THE ROLE OF LOCAL COMPLEMENT FACTOR H PRODUCTION IN COMPLEMENT-MEDIATED RENAL DISEASE

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DECLARATION OF ORIGINALITY

I certify that the work in this thesis is my own and that all else is appropriately referenced.

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ABSTRACT

The aim of my research was to define the roles of local and hepatic-derived factor H, in particular the contribution of extra-hepatic factor H to plasma C3 regulation and spontaneous renal disease associated with factor H deficiency. Factor H is the major soluble regulator of the alternative pathway of complement activation, synthesised predominantly by the liver. To investigate the role of extra-hepatic factor H, I generated mice with hepatocyte-specific factor H deficiency (hepatocyte-*Cfh*$^{-/-}$) by intercrossing conditional factor H-deficient animals with mice expressing Cre recombinase under the control of the murine albumin promoter. Plasma factor H levels in hepatocyte-*Cfh*$^{-/-}$ mice were 19% of wild-type levels and were associated with a secondary reduction in plasma C3 levels. Higher plasma C3 levels than in *Cfh*$^{-/-}$ animals demonstrated that extra-hepatic factor H contributes to plasma C3 regulation, confirmed by tubulointerstitial C3 staining in hepatocyte-*Cfh*$^{-/-}$ mice. Extra-hepatic factor H prevented the GBM C3 deposition typical of complete factor H deficiency and was associated with spontaneous mesangial C3 deposition. To test the hypothesis that underlying dysregulation of the alternative pathway predisposes to exaggerated renal injury in the presence of additional complement activation, I assessed the response of hepatocyte-*Cfh*$^{-/-}$ mice to accelerated serum nephrotoxic nephritis. Injection of nephrotoxic serum led to the rapid onset of haemolytic uraemic syndrome. Subtotal deficiency of factor H in hepatocyte-*Cfh*$^{-/-}$ mice therefore provides a novel model of either C3 glomerulopathy or atypical haemolytic uraemic syndrome depending on the environmental milieu. This mouse model will enable the pathophysiological mechanisms involved to be further defined and new therapeutics investigated. I also examined the association between abnormalities in factor H-related protein 5 and C3 glomerulopathy. Minimal expression of normal factor H-related protein 5 within the kidney could not prevent CFHR5 nephropathy. Serum factor H-related protein 5 levels may be a marker of renal complement deposition.

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ABBREVIATIONS

AFU	arbitrary fluorescence unit
bp	base pair
BSA	bovine serum albumin
C3GN	C3 glomerulonephritis
C4BP	C4b-binding protein
cDNA	complementary deoxyribonucleic acid
CFA	complete Freund's adjuvant
Cfh$^{-/-}$	homozygous factor H-deficient mice
Cfh$^{+/-}$	heterozygous factor H-deficient mice
Cfi$^{-/-}$	homozygous factor I-deficient mice
DAF	decay-accelerating factor
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FHL	factor H-like
FHR	factor H-related
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GBM	glomerular basement membrane
GPI	glycosylphosphatidylinositol
HRP	horseradish peroxidase

HUVEC	human umbilical vein endothelial cell
LHR	long homologous repeat
MAC	membrane attack complex
MASP	mannan-binding lectin-associated serine protease
MBL	mannan-binding lectin
MCP	membrane cofactor protein
$MgCl_2$	magnesium chloride
MPGN	membranoproliferative glomerulonephritis
NTN	nephrotoxic nephritis
NTS	nephrotoxic serum
PAGE	polyacrylamide gel electrophoresis
PAS	Periodic Acid-Schiff
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
qPCR	quantitative PCR
RCA	regulators of complement activation
RNA	ribonucleic acid
RNase	ribonuclease
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
TAE	tris-acetate buffer
TMB	tetramethylbenzidine

CONTENTS

1. CHAPTER ONE: INTRODUCTION 17

1.1. THE COMPLEMENT SYSTEM 18

1.2. COMPLEMENT ACTIVATION 18

1.2.1. Classical Pathway 18

1.2.2. Lectin Pathway 19

1.2.3. Alternative pathway 20

1.2.4. Terminal pathway 20

1.2.5. Non-canonical activation 21

1.3. COMPLEMENT FUNCTION 23

1.4. COMPLEMENT REGULATION 25

1.4.1. Membrane-bound regulatory proteins 26

1.4.2. Fluid-phase regulatory proteins 29

1.5. The Complement factor H (FH) protein family 32

1.5.1. Overview 32

1.5.2. Complement factor H (FH) 35

1.5.3. Complement FH-like protein 1 (FHL-1) 37

1.5.4. Complement FH-related proteins 38

1.5.5. Murine complement factor H (mFH) and mFH-related (mFHR) proteins 43

1.5.6. The synthesis of FH and the FHR proteins 44

1.5.7. The function of extra-hepatic FH and the FHR proteins 47

1.6. Complement and renal disease 48
1.6.1. Complement activation in glomerular disease 49
1.6.2. Tubulointerstitial (TI) disease 52
1.6.3. Renal disorders associated with complement dysregulation 54
1.6.4. C3 glomerulopathy 55
1.6.5. Atypical haemolytic uraemic syndrome (aHUS) 62
1.7. Generation of a conditional FH-deficient mouse 66
1.8. Aims of my project 69
2. CHAPTER TWO: MATERIALS AND METHODS 70
2.1. Molecular biology techniques 71
2.1.1. Ribonucleic acid (RNA) extraction 71
2.1.2. Deoxyribonucleic acid (DNA) extraction from mouse tissue 71
2.1.3. Complementary DNA (cDNA) synthesis 71
2.1.4. Polymerase chain reaction (PCR) and quantitative PCR (qPCR) 72
2.1.5. Agarose gel electrophoresis 74
2.1.6. DNA purification from PCR mixtures and agarose gels and sequencing 74
2.2. Mice 75
2.3. Mouse phenotypic analyses 75
2.3.1. Preparation of mouse plasma 75
2.3.2. Measurement of mouse plasma FH levels 76
2.3.3. Measurement of mouse plasma C3 levels 76

2.3.4. Western blotting of mouse FH and C3 77

2.3.5. Plasma urea levels 79

2.3.6. Albuminuria 79

2.3.7. Urinalysis 80

2.3.8. Haematocrit 80

2.3.9. Red cell fragments 80

2.4. Histological studies 81

2.4.1. Light microscopy 81

2.4.2. Immunostaining for mouse C3, mouse IgG and sheep IgG 81

2.5. Serum nephrotoxic nephritis 84

2.6. Analysis of FHR5 84

2.6.1. Quantification of FHR5 by ELISA 84

2.6.2. Western blotting of FHR5 85

2.7. List of antibodies 86

2.8. Statistical analysis 87

3. CHAPTER THREE: GENERATION OF HEPATOCYTE-SPECIFIC FACTOR H-DEFICIENT MICE 88

3.1. Introduction 89

3.2. Inter-crossing conditional FH-deficient animals with mice expressing *Cre* recombinase under the control of the mouse albumin promoter 91

3.3. Spontaneous complement profile in hepatocyte-specific FH-deficient mice 94

3.3.1. Plasma FH 94

3.3.2. Plasma C3 97

3.4. Spontaneous renal phenotype in hepatocyte-specific FH-deficient mice 99

3.4.1. Renal function and haematological parameters 100

3.4.2. Renal histology – Light microscopy 103

3.4.3. Renal histology – Immunofluorescence studies for C3 and IgG 105

3.4.4. Renal histology – Immunofluorescence studies for C3d and mouse FHR proteins 110

3.4.5. Renal histology - Electron microscopy 114

3.5. Discussion 116

3.6. Conclusions 120

4. CHAPTER FOUR: RESPONSE OF HEPATOCYTE-SPECIFIC FACTOR H-DEFICIENT MICE TO EXPERIMENTAL NEPHRITIS 121

4.1. Introduction 122

4.2. Overview of NTN experiments 124

4.3. Survival 125

4.4. Renal function: urinalysis 126

4.5. Renal function: serum urea 129

4.6. Haematological parameters 131

4.7. Renal histology 134

4.7.1. Glomerular light microscopy 134

4.7.2. Glomerular immunofluorescence for mouse C3 137

4.7.3. Glomerular immunofluorescence for mouse IgG 139

4.7.4. Glomerular immunofluorescence for sheep IgG 141

4.8. Immune response in hepatocyte-specific FH-deficient mice 144

4.9. Discussion 146

4.10. Conclusions 149

5. CHAPTER FIVE: THE ROLE OF FACTOR H-RELATED PROTEINS IN C3 GLOMERULOPATHY 150

5.1. Introduction 151

5.2. Recurrence of familial C3 glomerulonephritis in a renal transplant 155

5.2.1. Outline of the case 156

5.2.2. Diagnosis of CFHR5 nephropathy 158

5.2.3. Renal transplantation 160

5.2.4. Summary 163

5.3. Persistent glomerulonephritis following streptococcal infection in an individual with heterozygous FHR5 deficiency 164

5.3.1. Outline of the case 164

5.3.2. Assessment of serum factor triggering C3 activation 168

5.3.3. Complement genetic investigations 169

5.3.4. Assessment of FHR5 172

5.3.5. Summary 176

5.4. Synthesis of FH and FHR proteins by the kidney 177

5.4.1. Absolute expression of *CFH* and *CFHR* genes in liver and kidney tissue 177

5.4.2. Relative expression of *CFH* and *CFHR5* genes in liver and kidney tissue 179

5.5. Discussion 180

5.6. Conclusions 184

6. CHAPTER SIX: DISCUSSION 185

7. CHAPTER SEVEN: REFERENCES 196

8. CHAPTER EIGHT: APPENDICES 214

8.1. Appendix 1: Demonstration of hepatocyte-specific Cre-mediated recombination 215

8.2. Appendix 2: Summary of NTN experiments 1-3 (see Chapter 4, page 123) 216

Summary of NTN experiments 1-3 (continued) 217

9. CHAPTER NINE: PUBLICATIONS 218

9.1. Publications arising from this thesis 219

LIST OF FIGURES

Figure 1.1 **Complement activation and regulation.**.....22

Figure 1.2 **The human FH protein family.**.....34

Figure 1.3 **Schematic depiction of the human CFH-CFHR gene locus**. 42

Figure 1.4 **Mechanisms of C3 dysregulation associated with DDD.**. 57

Figure 1.5 **Generation of conditional FH-deficient mouse.** 67

Figure 1.6 **Deletion of exons 2 and 3 of the mouse *cfh* gene.**.....68

Figure 3.1 **Genotyping PCR in hepatocyte-*Cfh*$^{-/-}$ mice**.....92

Figure 3.2 **PCR on cDNA generated from RNA from livers of hepatocyte-*Cfh*$^{-/-}$ (lanes 2-4) and Alb-Cre mice (lanes 5-7)**.. 953

Figure 3.3 **Plasma FH levels in 12 week old Alb-Cre (n=6), *Cfh*$^{loxP/loxP}$ (n=8) and hepatocyte-*Cfh*$^{-/-}$ (n=7) mice**……...95

Figure 3.4 **Plasma FH western blot.** 96

Figure 3.5 **Plasma C3 levels in 12 week old Alb-Cre (n=6), *Cfh*$^{loxP/loxP}$ (n=8), hepatocyte-*Cfh*$^{-/-}$ (n=7) and *Cfh*$^{-/-}$ (n=13) mice.**.....98

Figure 3.6 **Plasma urea in 9 month old Alb-Cre, *Cfh*$^{loxP/loxP}$, hepatocyte-*Cfh*$^{-/-}$ and *Cfh*$^{-/-}$ mice.**.....101

Figure 3.7 **Haematocrit (A) and red cell fragments (B) in Alb-Cre, *Cfh*$^{loxP/loxP}$, hepatocyte-*Cfh*$^{-/-}$ and *Cfh*$^{-/-}$ mice.**.....102

Figure 3.8 **(A) Glomerular cellularity in 9 month old Alb-Cre, *Cfh*$^{loxP/loxP}$, hepatocyte-*Cfh*$^{-/-}$ and *Cfh*$^{-/-}$ mice.**.....104

Figure 3.9 **Immunostaining for C3 and IgG in 9 month old Alb-Cre, *Cfh*$^{loxP/loxP}$, hepatocyte-*Cfh*$^{-/-}$ and *Cfh*$^{-/-}$ mice.** 107

Figure 3.10 **Glomerular capillary wall (A) and mesangial (B) C3 scores in hepatocyte-*Cfh*$^{-/-}$ and *Cfh*$^{-/-}$ mice.** 108

Figure 3.11 **Quantitative immunofluorescence staining for IgG in hepatocyte-*Cfh*$^{-/-}$ and *Cfh*$^{-/-}$ mice.** 109

Figure 3.12 **Dual staining using anti-mouse C3 (green) and anti-mouse C3d (red) antibodies in *Cfh*$^{-/-}$ and hepatocyte-*Cfh*$^{-/-}$ mice.** 112

Figure 3.13 **Dual staining using anti-mouse C3 (green) and anti-human FH (red) antibodies in *Cfh*$^{-/-}$ and hepatocyte-*Cfh*$^{-/-}$ mice.** 113

Figure 3.14 **Electron microscopy images.**. 115

Figure 4.1 **Haematuria in wild-type, *Cfh*$^{loxP/loxP}$ and hepatocyte-*Cfh*$^{-/-}$ mice at day 1 (A) and 3 (B) following NTS injection.** 127

Figure 4.2 **Proteinuria in wild-type, *Cfh*$^{loxP/loxP}$ and hepatocyte-*Cfh*$^{-/-}$ mice at day 1 (A) and 3 (B) following NTS injection.** 128

Figure 4.3 **Serum urea in wild-type, *Cfh*$^{loxP/loxP}$ and hepatocyte-*Cfh*$^{-/-}$ mice at day 3 following NTS injection.** 130

Figure 4.4 **Haematocrit (A) and red cell fragments (B) in wild-type, *Cfh*$^{loxP/loxP}$ and hepatocyte-*Cfh*$^{-/-}$ mice on day 3 following NTS injection**. 132

Figure 4.5 **Peripheral blood film examination in wild-type, *Cfh*$^{loxP/loxP}$ and hepatocyte-*Cfh*$^{-/-}$ mice 3 days following NTS injection.** 133

Figure 4.6 **Glomerular thrombosis in wild-type, *Cfh*$^{loxP/loxP}$ and hepatocyte-*Cfh*$^{-/-}$ mice at day 3 following NTS injection.** 135

Figure 4.7 **Representative images of renal sections in wild-type, *Cfh*$^{loxP/loxP}$ and hepatocyte-*Cfh*$^{-/-}$ mice at day 3 following NTS injection.** 136

Figure 4.8 **Glomerular C3 immunofluorescence studies in wild-type, *Cfh*$^{loxP/loxP}$ and hepatocyte-*Cfh*$^{-/-}$ mice at day 3 following NTS injection**. 138

