been reported in several diseases that associate with excess complement activation. Many are type 1 mutations and are significant. The study of N-terminal variants in *CFH* in this chapter in the setting of FH SCR1-4 has contributed to previous functional studies of type 2 mutations that have been described in MPGN/C3G and aHUS. Combined with my studies, it is clear from functional studies of mutant proteins in the setting of FH SCR1-4 that complete loss function due to several type 2 mutations, R53C, R53H, R78G, Q81P and R83S can be demonstrated and like type 1 mutations should be considered significant in disease.

It is also clear from functional studies of mutant proteins in the setting of FH SCR1-4 that complete loss function is not evident for all rare genetic variants identified in this functional domain. These variants, that include the common polymorphism I62V, may be associated with disease but not causative. This highlights the need for such functional studies.

Furthermore, rare genetic variants in *CFH* can be found in a range of different disease phenotypes and include those that are functionally significant and those that are associated with disease. This suggests that abnormalities alone in FH, even in those with a functionally significant variant may not be sufficient to cause aHUS, AMD or MPGN/C3G and further studies are required to address this.

Finally, further studies to understand the role of rare genetic variants in *CFH* are required. These include correlation of functionally significant variants in longitudinal studies in patient cohorts and verification of the significance of these studies in the setting of FH SCR1-4 in full length protein. The next chapter describes my study of functionally significant *CFH* variants in the setting of a full length FH protein.

Chapter 6 Functional Study of Disease-Associated Variants in FH

6.1 Introduction

FH is the major fluid phase regulator of AP. Rare genetic variants in *CFH* have been identified in 4-16.2% (Servais *et al.*, 2012; Bu *et al.*, 2015; Iatropoulos *et al.*, 2016) of patients in cohorts of MPGN/C3G and in a quarter of patients with aHUS (Noris *et al.*, 2010; Fremeaux-Bacchi *et al.*, 2013). Approximately half of variants reported in *CFH* in patients with aHUS (Fremeaux-Bacchi *et al.*, 2013) and in patients with MPGN/C3G (as shown in chapter 5) lead to FH deficiency. These are type 1 mutations and are considered pathogenic. In the remainder of cases, functional studies are required to confirm pathogenicity.

Functional studies have been performed on disease-associated rare genetic variants identified throughout *CFH*. Studies of rare variants in the N- and C-terminal functional domains of *CFH* are summarised (Table 50). These studies of rare variants include the use of recombinantly expressed mutant proteins that comprise the functional domains, SCR1-4 or SCR19-20 domains of FH. Other studies use patient serum containing mutant FH in FH-dependent haemolysis assays in which wildtype FH protects sensitised sheep erythrocytes from lysis (Sanchez-Corral *et al.*, 2002).

Recombinant mutant proteins that contain the SCR19-20 recognition domain have been studied to determine the effects on C3b, C3d, heparin and endothelial cell binding due to rare genetic variants in the C-terminal domain of *CFH* (Jozsi *et al.*, 2006; Ferreira *et al.*, 2009). Furthermore, these mutant proteins also been tested in competition assays with full length wildtype FH on the surface of erythrocytes in FH-dependent haemolysis assays.

Recombinant mutant proteins in the setting of SCR1-4 have been used to determine differences in effect on individual regulatory functions in C3b binding, decay and co-factor assays (Pechtl *et al.*, 2011; Wong *et al.*, 2014). An important advantage in studies using recombinant proteins is that they are not reliant upon patient sampling. Studies using recombinant fragments account for a high proportion of studies of different rare genetic variants in the N- and C-terminal domains of *CFH*. However, the consequences of these mutant proteins must be extrapolated to understand their significance in a full length protein that may lead to incorrect interpretation of the significance of a rare variant.

False negative results are an important consideration of haemolytic assays used to test FH from patient serum. These assays are dependent upon an active complement system to enable

lysis. Samples that are not properly manipulated or samples from individuals with low complement levels may fail to lyse cells resulting in false negatives (Sanchez-Corral 2002).

Use of purified full length mutant proteins account for a minority of studies of rare genetic variants reported in the N- or C–terminal domain of *CFH*. However, they enable a diverse range of functional testing that includes the detection of small functional differences of the polymorphic variant, I62V (Tortajada *et al.*, 2009) and the novel molecular insights resulting from bound albumin on the R1210C mutant protein (Recalde *et al.*, 2015).

In the study of R1210C, a variant associated with risk of aHUS, MPGN/C3G and AMD, it was shown that the free cysteine in the R1210C mutant protein bound to human serum albumin. This additional albumin resulted in impaired binding of 3 different monoclonal antibodies that each had specificity to a different functional domain of FH. This rendered the mutant FH protein functionally inert (Recalde *et al.*, 2015).

Collaborators in the Barlow laboratory, Edinburgh have optimised a protocol using *Pichia pastoris* to express and purify human full length FH (Schmidt *et al.*, 2011). Disease-associated variants can be introduced using site-directed mutagenesis and expressed for functional studies (Herbert *et al.*, 2015). Mutant FH can then be purified to homogeneity.

6.1.1. Chapter Aims

In this chapter I will contribute to the studies of rare genetic variants in the N- and C-terminal domain of *CFH* using full length FH proteins. Using mutant FH proteins recombinantly expressed in the Barlow laboratory, I will describe the use of sheep erythrocyte haemolytic assays to test the effect of

- One N-terminal disease-associated variant in full length FH and
- Two C-terminal disease-associated variants in full length FH.

Studies of these purified full length mutant proteins will allow me to validate the numerous studies that have been performed in fragments of FH, including SCR1-4 and SCR19-20.

I will then demonstrate the utility of the sheep erythrocyte haemolytic assays in a study of a novel hybrid FH/FHR3 protein identified in a patient with aHUS.

Variant	SCR	Recombinant FH (SCRs)t	Patient FH	C3b/d binding	Heparin Binding	Co-factor	Decay	Endothelial Cell Binding	Lysis Assay	Reference
R53C	1	1-4	N/A	↓	N/A	↓	↓	N/A	↓[1]	(Yu *et al.*, 2014)
R53H	1	1-4	N/A	↔	N/A	↓	↓	N/A	↓[1]	(Pechtl *et al.*, 2011)
I62V	1	1-4	Purified	↔	ND	↓	↔	ND	↓[1]	(Tortajada *et al.*, 2009; Pechtl *et al.*, 2011)
R78G	1	1-4	N/A	↓	N/A	↓	↓	N/A	↓[1]	(Pechtl *et al.*, 2011)
R83S	1	1-4	N/A	↓	N/A	↓	↓	N/A	↓[1]	(Wong *et al.*, 2014)
D90G	2	1-4	N/A	↔	N/A	↓	↔	N/A	ND	(Yu *et al.*, 2014)
W198R	3	N/A	Serum	ND	N/A	ND	ND	ND	↓[2]	(Szarvas *et al.*, 2016)
ΔK224	4	N/A	Purified	↓[3, 4]	↔	↓	↓[5]	↔	ND	(Licht *et al.*, 2006)
D1119G	19	19-20	ND	↔ / ↓	↔	N/A	N/A	↔[6]	↓[7]	(Ferreira *et al.*, 2009; Lehtinen *et al.*, 2009)
Y1142C	19	ND	Serum	ND	ND	N/A	N/A	ND	↓[2]	(Abarrategui-Garrido *et al.*, 2008)
W1157R	19	8-20	ND	↓	↓	N/A	N/A	ND	ND	(Jozsi *et al.*, 2006)
P1161T	19	ND	Serum	ND	ND	N/A	N/A	ND	↓[2]	(Szarvas *et al.*, 2016)
E1172X	20	ND	Purified	↓	↓	N/A	N/A	↓[8]	ND	(Manuelian *et al.*, 2003; Jokiranta *et al.*, 2005)
R1182S	20	19-20	ND	↓[9]	↓	N/A	N/A	ND	↓[7]	(Ferreira *et al.*, 2009; Kopp *et al.*, 2012)
W1183R	20	1-20[10] 19-20	Purified / Serum	↑[9]	↑	↔	N/A	ND	↓[2, 7]	(Sanchez-Corral *et al.*, 2002; Sanchez-Corral *et al.*, 2004; Ferreira *et al.*, 2009; Kopp *et al.*, 2012)
W1183L	20	8-20 19-20	ND	↓	↓	N/A	N/A	↓ [6/8]	↓[2,7]	(Jozsi *et al.*, 2006; Ferreira *et al.*, 2009; Lehtinen *et al.*, 2009)

T1184R	20	19-20	ND	↔ / ↑	↑	N/A	N/A	↑ [6]	↓[7]	(Ferreira *et al.*, 2009; Lehtinen *et al.*, 2009)
L1189R	20	19-20	ND	↔ / ↑	↔ / ↑	N/A	N/A	↑ [6]	↓[7]	(Ferreira *et al.*, 2009; Lehtinen *et al.*, 2009)
L1189F	20	19-20	ND	↑	↑	N/A	N/A	ND	↓[7]	(Ferreira *et al.*, 2009)
S1191L	20	19-20	Serum	↑	↔	N/A	N/A	ND	↓[2, 7]	(Heinen *et al.*, 2006; Ferreira *et al.*, 2009)
SV	20	18-20 19-20	Serum	↓ / ↑	↔	N/A	N/A	ND	↓[2]	(Heinen *et al.*, 2006; Ferreira *et al.*, 2009)
V1197A	20	8-20	Purified / Serum	↓	↓	↔	N/A	ND	↓[2, 7]	(Jozsi *et al.*, 2006) (Sanchez-Corral *et al.*, 2002; Sanchez-Corral *et al.*, 2004; Heinen *et al.*, 2006)
E1198A	20	19-20	ND	↔	↔	N/A	N/A	↔[6]	ND	(Lehtinen *et al.*, 2009)
E1198K	20	ND	Serum	ND	ND	N/A	N/A	↓[8]	↓[2]	(Vaziri-Sani *et al.*, 2006)
R1203W	20	ND	Serum	ND	ND	N/A	N/A	ND	↓[2]	(Szarvas *et al.*, 2016)
R1210C	20	8-20 19-20	Purified / Serum[11]	↓	↓	↔	N/A	↓[8]	↓[2, 7]	(Sanchez-Corral *et al.*, 2002; Manuelian *et al.*, 2003; Sanchez-Corral *et al.*, 2004; Jozsi *et al.*, 2006; Ferreira *et al.*, 2009; Recalde *et al.*, 2015)
R1215G	20	8-20 19-20	ND	↓	↓	N/A	N/A	↓[8]	↓[7]	(Manuelian *et al.*, 2003; Jozsi *et al.*, 2006; Ferreira *et al.*, 2009)
R1215Q	20	19-20	ND	↔	↓	N/A	N/A	↔[6]	ND	(Lehtinen *et al.*, 2009)
T1216del	20	ND	Serum	ND	ND	N/A	N/A	ND	↔[2]	(Szarvas *et al.*, 2016)
P1226S	20	8-20	ND	↓	↓	N/A	N/A	ND	ND	(Jozsi *et al.*, 2006)

Table 50 Functional studies of rare genetic variants identified in N- and C-terminal of CFH

Recombinant proteins were made in the setting of the short consensus repeats (SCR) stated, patient FH was tested either in serum or purified, some patients had contradicting results from different studies indicated by multiple arrows. The variant 'SV' = S1191L/V1197A. [1]Decay and co-factor activity of FH is tested on the surface of sheep erythrocytes before cells are lysed with serum depleted of FH, [2]Patient serum is used on sheep erythrocytes, [3]includes peptid spot assay, [4]includes C3d binding, [5]Patient serum is used on C3bBb formed on sheep erythrocytes, [6]Endothelial cell binding using mouse glomerular endothelial cell, [7]Recombinant FH proteins compete with wildtype in sheep haemolysis assay, [8]Endothelial cell binding using human umbilical vein endothelial cells, [9]Impaired pentraxin binding, [10]Expressed in COS cell line, [11]FH-bound to serum albumin.