Figure 4.9 **Glomerular mouse IgG immunofluorescence studies in wild-type, *Cfh*$^{loxP/loxP}$ and hepatocyte-*Cfh*$^{-/-}$ mice at day 3 following NTS injection.**..... 140

Figure 4.10 **Glomerular sheep IgG immunofluorescence studies in wild-type, *Cfh*$^{loxP/loxP}$ and hepatocyte-*Cfh*$^{-/-}$ mice at day 3 following NTS injection.**..... 142

Figure 4.11 **Representative immunofluorescence staining images for C3, mouse IgG and sheep IgG in wild-type, *Cfh*$^{loxP/loxP}$ and hepatocyte-*Cfh*$^{-/-}$ mice at day 3 following NTS injection.**. 143

Figure 4.12 **Immune response to sheep IgG in complete Freund's adjuvant in the absence of NTN in hepatocyte-*Cfh*$^{-/-}$, *Cfh*$^{loxP/loxP}$ and wild-type mice** 145

Figure 5.1 **Schematic depiction of abnormal FHR proteins associated with C3 glomerulopathy.** 154

Figure 5.2 **Native renal biopsy.**. 157

Figure 5.3 **Western blot of serum for FHR5 from the affected individual.** 159

Figure 5.4 **Renal transplant biopsy.** 161

Figure 5.5 **Renal transplant biopsy staining for C5b-9.**..... 162

Figure 5.6 **Kidney biopsies performed at 9 and 20 months post presentation**. 166

Figure 5.7 **Serum complement C3 and creatinine levels versus time**..... 167

Figure 5.8 **Sequence chromatogram from the index case**. 170

Figure 5.9 **Family pedigree**..... 171

Figure 5.10 **Western blot for FHR5 using sera from pedigree members**.. 173

Figure 5.11 **Serum CFHR5 levels in individuals with biopsy-proven C3 glomerulonephritis and no *CFHR5* mutation**. 175

Figure 5.12 **Expression of *CFH* and *CFHR1-5* in human liver and kidney tissue.** 178

LIST OF TABLES

Table 2.1.**List of primers** 73

Table 2.2 **List of antibodies**..... 86

Table 5.1 **Complement profile in the index case and other pedigree members where available**..... 174

Table 5.2 **Relative expression of *CFH* and *CFHR5* in liver and kidney tissue** . 179

1. CHAPTER ONE: INTRODUCTION

1.1. THE COMPLEMENT SYSTEM

The complement system has long been recognised as a mediator in the development of renal disease. The identification of altered concentrations of complement components and complement-activating factors in serum along with the presence of complement deposits within glomeruli, have all led to the association of complement with renal injury. Whilst individual diseases may be associated with complement activation through particular pathways, downstream injury mediated by C5 activation may be a common mechanism in disease pathogenesis. Complement plays a role not only in acute glomerular injury, but also contributes to fibrosis, scarring and progressive kidney disease as a result of proteinuria and the subsequent generation of complement activation products in the tubular lumen.

Complement was identified as a heat-labile component that augmented the anti-bactericidal activity of plasma. Its diverse roles include host defence, clearance of apoptotic cells and immune complexes and modulation of the adaptive immune response. It consists of a large group of proteins, including pro-enzymes (zymogens), and activation pathways that are triggered in distinct ways: the classical (CP), lectin (LP), and alternative (AP) pathways (figure 1.1). These three pathways converge on complement C3, whose activation results in the production of C3 effector functions and activation of the terminal (TP) pathway. Effector functions of complement include opsonisation, phagocyte recruitment and activation and cell surface damage through the generation of a pore-forming complex termed the membrane attack complex (MAC). Complement activation is controlled by proteins (complement regulators) that minimise damage to host tissues. I will now discuss in turn complement activation, function and regulation.

1.2. COMPLEMENT ACTIVATION

1.2.1. Classical Pathway

The major trigger of the CP is the interaction of complement C1q with antibody complexed with antigen (immune complex). Complement activation resulting from recognition of apoptotic cell structures by C1q has also implicated the CP in the clearance of apoptotic cells and cellular debris in addition to immune complexes

(Botto, Dell'Agnola et al. 1998). C1q consists of six globular heads each assembled from three different polypeptide chains (A, B and C) and linked by a collagen-like tail (Reid and Porter 1976). These surround two molecules of each of the zymogens C1r and C1s which together with C1q form the C1 complex (Lepow, Naff et al. 1963). A conformational change occurs within the $(C1r:C1s)_2$ complex when C1q binds to an immune complex. This results in enzymatic activation within C1r (autocatalysis) and subsequent cleavage of C1s by C1r (transactivation) to produce an active serine protease. C1 then cleaves C4 into C4a and C4b, exposing a thioester bond within C4b, which enables C4b to attach covalently to surfaces. C2 complexed with C4b (C4bC2) is cleaved by C1s releasing a small fragment (C2b) whilst the larger fragment (C2a) remains complexed with C4b and functions as an active protease (C4b2a). C4b2a is an enzyme complex termed a C3 convertase, since it cleaves C3 into C3b (an opsonin) and C3a (an anaphylatoxin).

1.2.2. Lectin Pathway

Mannan-binding lectin (MBL) and ficolins (ficolin-1, -2 and -3) activate the LP following interaction with their ligands, which include mannose and acetylated groups. The LP is complex and incompletely understood. MBL and ficolins are present in complexes containing serine proteases termed MBL-associated serine proteases 1, 2 and 3 (MASP-1, -2, -3) and non-enzymatic proteins termed MAp44 and MAp19. The *MASP-1* gene encodes MASP-1, MASP-3 and MAp44 whilst the *MASP-2* gene encodes MASP-2 and MAp19. Currently, it is thought that MBL/ficolins complexed with ligand trigger complement activation through MASP activation. These proteases have functional similarity to C1r and C1s. MASP-1 strongly activates MASP-2 under physiological conditions. MASP-2 can cleave both C4 and C2 whilst MASP-1 can cleave C2. The net result is the formation of a C3 convertase, C4b2a, identical in composition to that generated by classical pathway activation. Complete MASP-1 deficiency is associated with absence of the LP indicating its critical role in the activation sequence (Degn, Jensen et al. 2012). MBL can bind to aggregated IgA (Roos, Bouwman et al. 2001) which may be relevant to complement activation in IgA nephropathy.

1.2.3. Alternative pathway

The AP is in a continuous state of low-level activation due to the spontaneous hydrolysis of the thioester bond within C3 that results in a C3 molecule (C3[H_20]) with C3b-like activity in that it can bind the protease factor B (FB) to generate a pro-convertase, C3[H_20]B (Schreiber, Pangburn et al. 1978). When bound within the pro-convertase, FB is cleaved by factor D (FD) releasing the FB fragment, Ba, and generating the AP C3 convertase: C3[H_20]Bb. This C3 convertase, like C4b2a, cleaves C3 to generate C3b and C3a.

C3b generated through the three activation pathways can trigger further C3b generation through a feed-back loop that utilises FB and FD (C3b amplification loop, figure 1.1). C3b interacts with FB generating pro-convertase, C3bB. Cleavage of bound FB by FD results in formation of the C3 convertase, C3bBb.

Spontaneous activation of C3 through the AP is normally inefficient due to the presence of complement regulators in plasma and on cell surfaces. This prevents AP activation from causing either injury to host surfaces or C3 depletion. Complement regulators are discussed below.

1.2.4. Terminal pathway

TP activation is triggered by the formation of enzyme complexes that cleave complement C5. These complexes are termed C5 convertases and form when C3 convertases bind an additional molecule of C3b: C4b2aC3b and C3bBbC3b. Cleavage of C5 results in the formation of the anaphylatoxin C5a and C5b. C5b initiates the formation of the MAC. C5b sequentially binds C6 and C7 to form a hydrophobic complex (C5b67) that inserts into lipid bilayers. Subsequent interaction with C8 allows binding and polymerisation of C9 to generate the pore-forming MAC. The MAC has a hydrophobic outer and hydrophilic inner surface, which allows the free passage of water and solutes across the cell membrane resulting in loss of membrane integrity and cell damage. MAC formation is particularly important for host defence against meningococcal infection, which is frequently seen in TP deficiency

states and may develop during therapeutic inhibition of C5 if preventative measures are not adopted (Lambris, Ricklin et al. 2008).

1.2.5. Non-canonical activation

In addition to the activation pathways depicted in figure 1.1, other activation pathways have been reported but their *in vivo* significance is unclear. Examples include antibody-mediated complement activation that proceeds in the absence of C2 or C4 (Deguchi, Gillin et al. 1987), direct activation of C3 by MASP-1 (Matsushita and Fujita 1995) and C3-independent activation of C5 by thrombin (Huber-Lang, Sarma et al. 2006). C3 and C5 can be cleaved by many enzymes including thrombin and plasmin (Amara, Flierl et al. 2010) and leucocyte elastase (Lachmann, Pangburn et al. 1982). The generation of activated C3 and C5 by enzymes derived from inflammatory cells is very likely to augment canonical complement activation at sites of tissue injury.

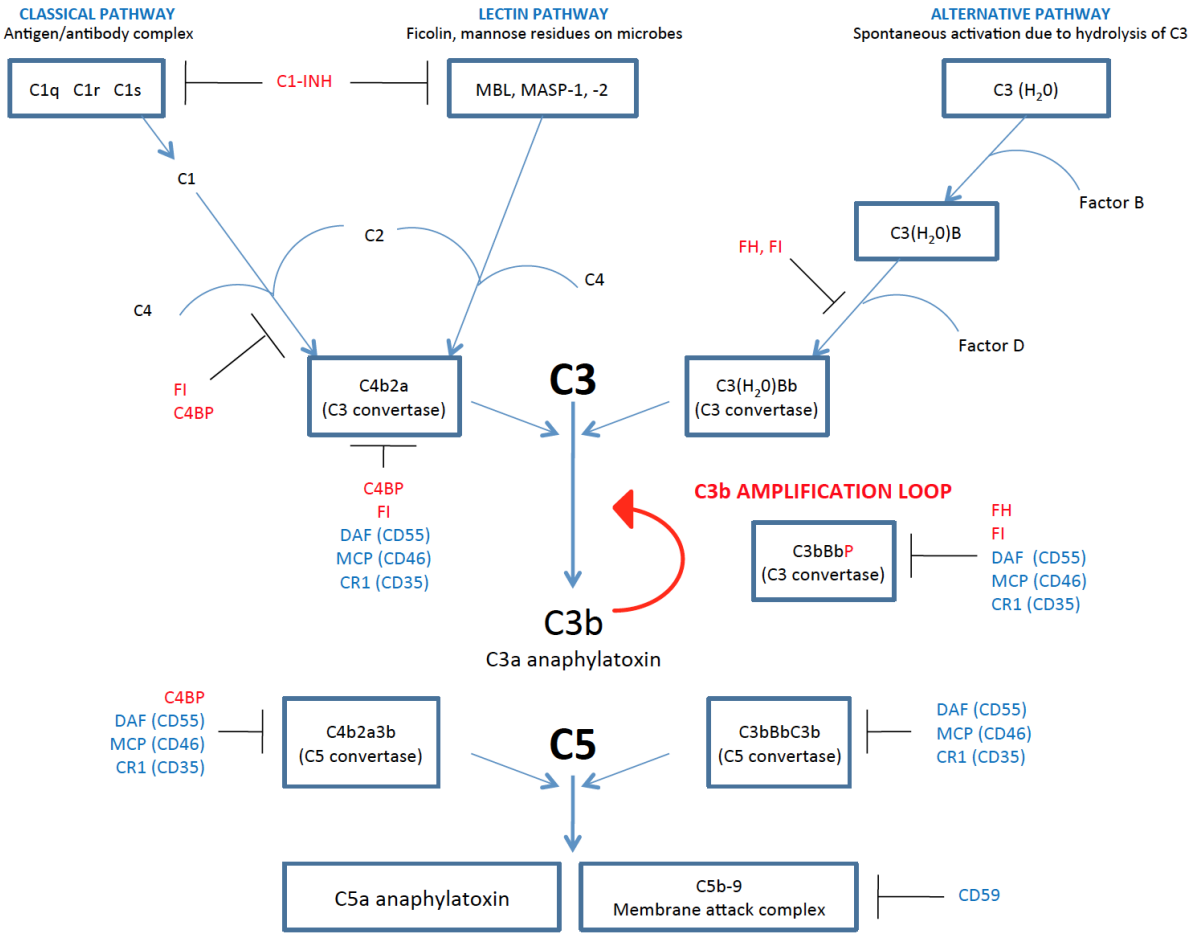


Figure 1.1 **Complement activation and regulation.** Complement activation generates C3 convertases which comprise C4b2a (classical and lectin pathway) and C3bBb (alternative pathway) and C5 convertases which comprise: C4b2aC3b (classical and lectin pathway) and C3bBbC3b (alternative pathway). Activation of the terminal pathway triggers the assembly of the membrane attack complex (MAC). The MAC is formed by the sequential addition to C5b of C6, C7, C8 and multiple C9 molecules. Complement regulatory proteins, in plasma (red) and on cell membranes (blue) include C4b-binding protein (C4BP) for the classical pathway; factor H (FH) for the alternative pathway and CD59 for the terminal pathway. Properdin (P), a positive regulator, stabilises the C3 convertase. MBL = mannose-binding lectin; MASP = MBL-associated serine protease; C1-INH = C1 inhibitor; DAF = decay-accelerating factor (CD55); MCP = membrane cofactor protein (CD46); FI = complement factor I; CR1 = complement receptor 1 (CD35).

1.3. COMPLEMENT FUNCTION

The established functions of complement include host defence against pathogens, the physiological removal of immune complexes and apoptotic cells (termed 'waste disposal') and a role as natural adjuvant. Effector mechanisms include opsonins (C3b, C4b), anaphylatoxins (C3a, C5a), MAC and interactions with complement receptors. More recently it has become evident that complement may contribute to tissue development and repair. For example, C3a and C5a were found to be necessary for liver cell survival during regeneration after liver resection (Markiewski, DeAngelis et al. 2009).

Complement activation on pathogens results in opsonisation of the pathogen with C3b, which facilitates phagocytosis, the production of the anaphylatoxins C3a and C5a, which facilitate inflammatory cell recruitment and changes in vascular permeability and MAC-mediated membrane damage. Acting through their respective G-protein-coupled receptors, C3a and C5a promote phagocytosis by attracting neutrophils, monocytes and macrophages to sites of complement activation, thereby facilitating interaction of opsonins with complement receptors. The G-protein-independent anaphylatoxin receptor, C5L2, may also act to modulate the inflammatory response. The interaction of C3b (or C4b) with complement receptor 1 (CR1) contributes to neutrophil-mediated phagocytosis. CR1 also acts as cofactor for the factor I (FI)-mediated cleavage of iC3b to C3dg. iC3b interacts with CR3 (CD11b/CD18) and CR4 (CD11c/CD18) on neutrophils and monocyte/macrophages and enhances phagocytosis.