6.2 Functional Study of Genetic Variants Using Recombinant Full Length FH

6.2.1 Selection of Genetic Variants

The three rare genetic variants selected for study have all been described in aHUS and previously studied in the setting of FH fragments (Figure 100). R53H is a rare variant in the N-terminal regulatory domain of *CFH* and has been previously described in the setting of FH SCR1-4. This study demonstrated a small reduction of C3b binding and co-factor activity. However, there was a profound loss of decay acceleration activity (Pechtl *et al.*, 2011). These differences are consistent with the effects of the disease-associated variant R53C that affects the same amino acid described in chapter 5 (Yu *et al.*, 2014).

D1119G is a rare genetic variant in the C-terminal recognition domain of *CFH* and has been previously studied in the setting of FH SCR19-20 (Ferreira *et al.*, 2009). A final variant is the result of a gene conversion event between exon 23 of *CFH* and exon 5 of *CFHR1* resulting in a C-terminal domain that differs by two amino acids, S1191L and V1197A (S1191L/V1197A). This variant has also been studied in the setting of FH SCR 19-20 (Heinen *et al.*, 2006; Ferreira *et al.*, 2009). In studies of both C-terminal variants, the mutant protein fails to regulate complement on host surfaces as shown in haemolytic assays. D1119G had the greatest impairment on cell surface assays in functional studies of a series of 16 aHUS-associated variants in the C-terminal domain of *CFH* (Ferreira *et al.*, 2009).

A clone expressing full length FH in *Pichia pastoris* has been optimised in the Barlow group in Edinburgh (Schmidt *et al.*, 2011; Herbert *et al.*, 2015). The wildtype clone carried the polymorphisms I62/Y402/D936 (IYD) commonly found in *CFH*. Wildtype and mutant sequence variants were expressed in *Pichia pastoris* in the setting of full length FH and purified in a protocol optimised by Dr. Herbert and Dr. Kerr from the University of Edinburgh. I then used the purified protein in sheep erythrocyte haemolytic assays.

A fourth ‘mutant’ protein was also expressed in a clone expressing full length FH. Two common polymorphic variants were introduced resulting in a mutant protein carrying the polymorphisms V62/Y402/E936 (VYE) also found in *CFH*. Previous studies have demonstrated a small but significant difference in co-factor activity between the I62 and V62 FH protein in studies of full length FH (Tortajada *et al.*, 2009) and FH SCR1-4 (Pechtl *et al.*, 2011).

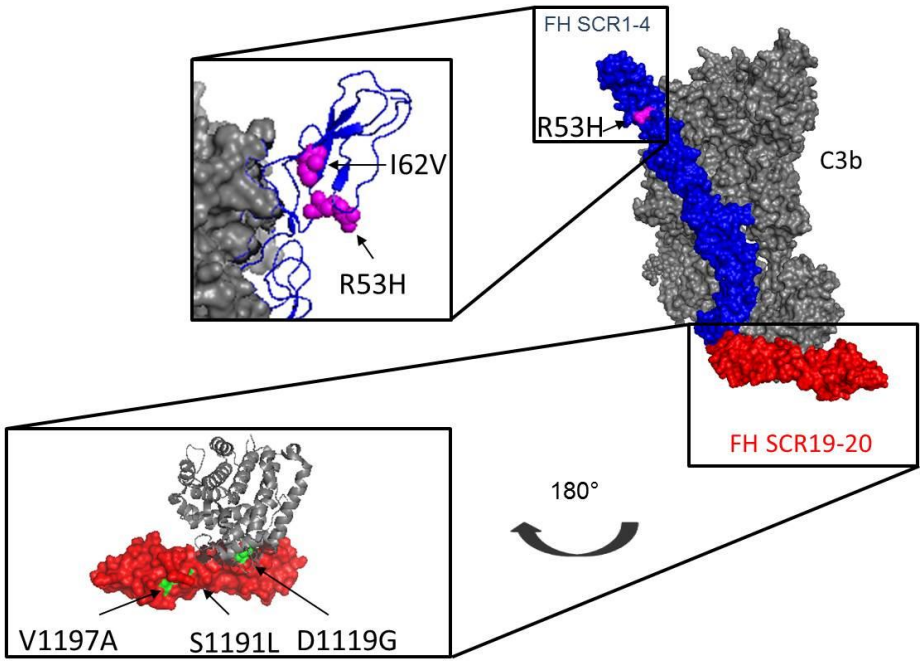

Figure 100 Co-crystal structures of FH SCR1-4:C3b and FH SCR19-20:C3b

The R53H variant (pink sphere) in the N-terminal domain of FH (blue) and 3 affected amino acids (green) in the C-terminal domain of FH (red) are modelled on a co-crystal structure of FH SCR1-4 and C3b (grey spheres) and FH SCR19-20 (grey ribbon) that have been aligned using Pymol. The common polymorphism I62V is also shown (pink sphere).

6.2.2 Determining Decay and Co-factor Activity in Sheep Erythrocyte Haemolysis Assays

Firstly, the function of recombinantly expressed full length FH was compared against plasma purified FH (obtained from Dr. Herbert). In a decay acceleration assay on the surface of sheep erythrocytes, the recombinantly expressed full length FH was only slightly less effective than plasma purified FH (Figure 101).

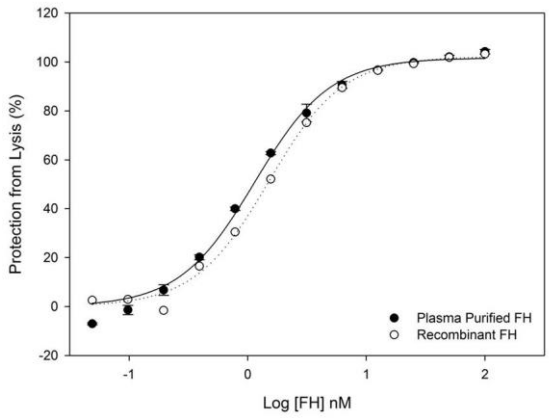

Figure 101 Decay activity on sheep erythrocytes – full length recombinant FH and plasma purified FH

FH was incubated with C3bBb formed on the surface of sheep erythrocytes. FH decays C3bBb and prevents lysis instigated by the addition of serum depleted of FB and FH. Recombinant full length FH (IC_{50} = 1.49nM)is 1.3-times less effective than plasma purified FH (IC_{50} = 1.21nM). Error bars represent 1 standard deviation from experiments performed in triplicate.

The ability of each of the disease-associated mutations to regulate complement on the surface of sheep erythrocytes was then tested in the haemolytic assay. 'Mutant' FH was tested alongside WT FH (IYD) in each case.

The VYE variant had no effect on decay activity on the surface of sheep erythrocytes (IC_{50} IYD 1.23nM vs IC_{50} VYE 1.20nM). All three aHUS associated genetic variants had an effect on decay activity on the surface of sheep erythrocytes. Compared to the wildtype FH, IYD, R53H required 4.7-fold higher concentration to achieve 50% protection from lysis, S1191L/V1197A required 1.7-fold and D1119G required 8.9-fold higher (Figure 102).

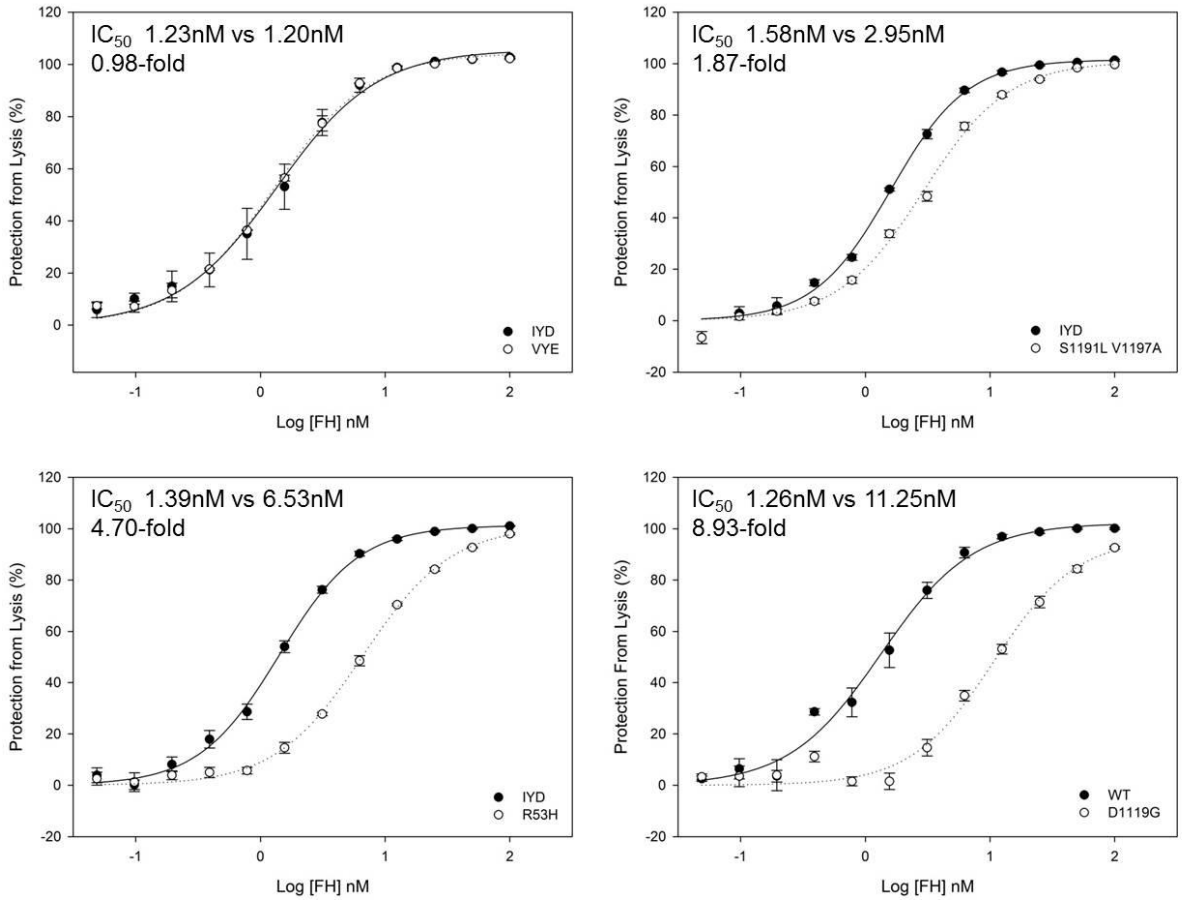

Figure 102 Decay activity on sheep erythrocytes – full length recombinant FH mutant proteins

FH and mutants were incubated with C3bBb formed on the surface of sheep erythrocytes. FH decays the C3bBb and prevents lysis instigated by the addition of serum depleted of FB and FH. The reference FH, IYD, is more effective than all three mutant proteins at protecting cells from lysis. The polymorphic variant VYE however showed no difference in decay activity compared to IYD. Error bars represent 1 standard deviation from experiments performed in triplicate.

There was only a small difference in co-factor activity on the surface of sheep erythrocytes between the VYE variant and IYD (IC_{50} IYD 8.13nM vs IC_{50} VYE 9.55nM – 1.17-fold difference). The three disease associated rare genetic variants had a large effect on co-factor activity on the surface of sheep erythrocytes. Compared to the IYD control FH, R53H required 2.5-fold higher concentration to achieve 50% protection from lysis, S1191L/V1197A required 2.6-fold and D1119G required >17-fold higher (Figure 103).

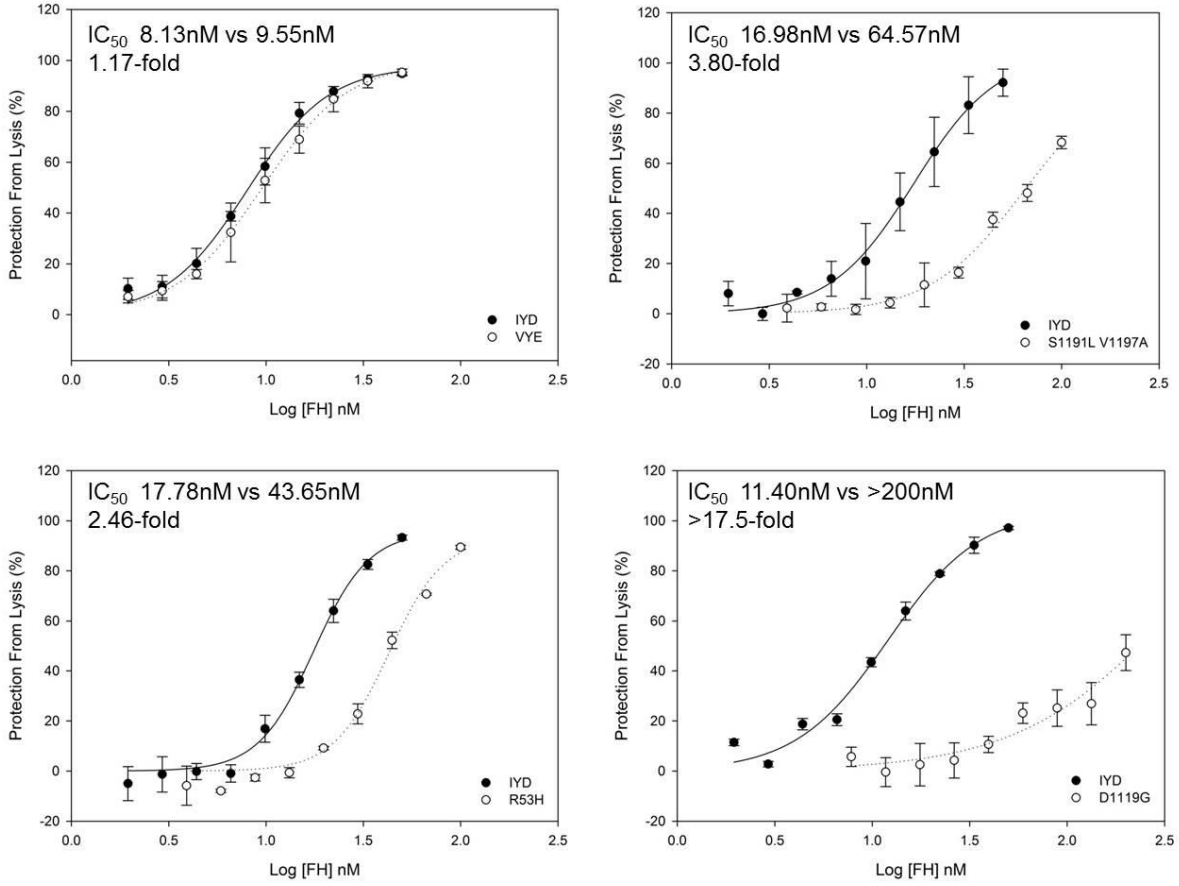

Figure 103 Co-factor activity on sheep erythrocytes – full length mutant recombinant FH mutant proteins

FH (and mutants) were incubated with sheep erythrocytes coated with C3b and factor I for 15 minutes. C3bBb was formed on intact C3b and lysis instigated by the addition of serum depleted of FB and FH. FH acts as a co-factor and inactivates C3b in the presence of factor I and prevents subsequent C3bBb formation and protects cells from lysis. The reference FH, IYD, is more effective than all three mutant proteins at protecting cells from lysis. The polymorphic variant VYE demonstrated a mild impairment in co-factor activity compared to IYD. Error bars represent 1 standard deviation from experiments performed in triplicate.

6.3 Functional Study of a Hybrid FH/FHR3 Protein Purified from Patient Serum

6.3.1 Clinical Case

A patient with aHUS was studied initially by Dr. Rachel Challis (Challis *et al.,* 2015). She used MLPA and Sanger sequencing to demonstrate that this patient with aHUS had a genetic deletion of exons 21 to 23 of *CFH*. This was predicted to result in an mRNA transcript that read from exon 20 of *CFH* to the next available splice acceptor site, predicted to be in exon 2 of *CFHR3*. The presence of this mRNA transcript, a hybrid between exons 1 to 20 of *CFH* and exons 2 to 6 of *CFHR3* was confirmed using cross gene polymerase chain reaction. Western blotting was used initially to confirm that this transcript led to a translated protein in the serum.

A wildtype FH protein from the normal allele and hybrid FH/FHR3 protein from the abnormal allele was initially co-purified using a FH SCR5 specific (OX24) immunoaffinity column. The identity of the 22 SCR hybrid protein, containing SCR1-17 of FH and SCR1-5 of FHR3 was confirmed on mass spectrometry. Figure 104 shows that the hybrid FH/FHR3 protein is larger than FH and that the hybrid FH/FHR3 protein lacks the critical C-terminal recognition domain of FH. This patient was also heterozygous for the Y402H polymorphism and in Dr. Challis' previous work, this was confirmed using specific antibodies, MBI6 and MBI7 to both the 402Y and 402H polymorphism respectively on Western blot. This showed that the wildtype FH was carried on the 402H allele and that the hybrid FH/FHR3 protein was carried on the 402Y allele (Figure 104). I undertook functional analysis of this hybrid FH/FHR3 protein to confirm its functional significance.

The work below describes the steps taken by me to purify the hybrid FH/FHR3 protein to homogeneity before characterisation of the hybrid protein in decay and co-factor assays on the surface of sheep erythrocytes and assays to test heparin binding and fluid-phase co-factor activity.

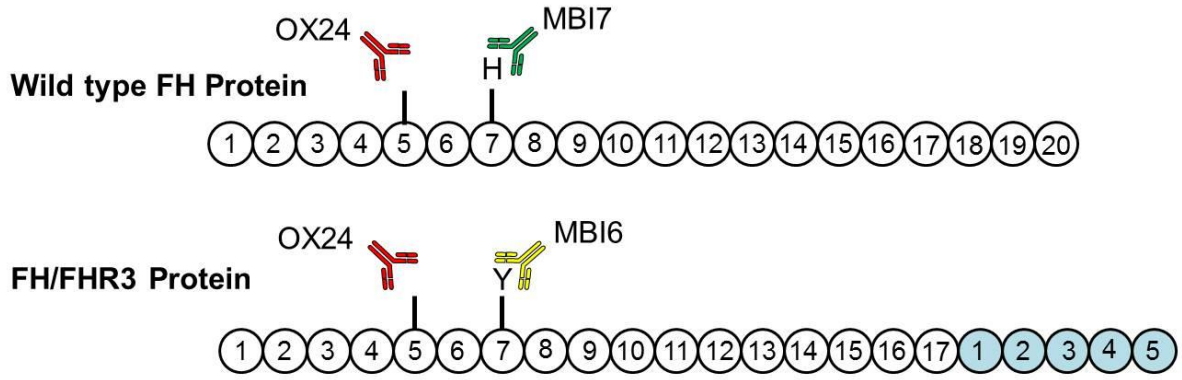

Figure 104 SCRs of FH and the hybrid FH/FHR3 protein

Adapted from Challis et al. The 20 SCRs of WT FH protein is represented by 20 numbered (unfilled) circles (1-20). Due to a gene deletion of exons 21-23 of CFH in one allele of CFH in the patient, an mRNA transcript is generated that leads to the expression of a 22 SCR hybrid protein. This hybrid FH/FHR3 protein contains 22 SCRs. The first 17 SCRs are identical to FH. SCRs 18-20 are missing and replaced by SCRs 1-5 of FHR3 shown by blue circles. OX24 is a monoclonal antibody to SCR5, common to both proteins. The patient has one copy of the normal CFH gene that is carried on the 402H allele. The WT FH protein is recognised by a FH 402H specific monoclonal antibody, MBI7. The patient has one copy of the hybrid CFH/CFHR3 gene that is carried on the 402Y allele. The FH/FHR3 hybrid protein is recognised by a FH 402Y specific antibody, MBI6.

6.3.2 Purification of Y-402 Specific FH from Serum

An antibody specific to the 402Y variant of SCR7 was obtained from the Harris/Morgan laboratories at the University of Cardiff. Specificity of this antibody (MBI6) was confirmed using Western blotting against genotyped controls (Figure 105). This antibody recognised the FH/FHR3 hybrid protein (and a putative degradation product) in patient sera and FH in control sera. Therefore the MBI6 antibody was used to produce a purification column that was specific to FH 402Y (MBI6 column) and used to purify the hybrid FH/FHR3 protein for functional studies.

Serum from a donor control homozygous for FH 402Y was used to optimised the purification of FH from the MBI6 column. Standard elution using 0.1M glycine pH 2.7 and subsequently pH 2.3 and 2.2 failed to elute any FH (Figure 106).

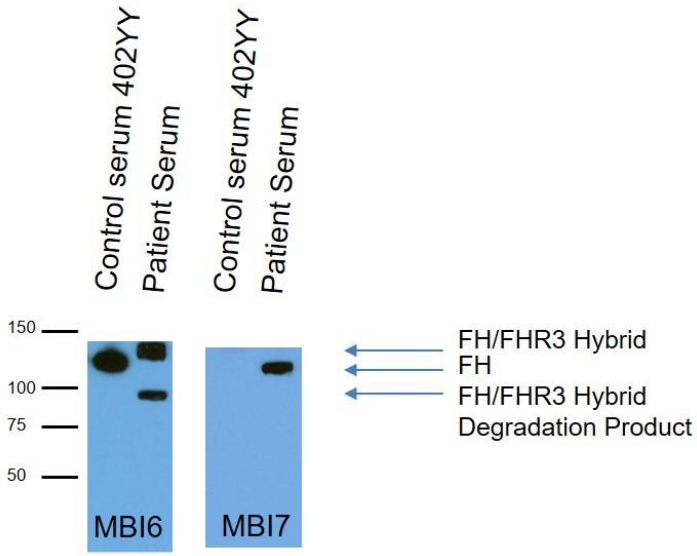

Figure 105 Western blot confirming specificity of MBI6 antibody to the hybrid FH/FHR3 protein

Serum (1:100) from patient and a genotyped control (CFH 402YY) was separated by 6% SDS-PAGE under non-reducing conditions and transferred onto nitrocellulose membrane. Following incubation with the mouse monoclonal antibodies MBI6 (1:2000) or MBI7 (1:2000) on separate blots followed by the secondary antibody sheep-anti mouse HRP (1:4000), FH 402Y in control serum was detected when using MBI6 as the primary antibody. Also detected was a larger protein consistent with the hybrid FH/FHR3 protein and a smaller protein consistent with a degradation product. When using the 402H specific (MBI7) antibody as a primary antibody, only the wildtype FH protein from patient serum carried on the 402H allele can be detected.