The complement receptor of the immunoglobulin family (CRIg) found on tissue-resident macrophages also promotes phagocytosis by binding target-bound C3b and iC3b (Helmy, Katschke et al. 2006), and is also believed to regulate the C3b-containing C3 and C5 convertases. Interaction of complement with the coagulation system also acts to prevent the spread of infection by promoting local coagulation and inhibiting fibrinolysis (Markiewski, Nilsson et al. 2007).

Complement also forms a link with adaptive immunity (Dunkelberger and Song 2010). Both iC3b and C3dg acting as ligands for CR2 (CD21) promote B cell activation and differentiation (Carroll 2008). Anaphylatoxins exert regulatory effects on B cells (Fischer and Hugli 1997, Ottonello, Corcione et al. 1999). The threshold for Fcγ receptor activation may also be lowered by C5a receptor signalling as a result of upregulation of the activating Fcγ receptors FcγR I and III and downregulation of the inhibitory FcγRIIB, whilst C5 synthesis itself may also be promoted by FcγR activation (Kumar, Ali et al. 2006). The effects of complement deficiency also suggest that complement is involved in the T cell response (Kopf, Abel et al. 2002), and may influence the polarisation of T cells to Th1 or Th17 cells (reviewed in (Ricklin, Hajishengallis et al. 2010)).

The complement system also plays a critical role in removing immune complexes and apoptotic cells, not only contributing to the resolution of inflammation but also promoting tissue repair (Botto and Walport 2002, Flierman and Daha 2007, Markiewski, DeAngelis et al. 2009). The failure to successfully remove cellular debris has been implicated in the development of systemic lupus erythematosus (reviewed in (Manderson, Botto et al. 2004)). C3b- and/or C4b-opsonised immune complexes interact with CR1 on red cells, which enable immune complexes to be transferred to the phagocytic cells within the liver and spleen. Cells undergoing apoptosis are able to bind complement and become opsonised as a result of changes to cell surface molecules. The loss of CD46 and CD59 permits complement activation, deposition of C3b and C4b and removal by phagocytosis (Cole, Hughes et al. 2006, Flierman and Daha 2007). The prevention of excessive complement activation and inflammation by remaining surface regulators and those in plasma together with changes such as the down-regulation of interleukin 12, ensure a more measured response than that elicited following foreign invasion (Kim, Elkon et al. 2004). The recruitment of C-reactive protein (CRP) to the apoptotic cell surface facilitates the binding of C1q and CP activation, whilst binding of the AP regulator, complement factor H (FH), inhibits AP amplification and downstream inflammation and lysis (Gershov, Kim et al. 2000). The importance of the CP is demonstrated by the reduction in apoptotic cell uptake

by macrophages from individuals deficient in components C1q, C4 and C2 (Botto, Dell'Agnola et al. 1998, Gullstrand, Martensson et al. 2009).

1.4. COMPLEMENT REGULATION

Complement activation is regulated by a series of soluble and membrane-bound proteins termed complement regulators. Control of targeted complement attack is required to prevent consumption of components and unwanted injury to host tissues. The need for such regulation is clear from the effects of excessive complement activation seen in deficiency states. Regulation occurs at multiple steps within the activation pathways and both within plasma and on host surfaces. Regulation mechanisms include the prevention of convertase formation, promotion of convertase decay and proteolytic degradation of activated components. Six of the regulators (FH, C4b-binding protein [C4BP], membrane cofactor protein [MCP], decay-accelerating factor [DAF], CR1 and CR2) are encoded by genes within the regulators of complement activation (RCA) gene cluster on chromosome 1. Each of these proteins consists of a number of repeating cysteine-rich modules of approximately 60 amino acids, termed short consensus repeat (SCR) domains. I will first summarise the key regulatory proteins and then discuss the major proteins in two groups: the membrane-bound and soluble complement regulators. FH, a major plasma complement regulator, is discussed in detail, as the biology of this protein was central to my thesis.

C1 inhibitor (C1-Inh) blocks the active site of C1r and C1s dissociating them from C1q and preventing CP activation. C4BP interacts with C4b to prevent the formation of the CP and LP C3 pro-convertase (C4bC2). C4BP also accelerates the spontaneous decay of the convertase (C4b2a) and will act as a cofactor for the FI-mediated cleavage of C4b. The AP and C3b amplification loop are regulated by FH, which will interact with C3b to prevent pro-convertase formation (C3bB) and accelerate convertase decay (C3bBb). It is also a soluble cofactor for the FI-mediated cleavage of C3b to iC3b. On host surfaces, DAF facilitates dissociation of

the convertases whilst MCP has cofactor activity for the FI-mediated cleavage of C3b and C4b. Membrane-bound CD59 prevents MAC assembly through its interaction with C8 and C9. The soluble C5b-7 complex may be prevented from inserting into membranes by interaction with vitronectin, clusterin and C8 in plasma. The anaphylatoxins C3a, C4a and C5a are inactivated by plasma carboxypeptidase-N through its cleavage of the C-terminal arginine residue exposed during their generation.

1.4.1. Membrane-bound regulatory proteins

1.4.1.1. Membrane cofactor protein (MCP, CD46, measles virus receptor)

MCP is a type 1 membrane glycoprotein expressed as four common isoforms (MCP-BC1, MCP-BC2, MCP-C1, and MCP-C2) derived from alternative splicing, and appearing as 'upper' (59-68kDa) and 'lower' (51-58kDa) bands on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). All isoforms share the same amino-terminal consisting of four contiguous SCR domains containing the binding sites for C3b and C4b. The alternatively spliced STP domain rich in serines, threonines and prolines follows this, and is O-glycosylated to generate the BC and C isoforms. Twelve amino acids with unknown function then precede the transmembrane domain and intracytoplasmic anchor. Two distinct cytoplasmic tails of 16 or 23 amino acids, Cyt-1 or Cyt-2, also result from alternative splicing. MCP is a cofactor for the FI-mediated cleavage of C3b and C4b, and as a membrane-bound regulator, only inactivates C3b and C4b deposited on the cell on which it is expressed.

1.4.1.2. Decay-accelerating factor (DAF, CD55)

DAF is a widely-distributed single-chain glycosylphosphatidylinositol (GPI)-anchored membrane glycoprotein. The four SCR domains making up the amino terminus are followed by a serine and threonine-rich region heavily modified by post-translational O-linked glycosylation. DAF accelerates the decay of the classical and alternative pathway C3 and C5 convertases by causing the rapid release of C2a and Bb from their binding sites on C4b and C3b respectively (Fujita, Inoue et al. 1987).

Interestingly the binding of DAF to the C3 convertase appears stronger than to C3b or C4b alone (Pangburn 1986). It is expressed on all haematopoietic cells and most other cell types including endothelial and epithelial cells. Deficiency of DAF on erythrocytes in paroxysmal nocturnal haemoglobinuria renders the affected cells susceptible to lysis by complement. This commonly results from a mutation in the phosphatidylinositol glycan A gene leading to defective GPI assembly and impairment of expression of GPI-linked proteins such as DAF.

1.4.1.3. CD59

CD59 is an 18-20-kDa GPI-anchored membrane single-chain glycoprotein encoded by a gene on chromosome 11 in humans (and chromosome 2 in mice), which inhibits MAC formation. Synthesised as a precursor including an N-terminal signal peptide, the carboxyl terminus has a hydrophobic sequence, which like DAF, is replaced post-translationally by a GPI anchor. It is widely distributed in humans including on all circulating cells, endothelial cells and epithelial cells but weakly expressed in the central nervous system. CD59 binds to C5b-7 complex in membranes preventing the interaction of C8 with the C5b-7 complex. With binding sites for both the α-chain of C8 and the b-domain of C9 (Ninomiya and Sims 1992), CD59 may also bind C9 in partially assembled MAC. Loss of CD59 from the erythrocyte cell membrane in paroxysmal nocturnal haemoglobinuria also contributes to increased susceptibility to complement-mediated lysis.

1.4.1.4. Complement receptor type 1 and type 2

CR1 (C3b/C4b receptor, CD35, immune adherence receptor) is a type 1 integral membrane glycoprotein of between 190-280kDa depending on variable N-linked glycosylation. Present on all peripheral blood cells (except natural killer cells and platelets) and also glomerular podocytes, CR1 contains a signal peptide and extracellular, transmembrane and cytoplasmic domains. The duplication or deletion of a large segment of genomic DNA has generated four major structural allotypes in humans, of which the most common is CR1*1 which has 30 extracellular SCR domains. The N-terminal 28 SCRs are arranged into larger long homologous repeats (LHRs) containing 7 contiguous SCRs each, with ligand binding sites residing in each of the first three LHRs. CR1*1 possesses two binding sites for C3b and three

for C4b demonstrating that each CR1 molecule can interact with complexes containing multiple C3b and C4b molecules. Aside from its role in immune complex processing, CR1 functions as a complement regulator. It has both cofactor and decay-accelerating activities. It is a cofactor for the FI-mediated cleavage of C3b to iC3b, iC3b to C3dg and of C4b to C4d. It accelerates the spontaneous decay of the CP/LP and AP C3 and C5 convertases.

CR2 (CD21) plays a major role in the immune response but has also been shown to have weak FI cofactor activity although the physiological significance of this is unclear.

1.4.1.5. The rodent-specific complement regulator: Crry

Whilst rodents possess MCP and DAF homologues, they express an additional complement regulator, Crry (Holers, Kinoshita et al. 1992). The presence of this rodent-specific regulator is important in interpreting murine phenotypes of complement dysregulation. It is a 65-kDa transmembrane glycoprotein expressed in most tissues, although the level of expression varies amongst different tissues along with tissue-specific isoforms. Crry regulates C3 activation in the same way as human MCP and DAF, acting as a cofactor for the FI-mediated inactivation of mouse C3b and C4b and possessing decay-accelerating activity for the CP and AP C3 convertases (Kim, Kinoshita et al. 1995). Crry deficiency is embryonically lethal as a result of the failure to regulate maternal complement activation at the feto-placental interface. Viable Crry-deficient animals can be generated if the mother is rendered C3 deficient (Xu, Mao et al. 2000). Crry is critical in the mouse as it is the only ubiquitously-expressed membrane-bound regulatory molecule. Crry-expressing cells *in vitro* are protected from C3b deposition whilst antibody-mediated blockade of rat Crry led to increased C3b deposition on glomerular endothelial and mesangial cells (Molina, Wong et al. 1992, Quigg, Morgan et al. 1995). Antibody-induced blockade of Crry *in vivo* in rat kidney resulted in C3 activation in the vicinity of glomeruli, tubules

and perivascular capillaries associated with inflammation and tissue injury (Nomura, Nishikawa et al. 1995).

1.4.2. Fluid-phase regulatory proteins

1.4.2.1. C1 Inhibitor

Control of the cleavage of C4 to C4b by C1s following activation of the classical pathway is mediated by the plasma serine protease inhibitor, C1-Inh. Heavily glycosylated, C1-Inh is a 105-kDa single-chain member of the serpin family of serine protease inhibitors, present in plasma at a concentration of 120-200μg/ml. By covalently binding to the catalytic site on the β-chains of activated C1r and C1s, C1-Inh causes their disassembly from C1q, which remains bound to antibody. Whilst preventing excessive complement activation on a target and activation of C1 in the fluid-phase, C1-Inh does not inhibit appropriate complement activation of C1 by IgM or IgG on a microbial surface.

1.4.2.2. C4b-binding protein (C4BP)

C4BP is an oligomeric plasma protein that exists in three isoforms in humans. The predominant isoform (*α7β1*) consists of seven identical 75-kDa α-chains (C4BPα) and one 45-kDa β-chain (C4BPβ), linked by disulphide bonds in their carboxyl terminal regions and which may be more compact in the soluble form. Minor populations exist of the other isoforms, α7/β0 and α6/β1. Mouse C4BP has no β chain and no inter-chain disulphide bonds.

Both α- and β-chains contain SCR domains similar to other complement regulatory proteins, and whilst SCRs 1-3 of the α-chain are critical for C4b binding (Ogata, Mathias et al. 1993), the β-chain is responsible for binding protein S. The *C4BPA* and *C4BPB* genes are closely linked in tandem within the RCA cluster on chromosome 1 with *C4BPA* lying downstream of *C4BPB*. Primarily synthesised by the liver, C4BP is present in plasma at a concentration of 150-300μg/ml.

C4BP has both decay-accelerating and cofactor activity. Accelerating the decay of the classical pathway C3 and C5 convertases, multivalent binding of C4b can prevent C2 binding and act as a cofactor for the FI-mediated cleavage of C4b. The classical pathway C3 convertase is inherently unstable without the properdin-stabilisation of the AP C3 convertase, and once dissociated, C2a cannot rebind. In contrast to the FI-mediated cleavage of C3b at two sites generating iC3b and C3f, iC4b is generated transiently from cleavage at a single site before being cleaved to C4c and C4d. The second cleavage of iC4b is required for its inactivation due to its ability to form an active C3 convertase with C2 (Yamazaki, Ichihara et al. 1986). C4BP may also bind C4i and weakly to C4c, but not C4d. It also shows weak affinity and cofactor activity for C3b.

1.4.2.3. Factor I

Following activation of native C3 or C4 by hydrolysis or proteolysis, FI mediates the subsequent proteolytic degradation of the products. C3i (or C4i) generated by the spontaneous or induced hydrolysis of the parent molecule and possessing an inactivated thioester group, is degraded in the same way as C3b (or C4b) but still retains the C3a (or C4a) domain. FI is constitutively active but in humans requires a cofactor for all cleavage reactions it mediates. FH and C4BP provide cofactor activity in plasma whilst MCP and CR1 act as cofactors on host surfaces. The degradation of surface-bound C3 and C4 derivatives by FI also determines their specificity for cellular complement receptors such as CR1, CR2 and CR3. The human FI gene lies on chromosome 4 and FI is synthesized primarily by the liver but also by monocytes, fibroblasts and endothelial cells amongst others (Dauchel, Julen et al. 1990). FI is found in plasma at a concentration of 35 μg/ml but as an acute-phase protein is upregulated by interferon-γ (IFN-γ) in hepatocytes, fibroblasts and endothelial cells (Dauchel, Julen et al. 1990, Vyse, Morley et al. 1996). Derived from a single-chain precursor, FI consists of two chains linked by a disulphide bond. The 50-kDa heavy chain originates from the amino-terminal of the precursor and includes a number of structural modules, whilst the serine protease domain resides within the 38-kDa light chain and is very similar to β-trypsin. By cleaving the α'-chains of C3b and C4b in the presence of appropriate cofactors (FH, MCP or CR1 for C3b, and C4BP, MCP or CR1 for C4b), FI prevents the formation of both the C3 and C5 convertases. FI-

mediated cleavage of C3b at two positions in the α'-chain generates iC3b and C3f, a 17-amino acid peptide. Further degradation of iC3b with CR1 as a cofactor, leads to the release of C3c and the production of C3dg, which remains bound to the target. Possessing the surface-binding site, C3dg is a ligand for CR2. Cleavage of C4b meanwhile on either side of the thioester releases C4c and yields C4d that remains attached to the surface.