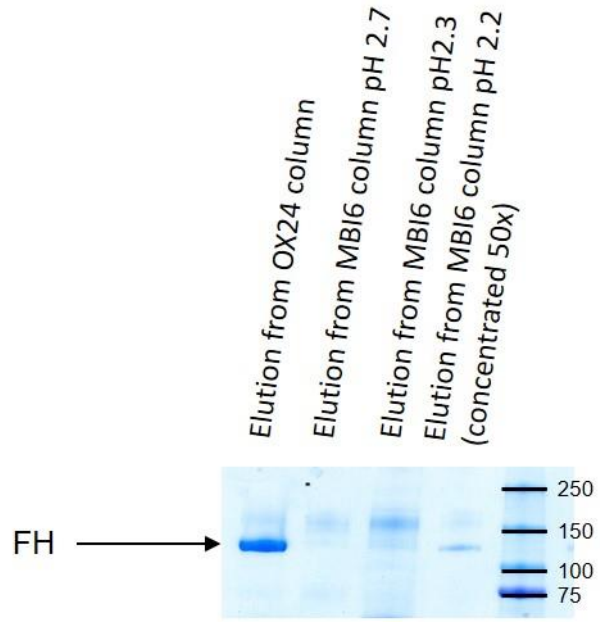

Figure 106 FH is not eluted from MBI6 column using 0.1M glycine

1ml of serum was loaded onto an OX24 (FH SCR5 specific) and also an MBI6 (FH SCR7 402Y specific) immunoaffinity column. FH is eluted using 0.1M glycine at pH 2.7 and is shown following 4-15% gradient SDS-PAGE migrating to its usual position between the 150kDa and 100kDa markers under non-reducing conditions. Attempts to elute FH using 0.1M glycine at pH 2.7, 2.3 and 2.2 were unsuccessful. A faint band suggestive of FH was visible when 0.1M glycine pH 2.2 was used as an elution buffer and only when extensively concentrated as shown.

Elution was then attempted successfully using PBS containing 0.05% diethylamine, pH 11.5. In these conditions, a large elution peak was observed and a pure protein was identified at the same size as FH from an OX24 column (Figure 107).

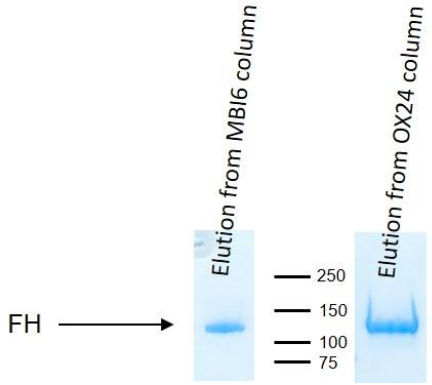

Figure 107 FH eluted from MBI6 and OX24 columns

FH is eluted from affinity column with specificity to FH 402Y (MBI6) using PBS containing 0.05% diethylamine, pH 11.5 and is shown following 4-15% gradient SDS-PAGE migrating to its expected size between the 150kDa and 100kDa markers under non-reducing conditions. FH eluted from an affinity column with specificity to FH SCR5 (OX24) using 0.1M glycine at pH 2.7 migrates to the same position.

Using the optimised elution conditions, hybrid FH/FHR3 protein was purified from patient serum and a control FH protein was purified from genotyped control serum using the MBI6 column. Purified protein free of aggregates or degradation products were obtained following size-exclusion and shown on SDS-PAGE gel in Figure 108. FH migrates to ~130kDa under non-reducing conditions. The purified FH/FHR3 hybrid is larger than the WT FH.

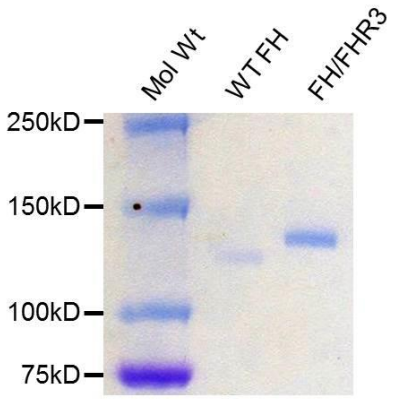

Figure 108 Purified WT FH and hybrid FH/FHR3 protein

Taken from Challis et al. WT FH from donor control serum and hybrid FH/FHR3 protein from patient serum was purified in two-steps using a SCR7 402Y specific immunoaffinity column followed by size exclusion. Following 4-15% gradient SDS-PAGE under non-reducing conditions, WT FH migrates at the expected size in between the 150kDa and 100kDa markers. FH/FHR3 as predicted is larger in size.

6.3.3 Determining Regulatory Activity in Sheep Erythrocyte Haemolysis Assays

The ability of the hybrid FH/FHR3 protein to regulate complement was tested on the surface of sheep erythrocytes in a series of haemolytic assays.

The hybrid FH/FHR3 protein was less effective than FH control in decay activity on the surface of sheep erythrocytes in a haemolytic assay. The IC_{50} for FH was 3.9nM vs 6.6nM for a hybrid FH/FHR3, a 1.7-fold difference (Figure 109).

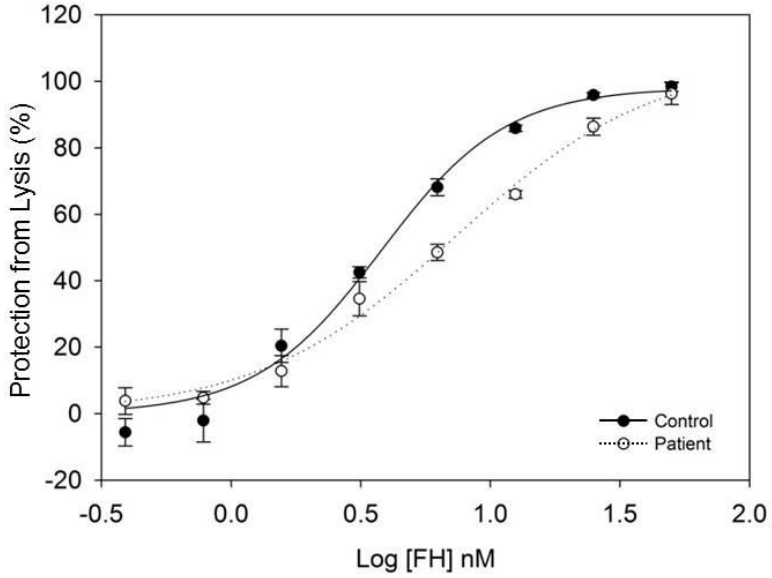

Figure 109 Decay activity on sheep erythrocytes - hybrid FH/FHR3 protein

Adapted from Challis et al. The hybrid FH/FHR3 and FH were incubated with C3bBb formed on the surface of sheep erythrocytes. FH decays the C3bBb and prevents lysis instigated by the addition of serum depleted of FB and FH. FH is 1.7-times more effective than FH/FHR3 hybrid at protecting cells from lysis (IC_{50} FH 3.9 nM vs FH/FHR3 hybrid 6.6 nM). Error bars represent 1 standard deviation from experiments performed in triplicate.

The hybrid FH/FHR3 protein was also less effective than the FH control in co-factor activity on the surface of sheep erythrocytes in a haemolytic assay. The IC_{50} for FH was 38.5nM. An exact IC_{50} could not be determined for the hybrid protein (Figure 110).

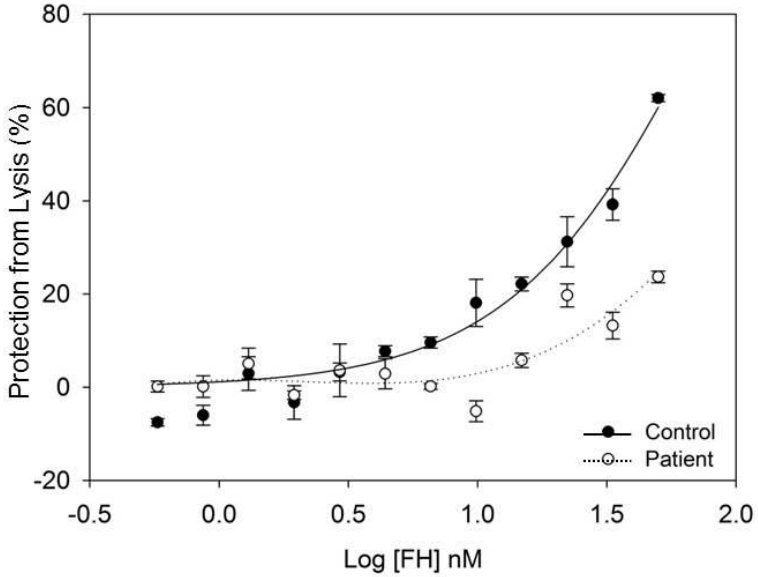

Figure 110 Co-factor activity on sheep erythrocytes - hybrid FH/FHR3 protein

Adapted from Challis et al. The hybrid FH/FHR3 or FH were incubated with sheep erythrocytes coated with C3b and factor I for 8 minutes. C3bBb was formed on intact C3b and lysis instigated by the addition of serum depleted of FB and FH. FH acts as a co-factor and inactivates C3b in the presence of factor I and prevents subsequent C3bBb formation and protects cells from lysis. FH is more effective than than FH/FHR3 hybrid at protecting cells from lysis. Error bars represent 1 standard deviation from experiments performed in triplicate.

6.3.4 Heparin Binding and Co-factor Activity

The hybrid FH/FHR3 protein lacks the C-terminal domain of FH that can bind heparin. Impaired binding to heparin compared to wildtype FH is demonstrated in (Figure 111). The other heparin domain in SCR6-8 of FH is unaffected. Fluid-phase co-factor activity, a function of the N-terminal domain was not impaired (Figure 112).

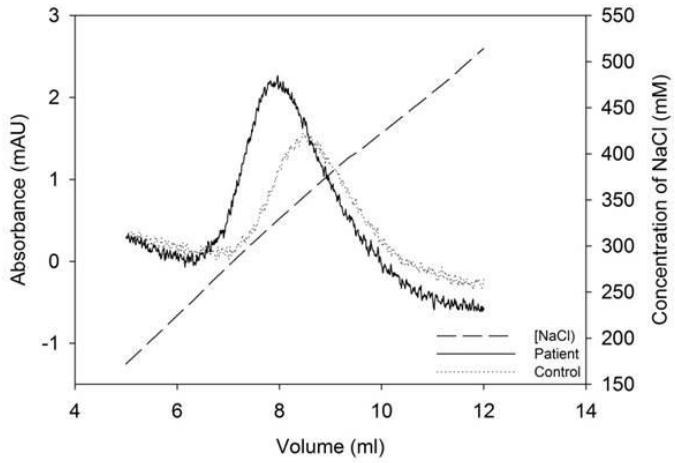

Figure 111 Heparin binding - hybrid FH/FHR3 protein

Taken from Challis et al. Purified proteins from patient (FH/FHR3) and control FH (402Y) were bound to a HiTrap heparin column and eluted with NaCl. The hybrid FH/FHR3 protein (black) eluted at 325mM and FH (grey) at 357mM, indicating reduced binding. Dashed line indicates gradient of NaCl.