1.4.2.4. Vitronectin and Clusterin

Vitronectin is a 75-80-kDa glycoprotein encoded by chromosome 17. Derived mainly from the liver and present in human plasma at 250-450μg/ml, it is also synthesized by platelets, megakaryocytes, macrophages and human umbilical vein endothelial cells (HUVEC), and is found in urine. The mature protein is generated from a single-chain polypeptide but allelic variation results in processing to either a single-chain or two-chain form. A heparin-binding domain at the carboxyl terminus facilitates vitronectin binding to sulphated polysaccharides contributing to its MAC regulatory activity. By binding to the metastable binding site of nascent C5b-7 complex, vitronectin prevents its insertion into the cell membrane (Podack, Kolb et al. 1977). Vitronectin contributes to the protection of 'self' cells from 'bystander' MAC attack since nascent MACs are not restricted to the surface on which complement is activated and can insert into any cell membrane. Any vitronectin-C5b-7 complexes that subsequently bind C8 and C9 are lytically inactive (Bhakdi and Tranum-Jensen 1983).

Clusterin (previously termed complement lysis inhibitor, apolipoprotein J) is an 80-kDa heterodimeric glycoprotein composed of two non-identical subunits (α and β) of approximately equal size, and is derived from the *CLI* gene on human chromosome 8. Widely distributed in a variety of human tissues and cells (except T lymphocytes), clusterin is present in plasma associated with lipoproteins at a concentration of 100-300 μg/ml (Hogasen, Mollnes et al. 1993) and is also found in urine, cerebrospinal fluid and semen. Similar to vitronectin, clusterin incorporates into nascent C5b-7 preventing its insertion into the cell membrane and producing complexes that are soluble and lytically inactive. This inhibitory function is dependent on a

thrombospondin-like region at the amino terminus enabling clusterin to bind to C7, the β-chain of C8 and the b domain of C9 (Tschopp, Chonn et al. 1993). Clusterin also prevents C9 polymerisation.

1.5. The Complement factor H (FH) protein family

1.5.1. Overview

FH is a 155-kDa single polypeptide chain glycoprotein found in plasma, first identified as β1H by Nilsson and Müller-Eberhard (Nilsson and Mueller-Eberhard 1965). It is composed of 20 SCR domains and its plasma concentration is approximately 500μg/ml although this is highly variable between individuals (figure 1.2). Each SCR domain contains 60 amino acid residues that follow a typical arrangement of highly conserved amino acids including four cysteines (forming intra-domain disulphide bridges), two prolines and one tryptophan (Barlow, Norman et al. 1992) and are characterised by a compact hydrophobic core wrapped in β-sheet, with multiple stranded anti-parallel β-sheets linked by loops and turns (Perkins, Haris et al. 1988, Norman, Barlow et al. 1991, Barlow, Norman et al. 1992, Barlow, Steinkasserer et al. 1993). FH is encoded by the *CFH* gene within the regulation of complement activation (RCA) gene cluster on chromosome 1q32 (Rodriguez de Cordoba and Rubinstein 1986). The *CFH* gene spans 94kb of genomic DNA and consists of 23 exons, each of which encodes a single SCR except exons 3 and 4 that encode SCR2 (Male, Ormsby et al. 2000, Perez-Caballero, Gonzalez-Rubio et al. 2001). Exon 10 contributes to the terminal amino acids of an alternative splice product of the *CFH* gene termed FH-like protein 1 (FHL-1; figure 1.2) (Estaller, Schwaeble et al. 1991). Removal of exon 10 from the FH mRNA by splicing enables exons 9 and 11, coding for SCRs 7 and 8, to be joined. Post-translational modifications include disulphide bond formation and glycosylation, with seven consensus sites for N-linked glycosylation. Adjacent and downstream of the *CFH* gene are five genes that encode FH-related proteins. The genomic order of these genes is *CFHR3, CFHR1, CFHR4, CFHR2* and *CFHR5* that encode for complement FH-related proteins 3, 1, 4, 2 and 5 respectively (FHR3, -1, -4, -2, -5; figure 1.2). The FH protein family therefore consists of FH, FHL-1 and the five FHR proteins: FHR1,

FHR2, FHR3, FHR4 and FHR5. FH is an established major inhibitor of AP activation and the C3b amplification loop. The biological role of the FHR proteins has been elusive. Currently, they are thought to act as competitive antagonists (deregulators) of FH. In this section I discuss FH, FHL-1 and the FHR proteins. I also include sections on extra-hepatic synthesis and our present understanding of its biological relevance.

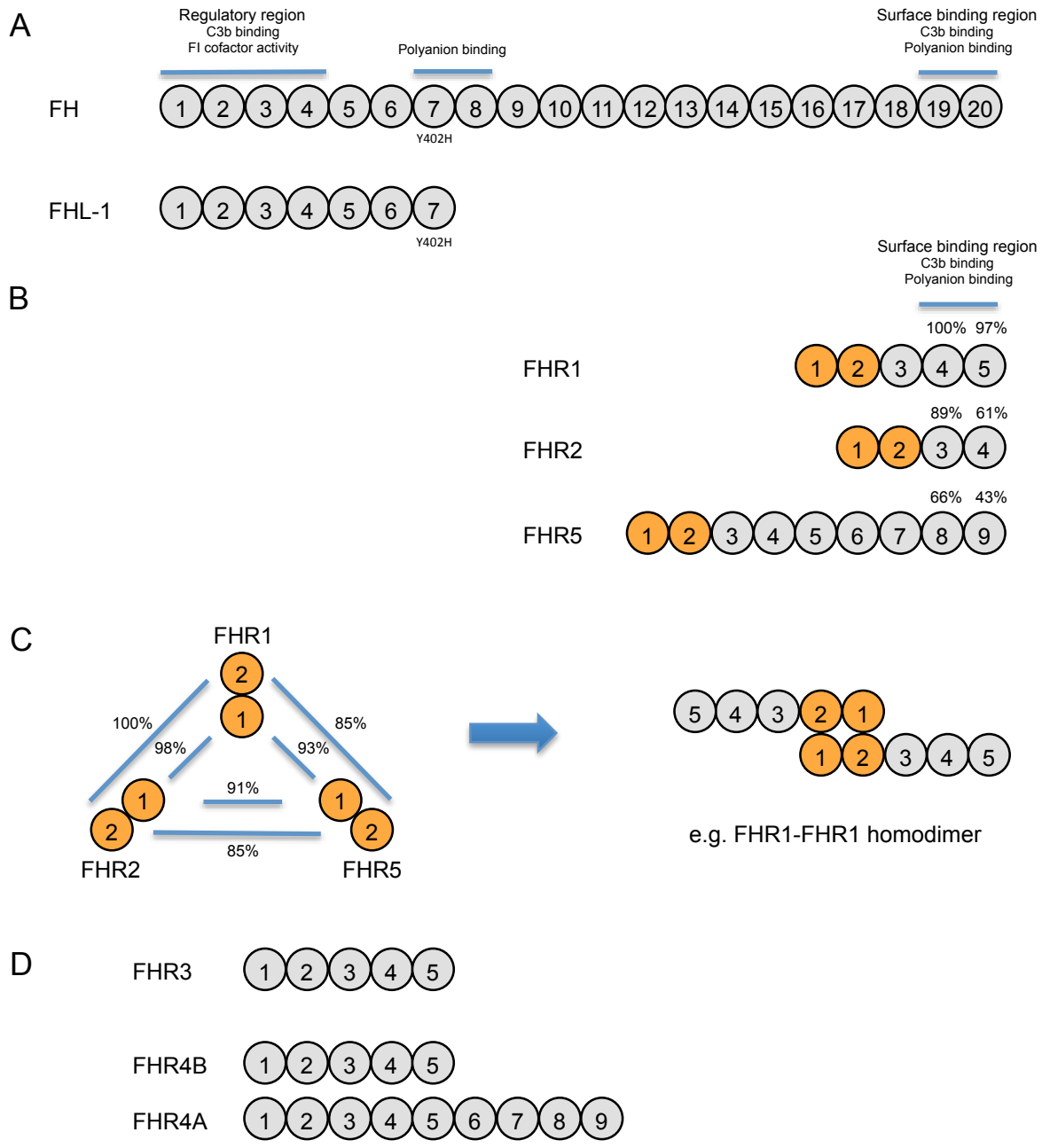


Figure 1.2 **The human FH protein family.** (A) FH consists of 20 SCR domains. Regulatory and surface binding region domains are indicated. FHL-1 is a splice variant comprised of the initial 7 FH domains. (B) and (C) FHR1, FHR2 and FHR5. These FHR proteins have high amino acid sequence similarity with the two carboxyl terminal domains of FH (indicated by percentages) and contain highly similar amino terminal domains which mediate obligate 'head to tail' dimerization (C) enabling the formation of homodimers and heterodimers. (D) FHR3 and FHR4. FHR4 has two splice variants termed FHR4A and FHR4B.

1.5.2. Complement factor H (FH)

FH regulates complement activation in three ways; by inhibiting formation of the AP C3 convertase through competition with FB for binding to C3b, by accelerating the decay of the AP C3 convertase (Whaley and Ruddy 1976) and by acting as a cofactor for the FI-mediated proteolytic inactivation of C3b to iC3b (Whaley and Ruddy 1976, Pangburn, Schreiber et al. 1977). These complement regulatory activities of FH all reside within SCRs 1-4. Rapidly able to inactivate C3b in plasma, the ability of FH to inactivate surface-bound C3b is influenced by the surface involved. The affinity of FH for surface-bound C3b may be less than fluid-phase C3b (Pangburn and Muller-Eberhard 1983) but may be enhanced in the presence of sialic acids and other polyanions such as heparin and glycosaminoglycans (Fearon 1978), likely due to concurrent recognition of C3b and sialic acids by the same FH molecule. Multiple binding sites for C3b and polyanions have been identified in FH, and the deposition of increasing amounts of C3b and iC3b on surfaces lacking polyanions also promotes FH-mediated inactivation of newly deposited C3b on these surfaces (Pangburn and Muller-Eberhard 1983). Surfaces on which bound C3b is relatively well protected from FH are susceptible to AP activation, as are antigen-IgG complexes with bound C3b (Pangburn and Muller-Eberhard 1978, Fries, Gaither et al. 1984).

The affinity with which FH binds the C3 convertase C3bBb, is similar to that with C3b alone (Pangburn 1986). Binding of FH to C3bBb results in irreversible displacement of the Bb fragment and loss of the active convertase, as occurs with DAF and CR1 binding on the cell surface. A specific interaction between Bb and the regulator or an allosteric effect on the conformation of C3b may be responsible (Pangburn 1986). The affinity of FI for C3b is greatly increased by FH thereby facilitating effective inactivation of C3b generated by tick over (Pangburn and Muller-Eberhard 1978). In addition to preventing the formation of further C3 convertase, the cleavage of C3b to iC3b on surfaces also provides a ligand for CR3 whilst further cleavage by FI in the presence of CR1 provides a ligand for CR2 in the form of C3dg.

FH possesses three distinct binding sites for both C3b and polyanions such as sialic acid and heparin. C3b binding occurs at SCR1-4, SCR12-14 and SCR19-20 whilst

heparin binding sites exist in SCR7, SCR13 and SCR19-20. FH also interacts with other plasma proteins such as CRP through SCR7, and with proteins derived from a variety of microorganisms at a number of different sites. Critical for its role as a cofactor in the FI-mediated inactivation of C3b is the C3b-binding site located in SCR1-4 (Sharma and Pangburn 1996), whilst the C3b- and polyanion binding sites in SCR19-20 are essential for regulating complement activation on host cells (Meri and Pangburn 1990, Pangburn 2000). The different roles mediated by the various functional domains of FH initially elucidated by techniques including site-directed mutagenesis and the use of monoclonal antibodies, have also become evident from the different phenotypes observed in the setting of FH dysfunction.

Many pathogenic organisms including *Neisseria meningitidis, Neisseria gonorrhoeae* and *Streptococcus pneumoniae* have evolved mechanisms to bind both FH and/or FHL-1 to make use of their AP inhibitory activities to prevent complement activation on their surface and thereby promote their survival (Ram, McQuillen et al. 1998, Hellwage, Meri et al. 2001, Jarva, Janulczyk et al. 2002, Meri, Hartmann et al. 2002, Huang, Qiao et al. 2008). The classic example of this is the binding of FH by the M6 protein and other M serotypes of *Streptococcus pyogenes*, leading to reduced surface deposition of C3b and macrophage and PMN-mediated phagocytosis (Horstmann, Sievertsen et al. 1988). The M protein binding site was found to be situated in SCR7 of FH close to the heparin and CRP binding sites (Sharma and Pangburn 1997), although FHL-1 has been subsequently shown to bind M proteins at this site with greater affinity than FH (Kotarsky, Hellwage et al. 1998) involving the hydrophobic tail specific to FHL-1 (Perez-Caballero, Alberti et al. 2000). Furthermore, the binding of FHL-1 to Fba proteins on the streptococcal surface may promote invasion via entry into epithelial cells (Pandiripally, Wei et al. 2003). Meningococcal strains possess a high affinity receptor, termed FH-binding protein, which has nanomolar affinity for human FH. This fhbp-FH interaction protects the pathogen from complement-mediated damage (Huang, Qiao et al. 2008).

Tumour cells may also exploit FH on their surface to prevent complement-mediated lysis and glioblastoma cells have been shown to inactivate surface-bound C3b to iC3b more effectively than other cell types (Junnikkala, Jokiranta et al. 2000, Fedarko, Jain et al. 2001). The apparent production of FH or a FH-like protein by

bladder tumour cells has also been utilised in immunoassays using patient urine (BTA*stat* and BTA TRAK) as an adjunct for diagnosing and monitoring bladder cancer alongside cystoscopy and urine cytology (Kinders, Jones et al. 1998, Thomas, Leyh et al. 1999). The urinary antigen identified as FH or a FH-like protein not only contains eight amino terminal residues identical to the corresponding amino acids in FH, but also binds C3b and accelerates cleavage of C3b in the presence of FI (Kinders, Jones et al. 1998).

1.5.3. Complement FH-like protein 1 (FHL-1)

FHL-1 is a 43-kDa human plasma protein encoded by an mRNA from the *CFH* gene, and contains the first 7 SCR domains present in FH along with four unique amino acid residues (Ser-Phe-Tyr-Leu) at its C-terminus (figure 1.2) (Schwaeble, Zwirner et al. 1987). The FHL-1 cDNA is 1644bp long representing 449 amino acids, the first 445 of which are identical to the N-terminal of FH. An additional exon (exon 10) present in the *CFH* gene is specific for the C-terminal region of this alternatively spliced 1.8kb FHL-1 transcript, and is located between the exons encoding for SCRs 7 and 8 (Sim, Kolble et al. 1993). Differing expression of FHL-1 and FH transcripts despite an identical promoter and transcription start site suggests regulation at the transcriptional and post-transcriptional level. Post-translational modifications include glycosylation with one consensus site for N-linked glycosylation in FHL-1.

Like FH, FHL-1 is constitutively synthesised by the liver (Schwaeble, Zwirner et al. 1987, Misasi, Huemer et al. 1989) but also by a variety of other cell types including peripheral blood monocytes, fibroblasts, human umbilical vein endothelial cells, oligodendrocytes and myoblasts (Friese, Hellwage et al. 1999). Expression may similarly be induced by a number of inflammatory mediators such as IFN-γ and anti-inflammatory agents such as dexamethasone reflected by the IFN-γ and glucocorticoid response elements identified in the gene promoter. Present in plasma at a concentration of 10-50μg/ml, FHL-1 also possesses cofactor and decay accelerating activity, promoting cleavage of C3b by FI and the dissociation of the C3 convertase respectively (Misasi, Huemer et al. 1989, Kuhn, Skerka et al. 1995, Kuhn and Zipfel 1996). Whilst displaying complement regulatory activity similar to FH, FHL-1 also has unique biological functions. Whilst SCR4 in both FH and FHL-1

contain the RGD (Arg-Gly-Asp) motif involved in the adhesive properties of matrix proteins such as fibronectin (Pierschbacher and Ruoslahti 1984), only FHL-1 acts as a matrix for cell attachment and spreading (Hellwage, Kuhn et al. 1997). The RGD domain of FHL-1 may be bound by an integrin receptor, a heterodimeric membrane glycoprotein, leading to loss of its complement regulatory activity mediated by SCRs 1-4. However by simultaneously binding C3b on target cells and integrin receptors on effector cells, FHL-1 (and FH) may promote the binding of phagocytic cells to their target surfaces. FHL-1 interacts with *Streptococcus pyogenes* where FHL-1 preferentially binds to streptococcal M protein compared to FH (Kotarsky, Hellwage et al. 1998). FHL-1 levels are altered in some disease states. For example, in rheumatoid arthritis it is expressed by synovial lining cells (Friese, Manuelian et al. 2003) and levels in synovial fluid are higher than those of FH (Friese, Hellwage et al. 1999).

1.5.4. Complement FH-related proteins

FHR1, FHR2, FHR3, FHR4 and FHR5 (Jozsi and Zipfel 2008) are a group of highly related proteins encoded by separate genes within the RCA gene cluster which, like FH, are composed of SCR domains exhibiting varying degrees of amino acid sequence identity to each other and to FH. FHR1 is composed of 5 SCRs and has two consensus sequences for N-linked glycosylation leading to two glycosylated (37- and 42-kDa) forms in human plasma. FHR1α and FHR1β have one and two carbohydrate side chains respectively (Skerka, Horstmann et al. 1991). FHR1 associates with high density lipoproteins (HDL) in the circulation and is reported to have a plasma concentration of 70-100μg/ml (Park and Wright 1996) (Skerka and Zipfel 2008). Comprised of 4 SCRs, FHR2 exists in two glycosylated forms in plasma at unknown concentration. At 24kDa and 28kDa respectively, the single and double glycosylated forms exist as part of HDL molecules like FHR1 (Skerka, Timmann et al. 1992, Park and Wright 1996). A number of glycosylated forms of FHR3 also exist, although the plasma concentration of this 35-56-kDa protein has not been determined. FHR4 is present in plasma in two isoforms derived from the *CFHR4* gene by alternative splicing. The nine SCR-containing FHR4A forms an internal doublet, with SCRs1-4 demonstrating a high level of sequence identity to SCRs 5-8, whilst FHR4B contains only 5 SCRs representing SCR1 and SCR6-9 of FHR4A. The

plasma concentrations of the 86-kDa FHR4A and 42-kDa FHR4B are not known although FHR4A is the major isoform in plasma. FHR4 also circulates in association with HDL and very low density lipoprotein particles (Skerka, Hellwage et al. 1997, Jozsi, Richter et al. 2005). At 65kDa, FHR5 is the largest of the FHRs with 9 SCR domains (McRae, Cowan et al. 2001). It is also glycosylated and associated with low and high density lipoproteins (Park and Wright 1996, McRae, Duthy et al. 2005), circulating in the plasma at a concentration of approximately 5µg/ml.

All five FHRs lack SCRs that are similar to the four N-terminal complement inhibitory domains of FH. However, there is high sequence similarity between the terminal carboxyl two domains within the FH and FHR proteins (Skerka and Zipfel 2008). The C-terminus of FH is the main site of surface recognition and binding to cell surface glycosaminoglycans, heparin, C3b and C3d (Manuelian, Hellwage et al. 2003, Oppermann, Manuelian et al. 2006). The C-termini of FH (SCR19-20) and FHR1 (SCR4-5) are the most alike in that FHR1 (SCR4-5) differs by only two amino acids, both located within SCR5. FH and FHR2 share 75% sequence identity compared to 50% for the C-termini of FHR3, FHR4 and FHR5 (Skerka and Zipfel 2008). The initial two SCR domains of FHR3 and FHR4 have amino acid sequence identity with FH SCR domains 6-7 of 79% and 66% respectively. In contrast, the initial two domains of FHR1, FHR2 and FHR5 are nearly identical and have recently been shown to mediate obligate dimerization (Goicoechea de Jorge, Caesar et al. 2013). This has revealed the existence of both homodimers and heterodimers within FHR1, FHR2 and FHR5.

FHR1, FHR3, FHR4 and FHR5 have all been reported to bind to C3b and may also bind C3d (Hellwage, Jokiranta et al. 1999, McRae, Duthy et al. 2005, Heinen, Hartmann et al. 2009).

FHR1 binds C3b, a number of microorganisms such as *Borrelia burgdorferi* and Group A *Streptococci* (Kotarsky, Gustafsson et al. 2001, Haupt, Kraiczy et al. 2007), and has been reported to possess C5 convertase inhibitory activity (Heinen, Hartmann et al. 2009). FHR1 inhibited C5 cleavage leading to a reduction in C5a generation and C5b deposition on sheep erythrocytes, and on binding C5, inhibited MAC formation (Heinen, Hartmann et al. 2009). FHR3 has been shown to bind to

C3b, C3d and heparin, and a complement modulatory role has also been proposed (Hellwage, Jokiranta et al. 1999). SCRs 4 and 5 of FHR4B bind the C3d region of C3b (Hellwage, Jokiranta et al. 1999), and in fact both isoforms have been shown to bind soluble C3b, iC3b and C3d (Hebecker and Jozsi 2012). FHR4A and FHR4B have also been reported to enhance FH cofactor activity (Hebecker and Jozsi 2012) (Hellwage, Jokiranta et al. 1999), although no cofactor activity was identified for FHR4A (Hebecker and Jozsi 2012) in contrast to reports of weak cofactor activity for FHR4B (Hellwage, Jokiranta et al. 1999). Native pentameric C-reactive protein has been identified as a ligand for FHR4, binding via an exposed site in SCR1 and resulting in enhanced opsonisation of necrotic cells through FHR4-mediated activation of C2/C4-dependent pathways (Hebecker, Okemefuna et al. 2010). FHR4 has also been reported to be able to activate the AP on surfaces where it can bind, via formation of C3 convertases from FHR4-bound C3b (Hebecker and Jozsi 2012). FHR4 has therefore been proposed as a promoter of complement activation and opsonisation, and such alternative forms of the C3 convertase may exhibit resistance against FH-mediated decay (Hebecker and Jozsi 2012). FHR5 has been reported to bind C3b and heparin and possess weak cofactor activity for the FI-mediated cleavage of C3b, along with fluid-phase C3 convertase inhibitory activity (McRae, Duthy et al. 2005). Notably, it was first identified as a component of glomerular immune deposits in a variety of renal pathologies where it was located in a similar distribution to C3 and C5b-9 (Murphy, Georgiou et al. 2002). The biological functions of the FHR family remain unclear although it appears from *in vitro* studies with FHR1, FHR2 and FHR5 that they do not possess any complement regulatory activities at physiological concentrations and may function as competitive antagonists of FH (Goicoechea de Jorge, Caesar et al. 2013, Tortajada, Yebenes et al. 2013).

The large genomic repeat regions along the *CFH-CFHR* gene locus predispose to *de novo* chromosomal recombination and duplication events in which whole genes or exons may be altered (figure 1.3) (Zipfel, Edey et al. 2007) (Heinen, Sanchez-Corral et al. 2006). The most frequent event has resulted in a *CFH-CFHR* allele in which recombination led to complete loss of the *CFHR3* and *CFHR1* genes (Δ*CFHR3-1*, figure 1.3) (Feifel, Prodinger et al. 1992). Disease-associated rearrangements have also been described and include a *CFH-CFHR1* hybrid gene in atypical haemolytic

uraemic syndrome (aHUS) (Venables, Strain et al. 2006) and an internal duplication in the *CFHR5* gene in familial C3 glomerulonephritis (termed CFHR5 nephropathy) endemic in Cyprus (Gale, de Jorge et al. 2010).

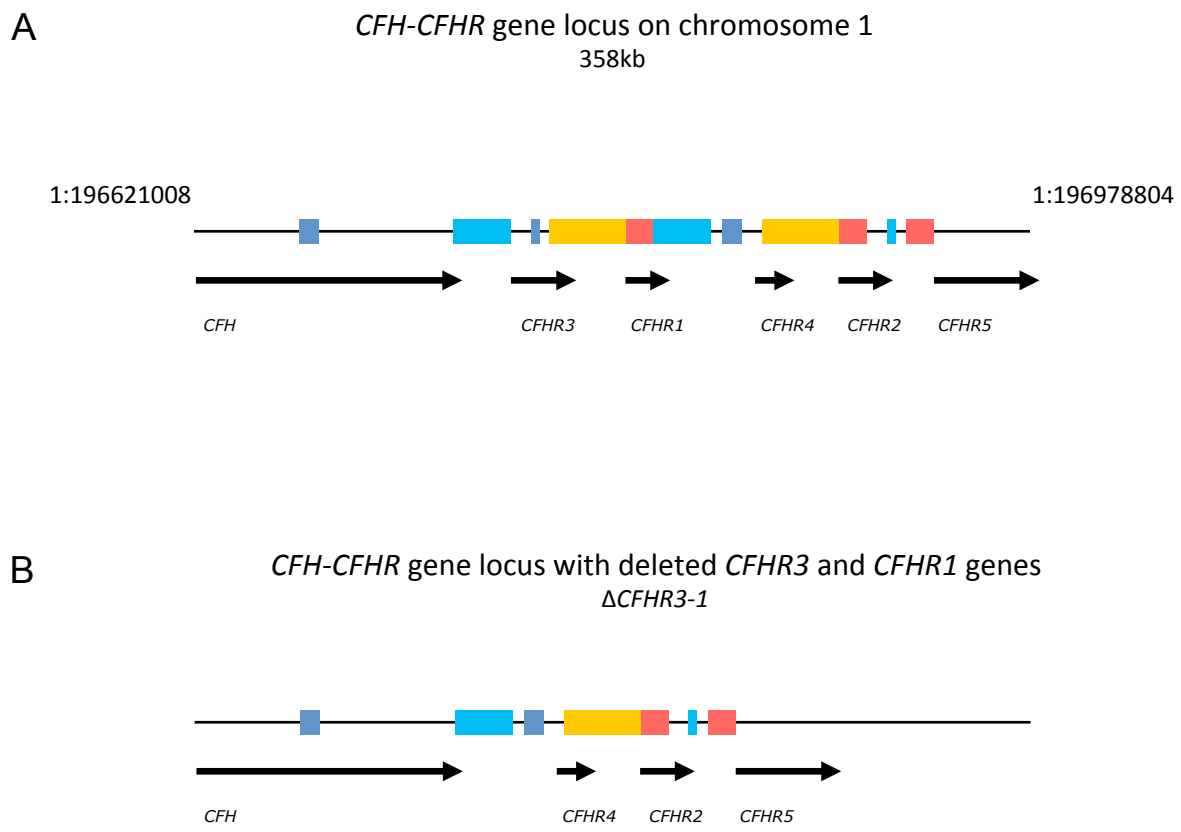


Figure 1.3 **Schematic depiction of the human *CFH-CFHR* gene locus**. This locus is situated on chromosome one and spans approximately 358 kb (A). It contains areas of long homologous repeats (depicted in colour), which predispose to non-allelic recombination events that can result in gene deletion or the formation of hybrid genes. Black arrows indicate position of the *CFH* and *CFHR* genes. Note the genomic order of the *CFHR* genes does not follow the numerical sequence i.e. the order is *CFHR3*, *CFHR1*, *CFHR4*, *CFHR2* and *CFHR5*. (B) Recombination between the inter-genic regions flanking the *CFHR1-CFHR3* region results in complete loss of these genes. This is a common polymorphism and denoted: *ΔCFHR3-1*. kb – kilobases.

1.5.5. Murine complement factor H (mFH) and mFH-related (mFHR) proteins

The sequence of mFH is reported to have 61% homology to human FH (Kristensen and Tack 1986) and is encoded by a ~100-kb gene (*cfh*) located on chromosome 1 (Munoz-Canoves, Tack et al. 1989). The total length of the *cfh* gene is approximately 24 times the length of its mRNA, although one quarter of this comprises the leader sequence exon and first intron (Vik, Keeney et al. 1988). mFH is encoded by 22 exons with the first exon encoding the 5'-untranslated region and the 18-amino acid leader peptide. Nineteen of the twenty SCRs in mFH are each encoded by a single exon whilst the coding sequence of SCR2 is split between two exons (Vik, Keeney et al. 1988). The exons vary in size from 77 to 210bp with an average of 178bp, and are grouped either in pairs (exons 2-11) or in a cluster (exons 12-20). Analysis of the exons at the nucleotide level revealed clustering into two major homology groups and one minor one. Exons 5 and 13-21 made up the first group, 6-11 and 22 the second group, and 2 and 12 the minor group, whilst exons 3 and 4 encoding SCR2, show no homology with any of the other exons. mFH also has a high cysteine and proline content, conserved amino acid residues, and eight potential sites for asparagine-linked glycosylation with a carbohydrate content of 4-18% by weight (Kristensen and Tack 1986). The RGD motif in FH is conserved in evolution and found in a corresponding position in murine and human FH (Kristensen and Tack 1986).

mFH-related proteins are present but poorly characterised in mice. Initial studies in the mouse identified three liver RNA species of 3.5kb, 2.8kb and 1.8kb using cDNA cloning and a full-length mFH cDNA probe (Vik, Munoz-Canoves et al. 1990). Three mFH-encoding transcripts from two distinct *cfhr* genes were subsequently found associated with two mFHR proteins in mouse plasma (Hellwage, Eberle et al. 2006). The cDNA sequences of these ~100- and 38-kDa proteins reported to represent murine FHR-C and FHR-B respectively, are similar to those clones reported previously (Vik, Munoz-Canoves et al. 1990). The *cfhr-c* gene encodes full-length mFHR-C and two splice variants. mFHR-C comprises 856 amino acids in 14 SCR domains and contains four consensus sites for N-linked glycosylation. SCRs 1-12

are homologous to SCRs 6-17 of mFH whilst SCRs 13 and 14 have sequence identity to SCRs 19-20 of mFH. The splice variant contains 13 SCRs lacking the mouse FH SCR 17-related domain. The *cfhr-b* gene encodes mFHR-B and another likely splice variant. Mature mFHR-B contains 309 residues arranged in 5 SCRs homologous to SCRs 5-7, 19 and 20 of murine FH, and contains four N-linked glycosylation sites. Recombinant mFHR-B was found to bind heparin and C3b. However whilst both native proteins bound heparin, mFHR-C bound C3b with greater affinity than mFHR-B. mFHR-C was also shown to bind to endothelial cells, a process mediated by polyanionic surface structures (Hellwage, Eberle et al. 2006). A rat FH-related protein containing 11 SCRs has previously been identified, the mRNA of which was upregulated in glomeruli exposed to complement activation (Ren, Doshi et al. 2002). The recombinant form of the protein was also found to bind rat C3b and inhibit AP activation.