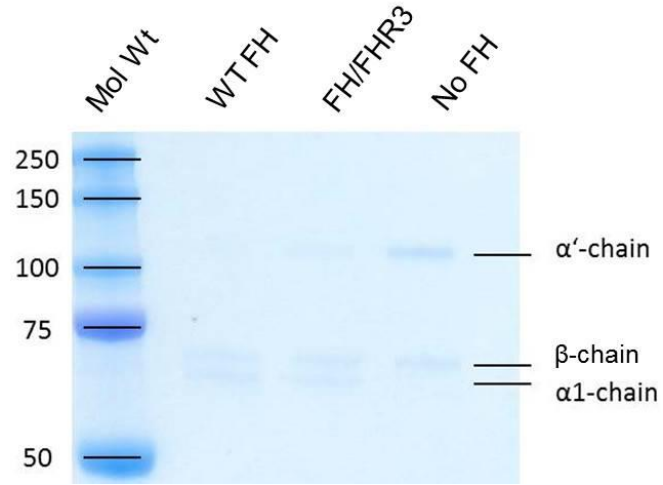

Figure 112 Co-factor activity in fluid-phase – hybrid FH/FHR3 protein

Adapted from Challis et al. Equimolar concentrations of wildtype FH and FH/FHR3 were used in a fluid phase co-factor assay. In the fluid phase the hybrid FH/FHR protein has comparable levels of co-factor activity compared to wildtype FH, as FI cleaves C3b to iC3b as seen by the loss of the α'chain and generation of the α_1 chain.

6.4 Discussion

6.4.1 Comparison of Studies in the Setting of Full Length and Fragments of FH

In this chapter, I have studied 3 disease-associated rare genetic variants and 1 disease-associated common polymorphism in the setting of recombinant full length FH. Clear differences in decay and co-factor activity were observed in the study of all three-disease associated mutant proteins when compared with the control, IYD. A small difference was observed in cofactor activity when comparing VYE and IYD. These differences are summarised in Table 51 and compared to the previously published studies of these mutant proteins.

R53H was selected for study as loss of regulatory activity had been previously demonstrated in the setting of the short fragment, FH SCR1-4. The effects on regulatory activity are confirmed in my studies of R53H in full length FH. In both fragment and full length studies, there was marked loss of decay activity and some loss of co-factor activity.

I62V has been studied previously in the setting of FH SCR1-4 and purified full length FH from serum. The finding of small functional effects on co-factor activity and not decay activity in my study is comparable to previous studies.

Studies of other variants on the interface of the N-terminal domain of FH and C3b will be useful to further validate the observations in full length FH. These should include variants such as R83S that resulted in complete loss of both decay and co-factor activity, or A161S where the effect on function was markedly less.

Previous studies of the two C-terminal variants, S1191L/V1197A and D1119G in FH SCR19-20 did not allow direct testing of decay or co-factor activity as the proteins used lacked the N-terminal domains. These two mutants were chosen for study because loss of function was demonstrated in assays testing cell surface binding. Impaired cell surface binding would result in a failure of FH to regulate complement on surface-bound C3b by decay and co-factor activity as was demonstrated in my studies using full length protein. The magnitude of the effect was greatest in both assays for D1119G and is consistent with the observations that D1119G had the greatest impairment on cell surface assays in previous studies (Ferreira *et al.*, 2009). In studies of both C-terminal variants in full length FH, impairment of co-factor activity appeared to be greater than decay as measured by differences in IC_{50}. This is an observation that could be tested in a larger sample of C-terminal variants.

Overall, the functional effects demonstrated in full length protein are consistent with those observed in previous studies using fragments of FH, such as SCR1-4 and SCR19-20. My studies confirm the functional effects observed in the original studies performed in short fragments.

Variant	Protein used	Difference in IC_{50} of mutant protein	
		Decay activity	Co-factor activity
R53H	Full Length	4.7-fold	2.46-fold
R53H	FH SCR1-4	40-fold	2.6-fold
VYE	Full Length	No difference	1.2-fold
I62V	Full Length	No difference	1.6-1.8-fold
I62V	FH SCR1-4	No difference	2-fold
S1191L/V1197A	Full Length	1.87-fold	3.8-fold
S1191L/V1197A	FH SCR19-20	N/A	N/A
D1119G	Full Length	8.9-fold	>17.5-fold
D1119G	FH SCR19-20	N/A	N/A

Table 51 Comparison of functional assays using full length and fragments of FH

Differences in IC_{50} of mutant proteins compared to WT or V62 to I62 are shown. The functional effects of R53H are greater in decay than co-factor activity in both full length and fragment studies. The functional effects of D1119G and S1191L/V1197A are greater in co-factor activity than decay both full length and fragment studies. The small effects on co-factor activity and not decay are observed in all studies of the I62V polymorphism. It should be noted that the full length protein containing the V62 variant also differed from I62 at the amino acid 936.

My studies of mutant full length FH proteins expressed in *Pichia pastoris* contribute to the small number of studies of mutant full length FH proteins previously described in the literature. Sanchez-Corral et al expressed the W1183L mutant in COS7 cells. They were able to demonstrate a loss of surface-bound C3b binding but no differences in fluid phase co-factor assays, consistent with the effects of a C-terminal variant. These findings were comparable to those using W1183L purified from serum (Sanchez-Corral *et al.*, 2002).

More recently, Mohlin et al expressed the Q950H mutant in HEK293 cells and reconstituted serum depleted of FH with mutant or wildtype protein in haemolytic assays. Using recombinant protein, they confirmed their earlier findings that the mutant protein had impaired regulation of complement in a sheep erythrocyte haemolytic assay. They then showed that the Q950H mutant recombinant protein had no impairment of co-factor activity in the fluid-phase, suggesting that the differences shown in haemolysis assays were due to loss of binding (Mohlin *et al.*, 2015).

In both of these studies, there was a comparison of recombinant and plasma purified FH. In my study, there was a small difference in decay activity between a recombinant and plasma purified wildtype FH protein. These differences were small, when compared to the differences in function that were observed due to the disease-associated rare variants (R53H, D1119G and S1191L/V1197A). However, it is possible that smaller differences of other variants may be masked. Further comparison of wildtype and mutant studies using both recombinant and plasma purified protein may be helpful, especially for the study of mutant proteins where the functional effects are smaller.

6.4.2 Methods of Purification of Full Length FH

I then went on to fully characterise a novel hybrid FH/FHR3 protein identified in Dr. Challis' studies of aHUS. In my experiments, the hybrid protein was purified from serum. FH was captured on a FH 402Y (MBI6) specific immunoaffinity column and eluted. The purified protein from serum was then used to determine the effect of the loss of the C-terminal recognition domain on decay and co-factor activity on the surface of sheep erythrocytes.

A number of different methods have been described for the purification of FH from serum (Table 52). The earliest methods involved polyethylene glycol 8000 precipitation, dissolution in PBS followed by multiple purification steps including heparin affinity, ion exchange followed by a final gel-filtration step.

More recently, immunoaffinity chromatography using a number of different monoclonal antibodies has been described. Antibodies specific to FH can be used to bind FH from serum and then eluted. The eluted FH would be a mixture of wildtype and mutant forms unless a variant was inherited in homozygosity or in the case of Sanchez-Corral et al, the V1197A variant was inherited with a type 1 mutation (Sanchez-Corral *et al.*, 2002). Given that most rare variants in *CFH* are inherited in heterozygosity, a method to separate wildtype from

mutant is required. The technique chosen by me in this chapter and that of others is to use an antibody specific to either form of the common polymorphism Y402H. One novel purification strategy that has been described involved the use of a purification column that binds albumin, exploiting the binding of the R1210C to albumin.

Variant	He/Ho	Purification technique	Reference
I62V	Ho	Multi-step	(Tortajada *et al.*, 2009)
DelK224	Ho	Multi-step	(Licht *et al.*, 2006)
Y402H	He	MBI6 and MBI7	(Francis *et al.*, 2012)
S890I	He	MBI7	(Tortajada *et al.*, 2012)
V1007L	He	MBI7	(Tortajada *et al.*, 2012)
E1172X	He	Multi-step	(Manuelian *et al.*, 2003)
W1183L	He[1]	Multi-step	(Sanchez-Corral *et al.*, 2002)
V1197A	He[2]	Anti-FH antibody column	(Sanchez-Corral *et al.*, 2002)
R1210C	He	Anti-FH[3]	(Sanchez-Corral *et al.*, 2002)
R1210C	He	Anti-FH[3]	(Recalde *et al.*, 2015)

Table 52 Purification techniques to purify FH from serum reported in functional studies

Variants were inherited in heterozygosity (He) or homozygosity (Ho) and purified for functional studies by a number of different techniques. [1]the purified protein was a mixture of wildtype and mutant protein. [2] due to the other allele being null, the purified protein was of the V1197A only, [3]further purification of the R1210C mutant protein covalently linked to albumin was performed by using an albumin-binding purification column.

Theoretically, other purification methods could be tried to separate a mutant protein from wildtype. The effect on a mutant protein might be altered ligand binding. If heparin binding is affected, a heparin-affinity column could be used. Alternatively, an individual amino acid substitution may lead to a sufficient change in electrostatic properties that ion exchange could separate two variants. Finally, the generation of additional high-specificity monoclonal antibodies against other common polymorphisms such as I62V or even the disease-associated variant itself may ultimately separate a wildtype and mutant protein.

6.4.3 Limitations of Study

My studies of 1 rare genetic variant in the N-terminal domain and 2 rare genetic variants in the C-terminal domain of *CFH* in the setting of a recombinantly expressed full length FH protein confirmed the previous observations from studies using short FH fragments. This suggests that data from previous demonstrating loss of function in short FH fragments can be extrapolated to a full length protein. Additional studies of mutant proteins in full length FH will strengthen this association and could include the use of FH purified from serum. Direct comparisons of mutant full length FH proteins from serum and recombinant expression should be performed to exclude the possibility that recombinant proteins, in *Pichia* may have additional modifications such as glycosylation that may confound results.

The use of FH purified from patient serum also allele specific quantification if the patient is heterozygous for the Y402H polymorphism (Hakobyan *et al.*, 2010). Modifications to the protein from serum, such as the binding of R1210C mutant to albumin can also be demonstrated. However, obtaining purified protein from serum does require access to patients, is invasive, and if in a child, presents as an additional ethical choice to ensure sufficient serum for preparation.

6.4.4 Future Studies

Studies of rare genetic variants that are not within the N-terminal and C-terminal domains of *CFH* are limited (Table 53). The polymorphic variants Y402H (Francis *et al.*, 2012) and S890I/V1007L (Tortajada *et al.*, 2012) were purified to homogeneity for functional studies that demonstrated no differences in complement regulatory function. Most recently, serum from patients carrying the variants Q950H and T956M were used in haemolysis assays (Szarvas *et al.*, 2016). A loss of regulatory effect was detected for the Q950H variant, and confirmed in studies using both patient's serum and recombinant full length FH (Mohlin *et al.*, 2015).

The functional testing and purification techniques described and discussed in this chapter are applicable to the study of MPGN and C3G-associated rare genetic variants that are not in the N- or C-terminal domains of *CFH* that were identified at the beginning of Chapter 5. Future functional studies using full length protein, both recombinantly expressed and purified from patient samples will improve the understanding of the role of FH in MPGN and C3G.