1.5.6. The synthesis of FH and the FHR proteins

Understanding the contribution of complement synthesised locally to that synthesised at a distant site is important and relevant for the soluble proteins. The contribution of the major synthesis site (e.g. the liver) versus local synthesis within tissues (e.g. by organ-specific cell populations like synoviocytes or podocytes) has been addressed using animal models or by comparing allotypic differences in humans who have undergone organ transplantation. For example, significant extra-hepatic synthesis has been demonstrated for the complement proteins C3 and C6. In a study of patients who had undergone bone marrow or liver transplantation in whom there was a mismatch for C3 allotypes, marrow-derived C3 was shown to contribute 0.2-5.2% of the total circulating C3 immediately following transplantation when inflammatory stimuli were likely to be present (Naughton, Botto et al. 1996). Monocytes and their precursors were deemed to be the source of the C3, but the drop in C3 levels after haematological recovery suggested that the contribution of myeloid-derived C3 in the resting state is negligible. Total extra-hepatic C3 synthesis meanwhile provided 5.6-11.4% of total circulating C3 in the liver transplant recipients and remained at this level for up to 1 year post transplant. Transplantation of livers from C6-deficient rats into C6-sufficient rats demonstrated that 30-40% of systemic C6 was extra-hepatic in origin for up to 100 days post-transplant (Brauer, Baldwin et

al. 1994). Bone marrow-derived C6 was shown to contribute 5% of circulating rat C6 levels for a similar period post-BMT, with monocytes and granulocytes responsible for the C6 synthesis observed.

The predominant site of synthesis of FH is the liver, which accounts for the vast majority of the plasma FH pool. In a single report of an individual who underwent hepatic transplantation, differences in recipient and donor FH allotypes enabled selective measurement of FH derived from the liver transplant and that derived from extra-hepatic FH synthesis in the host. This showed that almost all of the plasma FH was derived from the donor transplant (Brown, Tellez et al. 2012). Nevertheless, a diverse range of cell types including endothelial cells, fibroblasts, monocytes and oligodendrocytes have also been shown to synthesize FH (Schwaeble, Schwaiger et al. 1991) (Katz and Strunk 1988, Ripoche, Mitchell et al. 1988, Gasque and Morgan 1996).

Northern blot analysis and subsequent hybridisation with cDNA probes demonstrated expression of two different FH mRNA species (4.3kb and 1.8kb; produced by alternative splicing) in primary cell cultures of monocytes, fibroblasts and HUVEC, whilst a third 1.4kb FH mRNA was found *only* in liver tissue (Schwaeble, Schwaiger et al. 1991). The 4.3kb mRNA encodes FH whilst the 1.8kb mRNA encodes FHL-1. Not only did FH expression appear tissue-specific in terms of the nature and relative quantity of these different mRNA species, but also with regards to the response to cytokines. The addition of IFN-γ led to a six-fold increase in the abundance of the FH transcript in fibroblasts and a two-fold increase in HUVEC but had no effect on monocytes. Tumour necrosis factor-α (TNF-α) was able to induce expression of FH in the U937 pre-monocyte cell line. Identified previously in cultured HUVEC under standard conditions, stimulation with IFN-γ was also associated with a six- to seven-fold increase in both FH and FHL-1 transcript along with a rise in the secretion of FH itself (Ripoche, Mitchell et al. 1988). Expression of the FHL-1 transcript in HUVEC was only 20% that of the FH transcript, whilst in the liver expression was more comparable, with levels of FHL-1 up to 70% that of FH (Ripoche, Mitchell et al. 1988, Schwaeble, Schwaiger et al. 1991). Both FH and FHL-1 transcripts were identified in fibroblasts along with the corresponding proteins, all of which were increased by stimulation with IFN-γ (Katz and Strunk 1988).

Constitutive secretion of FH was also demonstrated at very low levels in a human oligodendrocyte cell line (HOG), and this was enhanced by stimulation with IFN-γ and TNF-α in a time- and dose-dependent manner (Gasque and Morgan 1996).

Renal synthesis of FH has been demonstrated in human mesangial cells and proximal tubular epithelial cells, and rodent podocytes (van den Dobbelsteen, Verhasselt et al. 1994, Gerritsma, Gerritsen et al. 1997, Ren, Doshi et al. 2003). FH was shown to be constitutively expressed by human mesangial cells in culture, and this was upregulated by IFN-γ in a dose- and time-dependent manner (van den Dobbelsteen, Verhasselt et al. 1994). Basal production of FH by human proximal tubular epithelial cell (PTEC) lines *in vitro* was also enhanced by incubation with IFN-γ, a finding not reproduced by incubation with other cytokines including TNF-α (Gerritsma, Gerritsen et al. 1997). In rats, mRNA of both glomerular epithelial cell FH and a rat FH-related protein with a similar structure to human FHR5 were found to be upregulated following exposure to complement activation both *in vitro* and *in vivo* (Ren, Doshi et al. 2002, Ren, Doshi et al. 2003). FH was found to be present on the plasma membrane of cultured glomerular epithelial cells (GEC), and FH mRNA expression increased over time in cultured GEC exposed to complement activation (Ren, Doshi et al. 2003). Passive Heymann's nephritis was used as the *in vivo* model of membranous nephropathy in which complement is activated in the proximity of the GEC, and the increased FH mRNA was also associated with a rise in FH expression at the protein level. Expression of FH has also been demonstrated in the retinal pigment epithelium/choroid in the eye (Hageman, Hancox et al. 2006).

The major site of synthesis of the FHRs is also presumed to be hepatic, but expression has been reported in other tissues. FHR1 is expressed in the retina (although at levels >250,000 fold lower than in liver (Hageman, Hancox et al. 2006)), and in the lining of blood vessels in the kidney, particularly larger arteries and in the afferent and efferent arterioles of glomeruli (Heinen, Hartmann et al. 2009). FHR1-5 were also detected in middle ear effusion fluid in children with otitis media (Narkio-Makela, Hellwage et al. 2001). FHR5 was identified in complement deposits within the kidney in a wide range of diseases although whether this was hepatic or renal-derived was not deduced (Murphy, Georgiou et al. 2002). Recurrence of CFHR5 nephropathy in an unrelated renal transplant demonstrates that normal renal-derived

FHR5 cannot prevent disease and I discuss this study further in my third results chapter (Vernon, Gale et al. 2011). Notably recurrence rates of FH-related aHUS in renal transplants are known to be high, again indicating that any renal-derived FH is insufficient to prevent disease (Kavanagh and Goodship 2011).

1.5.7. The function of extra-hepatic FH and the FHR proteins

In this section I discuss efforts to understand the biological roles of extra-hepatic FH and FHR production. In certain situations, the local production of complement proteins may play a more critical role than the circulating component. This has been postulated for C3 in the settings of allogeneic renal transplantation, cold ischaemia-induced renal injury and internalisation of uropathogenic bacteria into urinary epithelium (Springall, Sheerin et al. 2001, Pratt, Basheer et al. 2002, Farrar, Zhou et al. 2006). In mice, the transplantation of C3-deficient MHC-mismatched kidneys was associated with long-term graft function when compared to the transplantation of wild-type kidneys which were rapidly rejected (Pratt, Basheer et al. 2002). Graft survival was also much greater than that observed in C3-deficient recipients receiving wild-type grafts. In another renal isograft model, ischaemic C3-deficient kidneys transplanted into wild-type individuals exhibited only mild degrees of structural and functional disturbance, in comparison to widespread tissue damage and severe acute renal failure when C3-sufficient kidneys were transplanted into either C3-sufficient or C3-deficient recipients (Farrar, Zhou et al. 2006). These findings suggest that in this context at least, locally synthesized C3 appears to have a greater influence than the circulating component.

To date, the physiological importance of extra-hepatic FH and FHR synthesis is unknown. Reconstitution of irradiated FH-deficient mice with wild-type bone marrow was neither able to prevent the development of the spontaneous C3 glomerulopathy seen in FH-deficient mice or restore circulating plasma FH levels (Alexander, Aneziokoro et al. 2006). Plasma FH remained undetectable in FH-deficient mice that had received a single wild-type renal transplant kidney although some FH was detected within the kidney (Alexander, Wang et al. 2007). These data show that neither bone marrow-derived cells nor the kidney contribute to the circulating plasma

FH pool in mice, a finding consistent with the informative liver transplantation study discussed above (Brown, Tellez et al. 2012).

FH on the surface of platelets and podocytes in rodents appears to be involved in the processing of immune complexes through binding C3b in the complexes, in an analogous manner to CR1 on erythrocytes and podocytes in primates (Alexander, Hack et al. 2001, Alexander, Wang et al. 2007). The glomeruli of irradiated wild-type mice reconstituted with FH-deficient bone marrow and subjected to a model of immune complex injury (chronic serum sickness induced by apoferritin immunization) contain immune complexes. These are thought to accumulate due to failure of removal in plasma by platelet FH (Alexander, Hack et al. 2001, Alexander, Wang et al. 2007). In this chronic serum sickness model, greater renal injury was seen in wild-type hosts that received FH-deficient kidneys compared to those receiving wild-type kidneys. This may reflect pre-existing glomerular disease in the FH-deficient kidney but could also indicate that renal-derived FH was important in this model. Further data in this study argued that podocyte-derived FH acted to protect the kidney in this setting.

1.6. Complement and renal disease

Complement plays an important role in renal injury both within the glomerular and tubulointerstitial compartments. In the majority of cases, complement-mediated damage is one of several injurious mechanisms operating in a given renal pathology. However, in some distinct situations complement activation is the key driver of the renal pathology. Examples of these pathologies are aHUS and C3 glomerulopathy.

In this section, I will start with a general overview of the contribution of complement to glomerular disorders where additional non-complement mediated mechanisms are present. These include lupus nephritis, anti-neutrophil cytoplasmic antibody-associated vasculitis (AAV), membranous nephropathy, IgA nephropathy and diabetic nephropathy and tubulointerstitial injury. I will then focus on aHUS and C3 glomerulopathy. Both these pathologies are characterised by an inability to regulate complement and can be described as renal disorders associated with complement

dysregulation. This dysregulation may be inherited or acquired and in both pathologies defective function of FH is common.

1.6.1. Complement activation in glomerular disease

1.6.1.1. Lupus nephritis

Complement plays an important physiological role in the removal of immune complexes and apoptotic cells and defects in this function predispose to systemic autoimmunity. This is termed the 'waste disposal' hypothesis and apoptotic cells are considered to be the source of lupus autoantigens (reviewed in (Manderson, Botto et al. 2004)). C1q deficiency is associated with an SLE-like illness in humans and in an experimental model of murine C1q deficiency (Botto, Dell'Agnola et al. 1998).

Excessive complement activation within the glomerulus due to immune complex deposition contributes to renal injury in lupus nephritis. Anti-C1q antibodies are strongly correlated with renal disease in SLE, and can exacerbate antibody-mediated glomerular damage by augmenting the ability of immune complexes to activate complement (Trouw, Groeneveld et al. 2004). In experimental models, AP activation and C5 activation contribute to renal injury in SLE. Prevention of AP activation by introducing either FB (Watanabe, Garnier et al. 2000) or FD (Elliott, Jarmi et al. 2004) deficiency resulted in amelioration of renal injury in the spontaneous lupus mouse model (MRL/*lpr* (now termed MRL/MpJ-Tnfrsf6^{lpr}) mice). Consistent with these observations, targeted inhibition of complement using an artificial inhibitor (CR2-Crry) improved survival and renal function and histology (Atkinson, Qiao et al. 2008). Antibody-mediated C5 inhibition ameliorated renal injury in the NZB/NZW F1 lupus murine model (Wang, Hu et al. 1996) and a murine model of lupus nephritis induced by hybridoma-derived human IgG anti-DNA antibodies (Ravirajan, Wang et al. 2004).

Notably, deficiency of C3 in MRL/*lpr* mice was associated with enhanced albuminuria and glomerular IgG deposition (Sekine, Reilly et al. 2001). This illustrates that

complement plays a beneficial (immune complex removal) and deleterious (tissue injury through the AP and C5 activation) role in SLE.

1.6.1.2. Anti-neutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis (AAV)

AAV is characterised by a pauci-immune necrotizing crescentic glomerulonephritis. However, there is immunoglobulin and complement deposition in some biopsies and the presence of electron dense deposits in these individuals was associated with greater proteinuria (Neumann, Regele et al. 2003). ANCA IgG plays a critical role in the development of AAV (Xiao, Heeringa et al. 2002, Schlieben, Korbet et al. 2005). Induction of glomerulonephritis in mice by the intravenous injection of anti-myeloperoxidase IgG has also been shown to require neutrophils (Xiao, Heeringa et al. 2005) and factors released from activated neutrophils may activate complement (Vogt 1996, Wirthmueller, Dewald et al. 1997). In both the anti-MPO IgG and anti-MPO splenocyte transfer mouse AAV model, depletion of C3 by cobra venom factor completely prevented the induction of glomerulonephritis (Xiao, Schreiber et al. 2007). C5-deficient and FB-deficient mice were also protected from the induction of crescentic glomerulonephritis by anti-MPO IgG. The influx of neutrophils and monocytes was attenuated, likely secondary to the lack of C5 activation and generation of C5a. The demonstration of C3a production following complement activation by MPO-ANCA or PR3-ANCA IgG-stimulated human neutrophils, led the authors to hypothesize that necrotizing inflammation is mediated by activation of the AP by factors released from ANCA IgG-stimulated neutrophils. Pre-treatment with a monoclonal anti-C5 antibody again prevented disease, whilst treatment following disease induction led to more than 80% reduction in glomerular crescent formation (Huugen, van Esch et al. 2007). Mice with C4 deficiency were not protected excluding a role for the CP and LP in this model. Lack of involvement of the CP and LP in humans is supported by the absence of C1q, C4d and MBL in renal biopsy specimens from patients with MPO-ANCA positive glomerulonephritis (Xing, Chen et al. 2009).