Variant	SCR	Recombinant FH	Patient FH	C3b/d binding	Heparin binding	Co-factor	Decay	Endothelial Cell Binding	Haemolysis Assay	Reference
Y402H	7	SCR 6-8	Purified	↓[1]	↓[1]	ND	↔[2]	ND	↔[2, 3]	(Clark *et al*, 2006; Herbert *et al.*, 2007; Francis *et al.*, 2012)
S772X	13	N	Serum[4]	ND	ND	ND	ND	ND	↔[5]	(Szarvas *et al.*, 2016)
S890I	15	N	Purified	↔	ND	↔	ND	ND	↔[6]	(Tortajada *et al.*, 2012)
Q950H	16	SCR 1-20	Serum	ND	ND	↓	ND	ND	↓	(Mohlin *et al.*, 2015; Szarvas *et al.*, 2016)
T956M	16	N	Serum	ND	ND	ND	ND	ND	↔	(Szarvas *et al.*, 2016)
V1007L	17	N	Purified	↔	ND	↔	ND	ND	↔[6]	(Tortajada *et al.*, 2012)

Table 53 Studies of CFH variants not in the N- or C-terminal domains

Functional testing of recombinant FH, FH from patient serum and FH purified from patient serum. Tests used in the studies of N- and C-terminal domain variants are summarised in this table. [1]in setting of SCR6-8, [2]in setting of purified protein, [3]as described in (Tortajada et al., 2009) [4]low serum FH levels, [5]as described by (Sanchez-Corral et al., 2004), [6]Lysis initiated by serum from W1183L patient.

6.5 Summary

Functional studies of rare genetic variants in *CFH* are important to determine their importance in disease. A high proportion of studies, especially those of variants in the N-terminal domain are in the setting of short FH fragments. Whilst these studies have demonstrated clear differences in function, extrapolation of these effects to a full length protein is required.

Rare genetic variants in *CFH* that been previously studied in short fragments of FH were expressed recombinantly in full length FH. Experiments to test the effect of 1 mutant protein with an abnormal N-terminal domain and 2 mutant proteins with an abnormal C-terminal domain were performed on the surface of sheep erythrocytes. These experiments identified functional defects in complement regulation. These findings correlate with previous studies using short FH fragments.

The study of mutant FH proteins was extended to a novel hybrid FH/FHR3 protein identified in case of aHUS. In this study, mutant protein was purified directly from serum and functional defects were also demonstrated.

Expression of recombinant mutant FH proteins and purification of mutant FH proteins from patient serum can be used for the future study of other MPGN/C3G-associated rare genetic variants in *CFH*. This will improve the understanding of the role of FH in MPGN/C3G.

Chapter 7 Discussion

I had several main aims in this thesis.

1. Identification of acquired and genetic abnormalities of AP in two cohorts of MPGN/C3G.
2. Solving a long-standing conundrum in a case of familial MPGN.
3. Determining the range of functional effects of rare genetic variants in the N-terminal domain of *CFH* in MPGN/C3G.
4. Functional studies of rare genetic variants in full length FH to confirm the findings of previous functional studies performed in the regulatory domain of FH.

7.1 Identification of Acquired and Genetic Abnormalities of AP in MPGN/C3G

In this thesis, I have described a prospective and a retrospective cohort of patients with MPGN/C3G. Screening for rare genetic variants in *C3*, *CFB*, *CFH*, *CFI* and *CD46* in these cohorts revealed a low prevalence of genetic abnormalities (8.2% - 17.9%). Compared to the prevalence rate of C3 nephritic factor identified in this prospective cohort, and the prevalence rate of other autoantibodies identified in both the prospective and retrospective cohorts, it is clear that the predominant abnormalities in these cohorts are acquired, suggesting that MPGN/C3G is largely an autoimmune phenomenon, comparable with previous cohorts with data on both acquired and genetic abnormalities (Servais *et al.*, 2012; Iatropoulos *et al.*, 2016).

7.2 Functional Studies of Rare Genetic Variants in *CFH*

Whilst the prevalence of rare genetic variants in *C3*, *CFH*, *CFH*, *CFI* and *CD46* identified in my cohorts of MPGN/C3G was low, one genetic variant was of particular interest as it associated with a case of familial MPGN. The variant R83S in the N-terminal region of *CFH* segregated with all family members with the renal phenotype. Functional studies using short recombinant fragments of FH comprising the N-terminal domains of FH confirmed that the mutant protein expressed resulted in complete loss of regulatory function and solved a long standing conundrum in the field of complement-mediated renal disease.

I then reviewed all 43 rare genetic variants in MPGN/C3G identified in the literature and showed that many were functionally significant, including 17 that resulted in low FH levels and 4 (including my study of R83S) that resulted in a functionally defective mutant protein.

These abnormalities were all in keeping with the paradigm of the importance of fluid phase activation of complement in these diseases. I therefore undertook functional studies to confirm the significance of variants that had not been studied.

I characterised a further 8 disease-associated rare genetic variants that had been identified in the N-terminal domain of *CFH*, of which 3 had been described in MPGN/C3G. The other 5 had been described in aHUS and AMD, two other diseases that share complement risk factors with MPGN/C3G. It was evident that the functional effects of these genetic variants could be divided into two categories, those that resulted in complete loss of function, and those that do not. Initial interpretation of the results of these studies using short fragments of FH in the clinical context suggests that only those with complete loss of regulatory function can definitively be considered pathogenic.

The variants that resulted in complete loss of function were not exclusively in MPGN/C3G. Of the two variants that resulted in complete loss of function, one (Q81P) was in a case of aHUS and the other (R53C) was found in cases of MPGN/C3G, aHUS and AMD. Functional studies of variants in *CFH* are therefore important in all of these diseases that share complement risk factors. Small effects on regulatory function were observed in the remaining variants, though in contrast to those that resulted in complete loss of function, the functional significance of these remains uncertain and their role in disease pathogenicity on their own is unlikely.

Many of the functional studies in both MPGN/C3G and aHUS previously reported used short fragments of recombinantly expressed FH. A functional effect for many of these variants has been identified from functional studies including those of my own. To confirm that these effects can be extrapolated to full length protein, I used recombinantly expressed full length mutant proteins.

In these studies, I demonstrated that the variant, R53H, in the N-terminal domain of FH, had complete loss of decay activity (and some loss of co-factor activity) therefore confirming the previous findings from studies of R53H in short fragments of FH (Pechtl *et al.*, 2011). I also demonstrated that the variants, D1119G and S1191L/V1197A, in the C-terminal domain of FH had impairment of cell surface complement regulation by both decay and co-factor activity, again confirming the previous findings that both had impairment of cell surface complement regulatory activity (Ferreira *et al.*, 2009).

The study of full length mutant FH proteins was extended to include a novel hybrid FH/FHR3 protein identified in case of aHUS. In this study, mutant protein was purified directly from serum and functional defects were also demonstrated.

7.3 Future studies

Further studies are required to improve our understanding of MPGN/C3G. These studies should include the following.

- Longitudinal follow up of both MPGN/C3G cohorts to determine correlations of clinical outcomes with complement abnormalities.
- Given that MPGN/C3G is largely an autoimmune phenomena, characterisation of these abnormalities, especially C3 nephritic factor and autoantibodies to FH in a large prospective cohort is required.
- Functional studies of rare genetic variants in *C3* and *CFB*
- Functional studies of rare genetic variants in *CFH* - Studies that follow on directly from the work described in this thesis would involve the study of rare genetic variants that are not within the N- or C-terminal domains. Unlike those in the N- and C-terminal functional domains, these variants have not been extensively studied. These could be performed using full length mutant proteins, either expressed recombinantly or purified from serum.

7.4 Summary

Work in this thesis has identified a large number of abnormalities of AP, both acquired and genetic in two cohorts of MPGN/C3G. The majority of these are acquired and supportive of MPGN/C3G as largely an autoimmune phenomenon. Rare genetic variants, though individually fewer were not insignificant as demonstrated in the functional studies of mutant proteins that abolish regulatory function due to variants in the N-terminal domain of *CFH*. However, not all such variants lead to complete loss of function thus highlighting the importance of functional studies. Furthermore, previous studies performed in short fragments of FH describing both N- and C-terminal variants in *CFH* have been replicated in studies using full length protein.

In summary, the significance of rare genetic variants in *CFH* needs to be considered even in a cohort in which autoimmune abnormalities predominate. Future work to determine the functional and clinical significance of a larger number of acquired and genetic complement abnormalities of AP in MPGN/C3G is required.

Chapter 8 Appendices

8.1 Appendix 1 – Primers and DNA sequences

Variant	Forward Primer	Reverse Primer
R53C	cccaggctatctataaatgcTgccctggatatagatctcttgg	ccaagagatctatatccagggcAgcatttatagatagcctggg
G69E	ggtatgcaggaaggAagaatgggttgctcttaatccattaagg	ccttaatggattaagagcaacccattctTccttcctgcatacc
Q81P	ggaaatgtcCgaaaaggccctgtgg	ccacagggccttttcGgacatttcc
R83S	gggttgctcttaatccattaaggaaatgtcagaaaagTccctgtggacatcctggagatactcc	ggagtatctccaggatgtccacagggActttttctgacatttccttaatggattaagagcaaccc
D90G	ggccctgtggacatcctggaggtactccttttggtacttttaccc	gggtaaaagtaccaaaaggagtacctccaggatgtccacagggcc
DelE122-G128	gtaatgaggggtatcaattgctatgtgacacagatggatggacc	ggtccatccatctgtgtcacatagcaattgataccccctcattac
D130N	ggtgagattaattaccgtgaatgtAacacagatggatggacc	ggtccatccatctgtgtTacattcacggtaattaatctcacc
S159N	N/A[1]	N/A
A161S	ggaaaaattgtcagtagtTcaatggaaccagatcggg	cccgatctggttccattgAactactgacaatttttcc
M162V	cagtagtgcaGtggaaccagatcggg	cccgatctggttccaCtgcactactg

Table 54 Primers for site-directed mutagenesis

Mutagenesis primers were HPLC-purified from Integrated DNA Technologies. The mutated base pair has been capitalised.
[1]A clone of FH SCR1-4 in E.coli with the S159N mutation was a gift from Professor Alan Wright, University of Edinburgh.

	Primer
Forward	cgggttattgtttataaatac
Reverse	gtcgacggcgctattcagatcctc

Table 55 Primers for sequencing pPICZαB

8.2 Appendix 2 – Reagents

Stock solutions

10X YNB (13.4% yeast nitrogen base with ammonium sulphate without amino acids)

134g of yeast nitrogen base (YNB) with ammonium sulphate and without amino acids was dissolved in 1000 mL of dH_2O. Once completely dissolved, the solution was filter sterilised using a 0.22µm filter and stored at 4°C.

500X B (0.02% Biotin)

20mg biotin was dissolved in 100 mL of dH_2O. Once completely dissolved, the solution was filter sterilised using a 0.22µm filter and stored at 4°C.

10X D (20% Dextrose)

200g of D-glucose was dissolved in 1000mL of dH_2O. Once completely dissolved, the solution was filter sterilised using a 0.22µm filter and stored at 4°C.

10X Methanol (5% methanol)

50ml of methanol was diluted to a final volume of 1000ml of dH_2O. The solution was filter sterilised using a 0.22µm filter and stored at 4°C.

10X Glycerol (10% glycerol)

100mL of glycerol was diluted to a final volume of 1000ml of dH_2O. The solution was sterilised by autoclave and stored at 25°C.

1 M potassium phosphate buffer, pH 6.0

132mL of 1M K_2HPO_4 and 868 mL of 1M KH_2PO_4 were prepared and added together. The pH of the final solution was adjusted to 6.0 using phosphoric acid or KOH The solution was sterilised by autoclave and stored at 25°C.

YPD (Yeast Extract Peptone Dextrose Medium)

10g yeast extract and 20g of peptone were dissolved in 900mL of dH2O. 20g of agar was added for making YPD agar plates. The solution was autoclaved for 20 minutes on liquid cycle. After autoclaving, 100mL of 10X D was added. The liquid medium was stored at room temperature. Prior to pouring into plates, YPD agar was allowed to cool to ~55°C before Zeocin™ was added at the required concentration. The YPD agar plates were stored at 4°C.