1.6.1.3. Membranous nephropathy

Membranous nephropathy (MN) is an autoimmune disease characterised by deposition of immune complexes along the outer aspect of the glomerular basement membrane (GBM). Podocyte M-type phospholipase A_2 receptor is the antigen in the majority of cases of idiopathic MN. Heymann nephritis (HN) is a rat model of MN where the podocyte antigen is megalin. Proteinuria in HN has been reported to result from podocyte injury secondary to antibody-mediated complement activation and MAC formation (Cybulsky, Rennke et al. 1986). Complement-mediated changes to the slit diaphragm involving alterations in the distribution of nephrin and its association with actin also appear to play a role in the proteinuria observed (Saran, Yuan et al. 2003). Despite similar amounts of deposited sheep antibody and subepithelial deposits, complement-depleted rats maintained their slit diaphragm integrity and did not suffer the dissociation of nephrin from actin observed in complement-sufficient HN rats. In human MN the IgG deposited in the kidney is predominantly IgG4 which does not activate complement via the CP, with recent data suggesting that activation may be via the LP (Ma H 2011).

1.6.1.4. IgA nephropathy

Mesangial deposition of IgA1 characterises IgA nephropathy (IgAN), the commonest form of primary glomerulonephritis worldwide. Aberrant IgA1 glycosylation has been implicated in pathogenesis with variable amounts of these molecules detected in immune deposits (Giannakakis, Feriozzi et al. 2007) In some biopsies deposition of C3, C5b-9 and properdin in association with IgA may be seen (Bene and Faure 1987) implicating AP activation. Polymeric or chemically aggregated IgA can activate the LP through an interaction with MBL (Roos, Bouwman et al. 2001). Complement activation by the LP is only seen in a subpopulation of patients with IgAN (Roos, Rastaldi et al. 2006), although LP activation appears to be associated with more severe renal injury (Roos, Rastaldi et al. 2006). The presence of mesangial C4d also seems to predict a worse renal prognosis, with 10-year renal survival in C4d-positive patients reported as 43.9% compared to 90.9% in C4d-negative patients (Espinosa, Ortega et al. 2009). Recently, an unexpected association with the allele carrying deletion of both *CFHR1* and *CFHR3* (Δ*CFHR3-1*) has been described (Gharavi, Kiryluk et al. 2011). The presence of Δ*CFHR3-1* was associated with protection

against IgAN. How this works mechanistically is unclear but an attractive hypothesis is that lack of these FHR proteins reduces FH antagonism thereby enhancing the ability of FH to limit complement-mediated injury in IgAN (Malik, Lavin et al. 2012).

1.6.1.5. Diabetes mellitus

MAC deposition within the kidneys and blood vessels of diabetic patients (Falk, Sisson et al. 1987) led to the suggestion that complement activation may be involved in the development of proliferative diabetic complications. Through the release of growth factors from targeted endothelium, the MAC has been associated with stimulating smooth muscle cell, fibroblast and mesangial cell proliferation (Halperin, Taratuska et al. 1993). MAC triggered the production of extracellular matrix (ECM) proteins by mesangial cells. The glomerulosclerosis characteristic of diabetic nephropathy may thus be partially dependent on MAC-induced ECM expansion and vascular proliferation (Ziyadeh 1993).

The identification of a lysine residue that is not only susceptible to glycation but is also adjacent to a conserved amino acid essential for CD59 function, supported a hypothesis that glycation of CD59 in diabetes leads to its inactivation and the subsequent increased MAC deposition (Acosta, Hettinga et al. 2000). CD59 underwent glycation *in vivo* adversely affecting its function and site-directed mutagenesis of the lysine residue prevented sensitivity of human CD59 to glycation-inactivation (Acosta, Hettinga et al. 2000). Glycated human CD59 has also been shown to co-localise with MAC in diabetic, but not non-diabetic, kidney tissue (Qin, Goldfine et al. 2004). Glycation inactivation of CD59 and increased MAC deposition may also be exacerbated by antibodies directed against neo-antigens in glycated proteins (Orchard, Virella et al. 1999).

1.6.2. Tubulointerstitial (TI) disease

Although the glomerulus may be the site of initial injury, associated TI damage plays a major role in predicting renal prognosis (Nath 1998). Proteinuria is a strong predictor of progression in CKD with greater levels predicting a poorer outcome (Remuzzi, Benigni et al. 2006). Proteinuria is believed to be a significant cause of TI

injury as observed in animal models (Eddy 1989), and reducing proteinuria is associated with a more favourable prognosis. Whilst albumin and growth factors in urine of proteinuric patients have been suggested as possible mediators of renal damage (Olson, de Urdaneta et al. 1985, Honkanen, Teppo et al. 1997), complement proteins are also present and on activation within the tubular lumen generate complement activation products and the MAC (Morita, Ikeguchi et al. 2000). Evidence suggests that the AP is responsible for complement activation on the brush border of the proximal tubules (Camussi, Tetta et al. 1983). Furthermore, the lack of complement regulators on the apical surface of tubular cells (Ichida, Yuzawa et al. 1994) along with the additional formation of the AP convertase by amidated C3, may contribute to intraluminal complement activation. The MAC has been implicated in TI injury in proteinuric animal models, with C6-deficient rats exhibiting less severe TI injury than wild type rats with equivalent proteinuria (Nangaku, Pippin et al. 1999). Tubular C5b-9 deposition has also been associated with interstitial monocyte infiltration and volume expansion (Mosolits, Magyarlaki et al. 1997), and may promote peritubular myofibroblast accumulation in focal segmental glomerulosclerosis (FSGS) (Rangan, Pippin et al. 2004). Myofibroblast accumulation is reported to be a predictor of progression in a number of human glomerular diseases (Abbate, Zoja et al. 2002).

Patients with FSGS, characterised by nephrotic-range and non-selective proteinuria, have one of the highest rates of urinary C5b-9 excretion (Morita, Ikeguchi et al. 2000) and increased TI deposition of C5b-9 (Mosolits, Magyarlaki et al. 1997). Adriamycin-induced nephropathy in rodents is considered an analogue of human minimal change disease and FSGS, with long-term features typical of chronic progressive renal disease in humans (Okuda, Oh et al. 1986). The involvement of the AP in mediating injury in this model has been demonstrated using complement-deficient mice (Turnberg, Lewis et al. 2006, Lenderink, Liegel et al. 2007). Mice deficient in C3 and FD were protected from renal disease (Turnberg, Lewis et al. 2006) whilst complement activation in the glomeruli and TI was also reduced in FB-deficient mice (Lenderink, Liegel et al. 2007). FB inhibition using a monoclonal antibody in wild-type mice also led to the delayed onset of renal failure (Lenderink,

Liegel et al. 2007). Similarities in disease seen in C1q-deficient and wild-type mice demonstrated that complement activation in this model is not mediated by the CP. In contrast, mice lacking CD59 developed significantly greater glomerular and TI injury than controls (Turnberg, Lewis et al. 2006).

The generation of the anaphylatoxin C5a has also been implicated in TI damage and the production of profibrotic mediators (Boor, Konieczny et al. 2007), with inhibition of C5 (and C5a) in experimental models leading to a reduction in TI injury (Boor, Konieczny et al. 2007).

1.6.3. Renal disorders associated with complement dysregulation

The prototypic renal disease associated with complement dysregulation is dense deposit disease (DDD) (Fakhouri, Fremeaux-Bacchi et al. 2010). Subsequently, aHUS was recognised as a condition in which many cases were associated with defective AP regulation. In both conditions there are frequently either inherited or acquired defects of AP regulation (reviewed in (Smith, Alexander et al. 2007)). Since these conditions are pathologically distinct (DDD is characterised by glomerulonephritis and aHUS by renal thrombotic microangiopathy), a key question was: how does AP dysregulation give rise to these distinct phenotypes? Lessons from animal models and the characterisation of informative mutations have demonstrated that, although AP dysregulation is common to both DDD and aHUS, in DDD the dysregulation is within plasma (fluid-phase) whilst in aHUS it occurs along the renal endothelium. In DDD, such is the enormity of fluid-phase C3 activation and consumption of complement components, that systemic complement activity is frequently absent. The lesion is thought to occur because of the accumulation of C3 fragments along the GBM (Smith, Harris et al. 2011). In contrast in aHUS, impaired surface regulation along the renal endothelium occurs in the presence of functional complement regulation in the plasma (Pickering, de Jorge et al. 2007). The lesion is thought to occur because of an inability to control complement activation along the renal endothelium.

More recently, it is clear that DDD is part of a spectrum of glomerular pathologies in which there is predominant C3 accumulation. The term C3 glomerulopathy was coined to describe this group of glomerular pathologies (Fakhouri, Fremeaux-Bacchi et al. 2010). I will first discuss C3 glomerulopathy and then aHUS. I will particularly emphasise the role of models of FH deficiency since these have been particularly informative mechanistically and understanding their phenotypes is relevant to the interpretation of my thesis data.

1.6.4. C3 glomerulopathy

C3 glomerulopathy refers to glomerular pathology in which there is predominant C3 deposition over immunoglobulin (Fakhouri, Fremeaux-Bacchi et al. 2010). In the most extreme case, immunoglobulin is absent but this definition is too restrictive as in many cases of DDD scanty or 1+ immunoglobulin staining may be seen in the presence of predominant C3 staining. The most characterised entities within this pathological classification are DDD and C3 glomerulonephritis (C3GN).

The defining feature of DDD is the presence of intramembranous electron dense transformation of the GBM (Berger and Galle 1969). Historically DDD was re-named as a subtype of membranoproliferative glomerulonephritis (MPGN), termed MPGN type 2 (Habib, Gubler et al. 1975). However, when the original defining feature is used only approximately one third of biopsies have evidence of MPGN (Walker, Ferrario et al. 2007). Therefore viewing DDD as a category of MPGN is misleading and the term DDD should be used. The light microscopic appearances of DDD are highly variable and include: mesangial proliferative, crescentic and exudative in addition to MPGN (Walker, Ferrario et al. 2007).

DDD is commonly associated with the presence of hypocomplementaemia most often due to the presence of C3 nephritic factor (reviewed in (Smith, Alexander et al. 2007), an auto-antibody that stabilises the AP C3 convertase, C3bBb (Spitzer, Vallota et al. 1969, Daha, Fearon et al. 1976). The result is uncontrolled C3 activation through the AP. Rarer causes of uncontrolled C3 activation through the AP

and DDD include complete FH deficiency (Levy, Halbwachs-Mecarelli et al. 1986, Pickering and Cook 2008), autoantibodies to FH that result in functional FH deficiency (Meri, Koistinen et al. 1992, Jokiranta, Solomon et al. 1999), gain of function mutation in FB (Jokiranta, Solomon et al. 1999, Strobel, Zimmering et al. 2010) and gain of function mutation in C3 (Martinez-Barricarte, Heurich et al. 2010). These are summarised in figure 1.4.

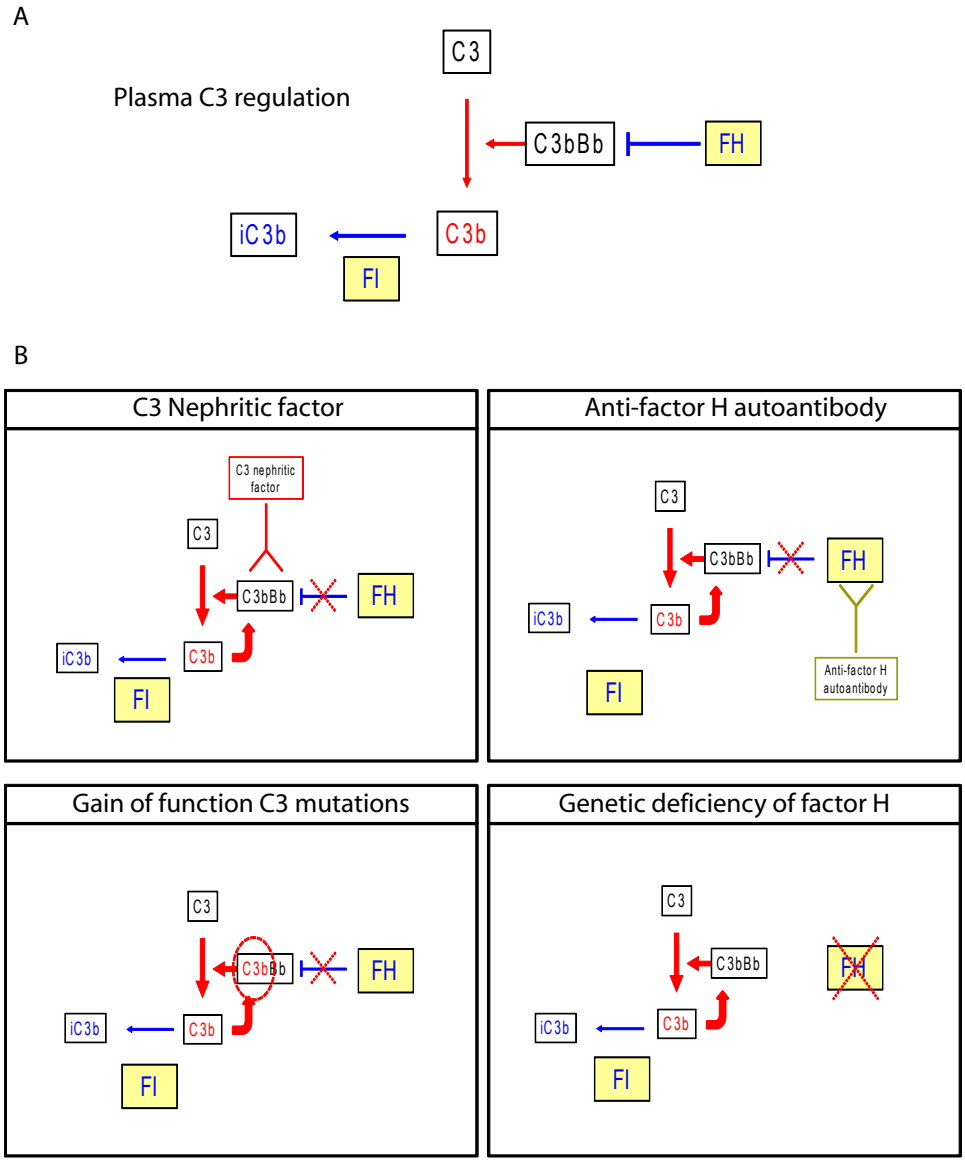


Figure 1.4 **Mechanisms of C3 dysregulation associated with DDD.** (A) In plasma the conversion of C3 to C3b by the AP convertase (C3bBb) is inhibited by FH. In addition, any C3b generated is proteolytically cleaved by FI to iC3b. The iC3b, unlike C3b, *cannot* contribute to further C3b activation via the C3b amplication loop. (B) Mechanisms that impair the FH-mediated inhibition of plasma C3 activation are associated with DDD. These include (i) C3 nephritic factor, an autoantibody that stabilises C3bBb preventing its inhibition by FH; (ii) FH autoantibodies that interfere with the binding of FH to the convertase; (iii) gain of function mutations in C3 that are resistant to inhibition by FH and (iv) genetic deficiency of FH (figures adapted from (Smith, Harris et al. 2011)).