LB (Luria-Bertani) medium

10g tryptone, 5g yeast extract and 10g NaCl were added to 1000ml of dH_2O. 15g of agar was added for making LB agar plates. The solution was autoclaved for 20 minutes on liquid cycle LB medium or agar was allowed to cool to ~55°C before Zeocin™ was added at the required concentration. The medium was stored at room temperature and LB agar plates stored at 4°C. For low salt LB, 5g instead of 10g NaCl was used.

Buffered Minimal Glycerol (BMG) or Buffered Minimal Methanol (BMM)

700mL of dH_2O containing 1% casamino acids powder (Fisher Chemical) was autoclaved for 20 minutes on liquid cycle. The solution was made up to 1L by the addition of 100 mL 1M potassium phosphate buffer, pH 6.0, 100mL 10X YNB, 2mL 500X B and 100mL 10X glycerol. For BMM, 100 mL 10X methanol was added instead of glycerol. The media was stored at 4°C.

BMG and BMM containing ^{15}N-ammonium sulphate as sole nitrogen source

BMG and BMM were prepared from alternative 10X stock solution by dissolving 34g of YNB without ammonium sulphate and amino acids in 1000 mL of dH_2O. Once completely dissolved, the solution was filter sterilised using a 0.22µm filter and stored at 4°C. From this, a 1X YNB without ammonium sulphate and amino acids solution was prepared, and supplemented with 2g of ^{15}N-ammonium sulphate (Isotec) per litre of BMG required and 1g of ^{15}N-ammonium sulphate (Isotec) per litre of BMM required.

Fermentation Basal Salt medium

26.7ml of 85% phosphoric acid, 0.93g calcium sulphate, 18.2g potassium sulphate, 14.9g magnesium sulphate-7H_2O, 4.13g potassium hydroxide, 25ml 100% glycerol and 10g of casamino acids powder were added to a final volume of 1L dH_2O per litre of media required.

PTM1 Trace Salts

PTM1 Trace Salts (Sunrise Science Products) comprising 6.0g cupric sulphate-5H_2O, 0.08g sodium iodide 3.0g manganese sulphate-H_2O, 0.2g sodium molybdate-2H_2O 0.02g boric Acid, 0.5g cobalt chloride, 20.0g zinc chloride, 65.0g ferrous sulphate-7H_2O and 0.2g biotin were dissolved in 5.0ml of sulphuric acid and dH_2O to a final of volume of 1L. Once completely dissolved, the solution was filter sterilised using a 0.22µm filter and stored at room temperature.

8.3 Appendix 3 - SDS-PAGE and Western Blotting

Molecular weight marker	Manufacturer
PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa	ThermoFisher Scientific
Prestained Protein Marker, Broad Range (7-175 kDa)	New England Biolabs Inc®
Precision Plus Protein™ Dual Color Standards	Biorad

Table 56 Molecular weight markers for SDS-PAGE

Protein target	FH SCR1-4[1]	FH[2]	FH[2]	FB[2]	α- and β-chain of C3b[3]
Size	30kDa	150kDa	150kDa	90kDa	40-110kDa
System	Biorad	Novex	Novex	Novex	Biorad
Gel percentage	4-15%	6%	6%	6%	10%
Serum dilution	N/A	1:100	1:100	1:100	N/A
Reduced or non-reduced	Non-reduced	Non-reduced	Non-reduced	Non-reduced	Reduced
Primary Antibody	Polyclonal Goat anti-FH (Calbiochem)	Polyclonal Goat anti-FH (Calbiochem)	MBI6/ MBI7 Monoclonal Mouse anti-402Y or H in SCR7 of FH (Gift from Professor Claire Harris, Cardiff University)	Polyclonal Goat anti-FB (R and D Systems)	Polyclonal Rabbit Anti-C3 (Abcam)
Concentration	1:14000	1:14000	1:2000	1:2000	1:5000
Secondary Antibody	Rabbit anti-Goat HRP (Calbiochem)	Rabbit anti-Goat HRP (Calbiochem)	Sheep anti-mouse IgG HRP (Jackson Immuno Research)	Rabbit anti-Goat HRP (Calbiochem)	Goat anti-Rabbit HRP (Abcam)
Concentration	1:3000	1:3000	1:4000	1:3000	1:5000

Table 57 Antibodies used to detect purified protein by Western blotting

Specific experiemental conditions for Western blotting are shown. [1]FH SCR1-4 was recombinantly expressed. [2]FH and FB were deteted in serum. [3]α- and β-chain of C3b detected following fluid-phase co-factor assay. N/A = not applicable, HRP = horse radish peroxidase.

8.4 Appendix 4 – Extinction Co-efficient of Proteins

Protein	Extinction co-efficient (M^{-1} cm^{-1})
FH SCR1-4	47870
FH	246800
FH/FHR3 hybrid	272170

Table 58 Extinction co-efficient to determine protein concentration from A_{280}

Extinction co-efficient calculated based upon amino acid sequence inputted into http://web.expasy.org/protparam/ - translate tool. Calculation of molar concentration of protein based upon formula of absorbance at 280nm (A_{280})/extinction co-efficient.

8.5 Appendix 5 – SNP Analysis in RaDaR Cohort

		C/C	C/G	G/G	Frequency of variant allele (G)	Odds Ratio (95% CI)	P (vs control)
C3	All	40	27	5	0.257	1.28 (0.87-1.85)	0.22
c.304C>G	Ig-GN	7	1	0	0.067	0.24 (0.03-1.85)	0.17
p. R102G	Ig-MPGN	15	10	4	0.310	1.64 (0.94-2.88)	0.07
rs2230199	DDD	2	10	1	0.462	3.14 (1.45-6.8)	0.004
	C3GN	16	6	0	0.136	0.57 (0.24-1.37	0.21
	Control[1]	2670	1459	171	0.209		
		C/C	C/T	T/T	Frequency of variant allele (T)	Odds Ratio (95% CI)	P (vs control)
C3	All	38	29	5	0.271	1.44 (1.00-2.09)	0.05
c.941C>T	Ig-GN	7	1	0	0.063	0.25 (0.03-1.96)	0.19
p.P314L	Ig-MPGN	16	11	2	0.259	1.36 (0.75-2.45)	0.31
rs1047286	DDD	4	8	1	0.385	2.43 (1.10-5.36)	0.03
	C3GN	11	9	2	0.295	1.63 (0.85-3.12)	0.14
	Control[1]	2702	1436	162	0.205		
		C/C	C/T	T/T	Frequency of variant allele (T)	Odds Ratio (95% CI)	P (vs control)
CFB	All	65	5	1	0.049	0.47 (0.22-1.03)	0.06
c.94C>T	Ig-GN	8	0	0	0.000	0.28 (0.02-4.66)	0.37
p.R32W	Ig-MPGN	27	2	0	0.034	0.33 (0.08-1.35)	0.12
rs641153	DDD	10	1	1	0.125	1.32 (0.39-4.43)	0.66
	C3GN	20	2	0	0.045	0.44 (0.11-1.82)	0.26
	Control[1]	2206	476	27	0.098		
		G/G	G/A	A/A	Frequency of variant allele (A)	Odds Ratio (95% CI)	P (vs control)
CFB	All	65	5	0	0.036	0.39 (0.16-0.95)	0.04
c.95G>A	Ig-GN	7	1	0	0.063	0.70 (0.09-5.34)	0.73
p.R32Q	Ig-MPGN	28	0	0	0.000	0.09 (0.01-1.51)	0.10
rs12614	DDD	11	1	0	0.045	0.46 (0.06-3.41)	0.45
	C3GN	19	3	0	0.107	1.27 (0.38-4.21)	0.70
	Control[1]	2260	429	20	0.087		
		C/C	C/T	T/T	Frequency of variant allele (T)	Odds Ratio (95% CI)	P (vs control)
CFH	All	25	21	7	0.330	1.22 (0.73-2.04)	0.45
c.-331C>T	Ig-GN	2	4	1	0.429	1.85 (0.61-5.60)	0.27
	Ig-MPGN	7	11	5	0.457	2.08 (1.07-4.03)	0.03
rs3753394	DDD	4	2	0	0.167	0.49 (0.10-2.33)	0.37
	C3GN	12	4	1	0.132	0.46 (0.18-1.17	0.10
	Control[2]	44	43	5	0.288		

		G/G	G/A	A/A	Frequency of variant allele (A)	Odds Ratio (95% CI)	P (vs control)
CFH	All	49	20	3	0.186	0.80 (0.52-1.23)	0.31
c.184G>A	Ig-GN	4	3	1	0.313	1.59 (0.55-4.59)	0.39
p.V62I	Ig-MPGN	22	7	0	0.121	0.48 (0.22-1.06)	0.07
rs800292	DDD	9	4	0	0.154	0.64 (0.22-1.85)	0.41
	C3GN	14	6	2	0.227	1.03 (0.51-2.09)	0.93
	Control[1]	2601	1489	210	0.222		
		T/T	T/C	C/C	Frequency of variant allele (C)	Odds Ratio (95% CI)	P (vs control)
CFH	All	18	39	15	0.090	0.47 (0.26-0.85)	0.01
c.1204T>C	Ig-GN	5	3	0	0.188	0.37 (0.11-1.31)	0.12
p.Y402H	Ig-MPGN	5	18	6	0.517	1.73 (1.03-2.90)	0.03
rs1061170	DDD	3	7	3	0.500	1.62 (0.75-3.49)	0.22
	C3GN	5	11	6	0.523	1.77 (0.98-3.20)	0.06
	Control[1]	1649	2014	637	0.382		
		A/A	A/G	G/G	Frequency of variant allele (G)	Odds Ratio (95% CI)	P (vs control)
CD46	All	10	18	10	0.500	1.62 (0.92-2.83)	0.09
c.-652A>G	Ig-GN	2	2	1	0.400	1.08 (0.29-3.99)	0.91
	Ig-MPGN	0	11	5	0.656	3.09 (1.39-6.88)	0.006
rs2796267	DDD	2	2	1	0.400	1.08 (0.29-3.99)	0.91
	C3GN	6	3	3	0.375	0.97 (0.40-2.37)	0.95
	Control[2]	30	34	12	0.382		
		A/A	A/G	G/G	Frequency of variant allele (G)	Odds Ratio (95% CI)	P (vs control)
CD46	All	11	21	6	0.434	1.31 (0.75-2.29)	0.34
c.-366 A>G	Ig-GN	2	2	1	0.400	1.14 (0.31-4.21)	0.84
	Ig-MPGN	2	12	2	0.500	1.71 (0.80-3.67)	0.17
rs2796268	DDD	2	2	1	0.400	1.14 (0.31-4.21)	0.84
	C3GN	5	5	2	0.375	1.03 (0.42-2.49	0.16
	Control	33	35	12	0.369		
		T/T	T/C	C/C	Frequency of variant allele (C)	Odds Ratio (95% CI)	P (vs control)
CD46	All	17	30	7	0.315	0.94 (0.58-1.51)	0.79
c.*4070T>C	Ig-GN	3	3	1	0.357	0.76 (0.24-2.34)	0.63
	Ig-MPGN	6	15	2	0.413	0.96 (0.50-1.84)	0.90
rs7144	DDD	2	4	2	0.500	1.36 (0.49-3.78	0.55
	C3GN	6	8	2	0.375	0.81 (0.38-1.76	0.61
	Control[2]	36	41	21	0.423		

Table 59 Association of common SNPs with MPGN and C3G

In an analysis of 10 SNPs, statistical significance taken as P<0.005. Control data from [1]European American population of evs.gs.washington.edu/, or [2](Fremeaux-Bacchi et al., 2005).

8.6 Appendix 6 – Less Common Polymorphisms in RaDaR cohort

Disease	Gene	Variant	Frequency[1]
C3GN	*CD46* *CFB*	A353V K565E	1.7% 1.6%
IC-MPGN	*CFB*	L9H	4.2%
C3GN	*CFB*	L9H	4.2%
IC-MPGN	*CFB*	G252S	3.9%
DDD	*CFB*	G252S	3.9%
IC-MPGN	*CD46*	A353V	1.7%
IC-MPGN	*CFB*	K565E	1.6%
IC-MPGN	*CFB*	K565E	1.6%
C3GN	*CFB*	K565E	1.6%

Table 60 Polymorphisms reported at 1-5% of control cohort

Genetic variants in C3, CFB, CFH, CFI and CD46 identified in RaDaR cohort with [1]frequency of 1-5% as reported in European American population of evs.gs.washington.edu/.

8.7 Appendix 7 – Lipodystrophy Genes

Gene Name	Symbol	Reference
Partial lipodystrophies associated genes		
Lamin A/C	*LMNA*	(Cao and Hegele, 2000)
Peroxisome proliferator-activated receptors γ	*PPARG*	(Hegele *et al.*, 2002)
Zinc metalloproteinase STE24	*ZMPSTE24*	(Agarwal *et al.*, 2003)
Perilipin	*PLIN1*	(Gandotra *et al.*, 2011)
Nuclear lamina protein lamin B2	*LMNB2*	(Hegele *et al.*, 2006)
Protease subunit, beta type 8	*PSMB8*	(Agarwal *et al.*, 2010)
Total lipodystrophy associated genes		
Seipin	*BSCL2*	(Magre *et al.*, 2001)
Lysophosphatidic acid acyltransferase	*AGPAT2*	(Agarwal *et al.*, 2002)
Caveolin1	*CAV1*	(Kim *et al.*, 2008)
Polymerase I and transcript release factor	*PTRF*	(Hayashi *et al.*, 2009)

Table 61 Lipodystrophy-associated genes

Taken from (Wong et al., 2014). The genes listed above have been associated with partial and complete lipodystrophies. Whole exome sequencing was performed to screen these genes however no genetic variants were identified which segregated with lipodystrophy in the family.

8.8 Appendix 8 – Rare Genetic Variants in *CFH* in MPGN and C3G

Variant	SCR	Disease	He/Ho	Type1 or 2	Reference
P26S	1	C3GN	He	2	Unpublished, Newcastle
R53C	1	C3GN	He	2	(Servais *et al.*, 2012)
R53C	1	MPGN	Ho	2	(Servais *et al.*, 2012)
R53C	1	MPGN[1]	He	2	(Janssen van Doorn *et al.*, 2013)
P76X	1	C3GN	He	1	(Servais *et al.*, 2007)
L77X	1	C3GN	He	1	(Servais *et al.*, 2012)
R78G	1	C3GN	Ho	2	(Iatropoulos *et al.*, 2016)
R83S[2]	1	MPGN	He	2	(Wong *et al.*, 2014)
P88T	2	MPGN	Ho	1[3]	(Alfandary and Davidovits, 2015; Iatropoulos *et al.*, 2016)
DelG122-E128	2	C3GN	He	2	(Servais *et al.*, 2012)
R127L	2	MPGN	Ho	1[3]	(Dragon-Durey *et al.*, 2004)
R127C	2	C3GN	He	NK	(Iatropoulos *et al.*, 2016)
D130N	2	C3GN	He	2	(Servais *et al.*, 2012)
V143I	2	DDD	He	1	(Servais *et al.*, 2012)
A161S	3	DDD	He	2	(Servais *et al.*, 2012)
IVS 11+5[4]	N/A	C3GN	He	2	(Servais et al., 2012)
S199G / E1172X	4/20	DDD	He[5]	NK	Unpublished, Newcastle
I216T	4	MPGN[1]	He	1	(Gnappi *et al.*, 2012)
DelK224[2]	4	C3GN	Ho	2	(Licht *et al.*, 2006)
R232X	4	C3GN	He	1	Unpublished, Newcastle
R232X	4	DDD	He	1	(Servais *et al.*, 2012)
G334A	6	MPGN	He	2	Unpublished, RaDaR
C431S	7	C3GN	He	1[6]	Unpublished, Newcastle
C431S	7	DDD	Ho	1[3]	(Dragon-Durey *et al.*, 2004; Servais *et al.*, 2012)
N516K	9	DDD	He	NK	(Zhang *et al.*, 2012)
C597R	10	C3GN	Ho	1[3]	(Servais *et al.*, 2012)

C597R	10	MPGN1	Ho	1[3]	(Servais *et al.*, 2012)
V609I	10	MPGN	He	NK	Unpublished, Newcastle
V609I	10	C3GN	He	NK	Unpublished, Newcastle
P621Y	10	MPGN[1]	Ho	1[2]	(Vaziri-Sani *et al.*, 2006)-
G650V	11	C3GN	He	2	(Servais *et al.*, 2007)
G650V	11	MPGN	He	2	Unpublished, RaDaR
C673R	11	DDD	He	1	(Servais *et al.*, 2012)
C673S	11	MPGN	Ho	1[3]	(Dragon-Durey *et al.*, 2004)
F717L[2]	12	MPGN	He	2	(Servais *et al.*, 2012)
M725X	12	C3GN	He	NK	(Sethi *et al.*, 2012a)
N767KfsX	13	aHUS[7]	He	1	(Boyer *et al.*, 2008)
V837I / E1145D	14/19	DDD	He[5]	NK	(Zhang *et al.*, 2012)
H878Y	15	MPGN	He	2	Unpublished, Newcastle
A892V	15	MPGN	He	2	Unpublished, RaDaR
Y899X	15	aHUS[7]	Ho	1	(Boyer *et al.*, 2008)
Q950H	16	C3GN	He	NK	(Iatropoulos *et al.*, 2016)
T956M	16	DDD	He	NK	(Zhang *et al.*, 2012)
T956M	16	MPGN	He	NK	(Sethi *et al.*, 2011)
T956M	16	C3GN	He	NK	(Iatropoulos *et al.*, 2016)
Y1008X	17	C3GN	Ho	1[3]	(Iatropoulos *et al.*, 2016)
C1043S	17	MPGN1	He	1	(Servais *et al.*, 2012)
W1096R[2]	18	DDD	Ho	1[3]	Unpublished, Newcastle
R1210V	20	C3GN	He	2	(Servais *et al.*, 2012)
R1210C	20	C3GN	He	2	(Servais *et al.*, 2007)
R1210C	20	DDD	He	NK	(Iatropoulos *et al.*, 2016)

Table 62 Rare genetic variants in CFH in cases of MPGN/C3G

Rare genetic variants in CFH identified in cases of MPGN/C3G from literature and cohort studies described in this thesis. The rare genetic variants in CFH for individual patients or pedigrees identified are represented in Figure 79. SCR = short consensus repeat, He = heterozygous, Ho = homozygous, NK= not known, N/A = not applicable.. [1]Later developed aHUS, [2]Familial, [3]Complete FH deficiency,[4]IVS11+5 = splice change, [5]Could be compound heterozygous,[6]Known type 1 mutation, but normal FH levels in this case, [7]Later developed C3GN in transplant.

8.9 Appendix 9 – Rare Genetic Variants in N-terminal Domain of *CFH* in aHUS

Variant	SCR	Disease	Type	Reference
R28Ifs5X	1	aHUS	1	(Warwicker *et al.*, 1998)
T30Nfs10X	1	aHUS[1]	1	(Bresin *et al.*, 2013)
I32X	1	aHUS	1	(Kavanagh *et al.*, 2013)
R53H	1	aHUS	2	(Pechtl *et al.*, 2011) Unpublished, Zipfel
R53S	1	aHUS	U	(Rodriguez de Cordoba *et al.*, 2014)
R53C	1	aHUS[1]	2	(Fakhouri *et al.*, 2010b)
S58A	1	aHUS	U	(Rodriguez de Cordoba *et al.*, 2014)
W71R	1	aHUS	2	Unpublished, Newcastle
R78G	1	aHUS	2	(Pechtl *et al.*, 2011) (Noris *et al.*, 2010)
Q81P	1	aHUS[1]	2	(Fakhouri *et al.*, 2010b)
V111E	2	aHUS	U	(Bresin *et al.*, 2013)
Y118IfsX4	2	aHUS	1	(Bresin *et al.*, 2013)
I124Mfs12X	2	aHUS	1	(Dragon-Durey *et al.*, 2004)
R127H	2	aHUS	1	(Falcao *et al.*, 2008)
W134R	2	aHUS	U	(Bresin *et al.*, 2013)
P139S	2	aHUS	U	(*Factor H HUS Mutation Database*, 2016)
S159N	3	aHUS	2	Unpublished Newcastle cohort
A161S	3	aHUS[1]	2	(Fakhouri *et al.*, 2010b)
A161S	3	aHUS	2	Unpublished, Newcastle
A161S	3	aHUS	2	(Loirat and Fremeaux-Bacchi, 2011)
M162V	3	aHUS	2	(Fremeaux-Bacchi *et al.*, 2013)
R166L	3	aHUS	U	(Chaudhary *et al.*, 2014)
W198$	3	aHUS	2	(Szarvas *et al.*, 2016)
G218E	4	aHUS[1]	1	(Fakhouri *et al.*, 2010b)
R232X	4	aHUS	1	Unpublished, Newcastle
Y235S	4	aHUS	U	(Rodriguez de Cordoba *et al.*, 2014)
C247G	4	aHUS	U	(*Factor H HUS Mutation Database*, 2016)
P258L	4	aHUS	1	(Rodriguez de Cordoba *et al.*, 2014)

Table 63 Rare genetic variants in N-terminal domain of CFH in aHUS

Rare genetic variants in N-terminal domain of CFH in aHUS identified in literature and unpubilshed from Newcastle Complement Genetics cohort. Type = Type 1 or Type 2 mutation, U = unknown. [1]Pregnancy-associated.

8.10 Appendix 10 – Rare Genetic Variants in N-terminal Domain of *CFH* in AMD

Variant	SCR	Variant	SCR
p.L3V[1]	1	p.R166W	3
p.P26S	1	p.R166Q	3
p.T46A	1	p.E167Q	3
p.R53C[4]	1	p.A173G[1]	3
p.R53H	1	p.R175Q[1]	3
p.S58A	1	p.R175P	3
p.G69E[2]	1	p.C192F	3
p.D90G[3]	2	p.S193L[1]	3
p.T91S	2	p.W198X	3
p.R127H	2	p.V206M	3
p.C129Y	2	p.I216T[1]	4
p.D130N	2	p.G218X	4
c.428-2A>G[1] Splice acceptor	3	p.I221V	4
p.S159N	3	p.R232Q	4
p.A161S[1]	3	p.M239T	4

Table 64 Rare genetic variants in N-terminal domain of CFH in AMD and Drusen

Rare genetic variants in N-terminal domain of CFH in AMD identified in literature All cases reported in (Triebwasser et al., 2015) unless indicated. Two additional splice site changes were also reported in (Triebwasser et al., 2015) within the N-terminal domain of CFH. [1](Duvvari et al., 2015), [2](Raychaudhuri et al., 2011), [3](Yu et al., 2014), [4]reported in both Triebwasser et al and Yu et al. SCR = short consensus repeat.

Chapter 9 References

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