C3GN is characterised by subendothelial electron dense deposits and the absence of the highly electron dense intramembranous change characteristic of DDD (Servais, Fremeaux-Bacchi et al. 2007). Like DDD, light microscopic appearances are heterogeneous (Fakhouri, Fremeaux-Bacchi et al. 2010). The importance of C3GN is that it tells us that there is a spectrum of C3 dominant glomerular pathologies and consequently it is likely that additional mechanisms beyond fluid-phase C3 dysregulation are present. This is most obvious from the observation that many patients with C3GN do not have systemic hypocomplementaemia (Fakhouri, Fremeaux-Bacchi et al. 2010). The most well characterised cause of C3GN derived from studies of familial C3GN in Cyprus (Gale, de Jorge et al. 2010). Affected individuals had an autosomal dominant C3GN characterised by normal plasma complement profile, absence of causative factors described for DDD (e.g. C3 nephritic factor), synpharyngitic macroscopic haematuria (leading in some cases to an erroneous diagnosis of IgAN) and more severe renal disease in males (Gale, de Jorge et al. 2010, Athanasiou, Voskarides et al. 2011). Genetic studies revealed that this disease was due to a heterozygous internal duplication in the *CFHR5* gene and led to it being termed CFHR5 nephropathy (Gale, de Jorge et al. 2010). This mutation does not alter the genetic architecture of the *CFH* gene so that FH function is ostensibly normal. As discussed earlier, the current biological role for FHR proteins including FHR5 is that they compete with FH for binding to ligand (Goicoechea de Jorge, Caesar et al. 2013, Tortajada, Yebenes et al. 2013). However, unlike FH they are not able to prevent C3b amplification, as they have no intrinsic complement regulatory activity at physiological concentrations (Goicoechea de Jorge, Caesar et al. 2013, Tortajada, Yebenes et al. 2013). The result is that they function as FH deregulators. In CFHR5 nephropathy, the current hypothesis is that the mutant FHR5 protein acts as a 'gain of function' FH deregulator and impairs the ability of FH to prevent accumulation of C3 along the GBM. I discuss this and further associations between familial C3GN and FHR mutations in Chapter 5.

1.6.4.1. Spontaneous porcine FH deficiency

Hereditary porcine membranoproliferative glomerulonephritis type II was described in piglets of the Norwegian Yorkshire breed that had developed spontaneous FH deficiency (Jansen, Hogasen et al. 1993, Hogasen, Jansen et al. 1995). Although healthy at birth, the piglets failed to thrive and died within 72 days from renal failure associated with rapidly progressive glomerulonephritis. Histological examination revealed thickening of the glomerular capillary wall, glomerular proliferation, and extensive dense deposits within a thickened GBM, all associated with capillary wall deposition of complement but not immunoglobulin. This lesion was therefore analogous to DDD and has been referred to as porcine DDD. Low plasma C3 levels and elevated terminal complement complex (TCC) were felt to reflect excessive fluid-phase complement activation. The increased C3 and reduced TCC levels witnessed in piglets transfused with plasma led to the subsequent fractionation of normal porcine plasma and the identification of a single protein responsible for the complement inhibitory activity. With a molecular weight of 150kDa, *in vitro* FI cofactor activity, immunologic cross-reactivity with human FH and an amino-terminal amino acid sequence similar to human, murine and rabbit FH, the single chain protein was confirmed as porcine FH.

The disease was found to follow an autosomal recessive pattern of inheritance and showed complete penetrance in homozygosity. Heterozygous carriers were healthy. FH levels in affected piglets were found to be ~3% compared to healthy littermates, whilst half normal levels were observed in their parents. Two non-synonymous single nucleotide changes were identified in the porcine factor H gene, one in SCR9 and one in SCR20 (Hegasy, Manuelian et al. 2002). FH expression at the mRNA and protein levels was found to be intact but the mutant protein was not secreted and accumulated within hepatocytes.

1.6.4.2. Gene-targeted FH-deficient mice

Mice rendered completely FH-deficient through gene targeting were also found to develop spontaneous renal disease (originally described as MPGN type 2 but now would be described as a C3 glomerulopathy) associated with increased mortality and

albuminuria (Pickering, Cook et al. 2002). Mice had severely reduced plasma C3 and C5 levels (Pickering, Cook et al. 2002, Pickering, Warren et al. 2006, de Jorge, Macor et al. 2011). Glomerular lesions consisted of mesangial matrix expansion, mesangial hypercellularity, capillary loop thickening and double contouring of the GBM. Mesangial cell interposition, new basement membrane formation and subendothelial electron dense deposits on electron microscopy were accompanied by capillary wall and mesangial C3 and C9 deposition. Subendothelial deposits contained C3 and C9 on immunoelectron microscopy, but no IgG. Glomerular deposition of C3 and C9 was found to precede the appearance of subendothelial deposits, and these were both followed by development of the light microscopic features. Notably, heterozygous FH deficiency did not result in the development of MPGN.

The absolute requirement for uncontrolled C3 activation in the development of the renal lesion was demonstrated by the lack of renal disease in FH-deficient mice that were unable to activate the AP due to the introduction of FB deficiency. Mice with combined deficiency of both FH and FB had normal plasma C3 levels and normal renal histology (Pickering, Cook et al. 2002).

The deleterious effect of glomerular C3 deposition alone was evident by comparing the spontaneous renal phenotype of mice with combined deficiency of both FH and C5 with mice that were deficient in FH alone (Pickering, Warren et al. 2006). Despite having significantly lower mortality and creatinine levels, 12-month old combined FH- and C5-deficient mice had comparable albuminuria to FH-deficient mice indicating that chronic C3 deposition along the GBM was sufficient to disrupt GBM permeability. Capillary wall double contours, GBM thickening and subendothelial electron dense deposits were still present in the FH- and C5-deficient mice, indicating that these develop in the absence of C5. However, the absence of C5 was associated with reduced glomerular cellularity, neutrophils and crescent formation (Pickering, Warren et al. 2006).

Mice with complete FH deficiency were also found to be more susceptible to renal injury induced by accelerated (Pickering, Cook et al. 2002) and heterologous (Pickering, Warren et al. 2006) serum nephrotoxic nephritis (NTN). The administration of nephrotoxic serum (NTS) to pre-immunised animals (accelerated NTN) led to haemoglobinuria and significantly greater albuminuria and mortality compared to wild-type animals. The importance of C3 activation through the AP in this phenotype was demonstrated by the prevention of the phenotype in mice with combined deficiency of FH and FB (Pickering, Cook et al. 2002). In heterologous NTN, FH-deficient mice exhibited an excess of glomerular neutrophils, additional C3 deposition and proteinuria in comparison to wild-type animals (Pickering, Warren et al. 2006). The increase in neutrophil numbers and proteinuria was shown to be dependent on C5 activation, in particular the formation of C5a rather than the MAC. Concomitant C6 deficiency failed to prevent the increased neutrophil numbers and proteinuria seen in FH-deficient mice, whilst these were abrogated by either genetic or antibody-mediated C5 deficiency (Pickering, Warren et al. 2006).

Whilst uncontrolled AP activation and isolated glomerular C3 deposition are common in human DDD there were differences between the lesions seen in the FH-deficient mice and what is typically seen in human DDD. The mice developed subendothelial electron dense deposits and not intramembranous deposits typical of DDD. So in current terms, the murine lesion would be better described as C3GN. The deposits stained directly for C3 and C9 in the mice whereas deposits in DDD characteristically stain for C3 at the margins only. On C3 staining this marginal staining results in mesangial deposits appearing as ‘rings’ and GBM C3 deposition as ‘rail-road tracks’ (Sibley and Kim 1984). Deposits are also common within Bowman’s capsule and the tubular basement membranes in DDD whereas deposits were not identified at this location in the FH-deficient mice. Interestingly, during periods of hypocomplementaemia, paramesangial C3 deposits have been described in DDD (West and McAdams 1995), which again are not seen in the FH-deficient mice.

1.6.5. Atypical haemolytic uraemic syndrome (aHUS)

aHUS is characterised by microangiopathic haemolytic anaemia, thrombocytopaenia and renal failure in the absence of infection with shigatoxin-producing bacteria like strains of *Escherichia coli*. The renal lesion is thrombotic microangiopathy. Unlike DDD, hypocomplementaemia is not typically seen in aHUS. Triggers include infection, pregnancy, malignancy, transplantation and drugs such as immunosuppressive and cancer therapies, and the oral contraceptive pill (Caprioli, Noris et al. 2006).

The role of uncontrolled AP activation in aHUS is now well recognised with 60% of patients possessing inherited or acquired abnormalities that result in AP dysregulation (reviewed in (Kavanagh and Goodship 2011)). Mutations are either loss of function changes in complement regulators: FH (Warwicker, Goodship et al. 1998, Caprioli, Bettinaglio et al. 2001, Perez-Caballero, Gonzalez-Rubio et al. 2001, Richards, Buddles et al. 2001, Sanchez-Corral, Perez-Caballero et al. 2002, Manuelian, Hellwage et al. 2003), FI (Fremeaux-Bacchi, Dragon-Durey et al. 2004, Kavanagh, Kemp et al. 2005) and MCP (Noris, Brioschi et al. 2003, Richards, Kemp et al. 2003), or gain of function changes in the AP activation proteins, FB (Goicoechea de Jorge, Harris et al. 2007) and C3 (Fremeaux-Bacchi, Miller et al. 2008). They are typically heterozygous and mutations in FH are most frequent. Acquired FH dysfunction due to anti-FH autoantibodies has been reported (Skerka, Zipfel et al. 2010). Interestingly, anti-FH autoantibodies are most frequently seen in patients with the Δ*CFHR3-1* deletion allele (Zipfel, Edey et al. 2007, Jozsi, Licht et al. 2008). aHUS in this setting has been termed ‘deficiency of FHR proteins and auto-antibody positive-HUS’ (DEAP-HUS), and it most commonly affects children (Skerka, Zipfel et al. 2010).

The molecular pathogenesis of aHUS is most clearly understood for FH mutations. The majority of FH mutations occur within the terminal SCR domains (SCR 19 and SCR 20). These mutations selectively impair the surface recognition domains of FH

since the complement regulatory domains reside within SCR domains 1-4. Mutations may be sequence variants within the exons encoding for these domains or a consequence of copy number variation. Examples of the latter are FH hybrid genes in which there are non-allelic recombination events that result in the terminal FH domains being replaced by exons from the downstream FHR proteins. This generates hybrid FH-FHR proteins that lack either both or one of the FH SCR 19 and 20 surface domains (Venables, Strain et al. 2006, Francis, McNicholas et al. 2012). Hybrid proteins described to date include FH-FHR1 (consists of FH SCR 1-18 linked to FHR1 SCR domains 4 and 5) (Venables, Strain et al. 2006) and FH-FHR3 (consists of FH SCR 1-19 linked to FHR3 SCR domains 1 to 5) (Francis, McNicholas et al. 2012).

Consistent with the selective targeting of aHUS-associated FH mutations to its surface recognition domains, functional analyses of many FH aHUS-associated mutations have demonstrated normal complement regulatory activity in fluid-phase (typically using FI cofactor assays) but impaired surface recognition activity (typically impaired binding to surface-bound C3b and polyanions with consequent limited ability to prevent complement-mediated cell lysis) (Pangburn 2002, Sanchez-Corral, Perez-Caballero et al. 2002, Manuelian, Hellwage et al. 2003). Similarly, FH autoantibodies in aHUS are directed against the surface recognition domains and result in an acquired defect of surface recognition without disrupting complement regulatory activity in plasma (Jozsi, Strobel et al. 2007).

Presently, aHUS is thought to require a combination of a complement mutation, an environmental trigger (e.g. pregnancy) and the presence of predisposing polymorphisms (Esparza-Gordillo, Goicoechea de Jorge et al. 2005, Esparza-Gordillo, Jorge et al. 2006). The latter include variants within MCP (Esparza-Gordillo, Goicoechea de Jorge et al. 2005) and certain haplotypes across the *CFH* gene locus. A FH haplotype (termed H3 and defined by five SNPs across the gene: -332T, c.184G, c.1204T, c2016G, and c.2808T) was significantly increased in patients with aHUS versus controls (Kavanagh and Goodship 2011). Interestingly, a distinct FH

haplotype (termed H1, -332C, c.184G, c.1204C, c.2016A and c.2808G) was associated with DDD. Furthermore, plasma FH levels vary widely within the general population but show consistency amongst individuals. In a Spanish population of 358 individuals from 21 pedigrees, plasma FH levels were found to vary over a five-fold range (116-562μg/ml). Of the phenotypic variability, 62% was found to be genetically determined and three quantitative trait loci, including one within the RCA gene cluster at 1q32 were detected (Esparza-Gordillo, Soria et al. 2004). Polymorphisms exist in FH for which the functional implications are not clear as they are neutral at the protein level and do not appear to be associated with altered FH levels. Three SNPs lacking any apparent effect on the protein or its circulating level have also been detected in aHUS patients more frequently than in controls (Caprioli, Castelletti et al. 2003). FH levels may influence the penetration of aHUS since inactivation of surface-bound C3b has long been known to be more sensitive to FH levels than inactivation of fluid-phase C3b (Schreiber, Pangburn et al. 1978).

The outcome for aHUS patients has been transformed by the availability of a therapeutic monoclonal antibody, termed Eculizumab that inhibits C5. This has recently been shown to be effective in both plasma-sensitive and plasma-resistant aHUS and its efficacy is independent of the presence or type of complement mutation (Legendre, Licht et al. 2013). The application of Eculizumab in this condition derived from mechanistic studies and these include the development of a mouse model of FH-associated aHUS in which the renal pathology was shown to be C5-dependent (de Jorge, Macor et al. 2011). I now discuss this model in detail.

1.6.5.1. A murine model of FH-associated aHUS

To develop a model of FH-associated aHUS, mice expressing mFH lacking the surface recognition domains were generated (Pickering, de Jorge et al. 2007). Like FH, complement regulatory domains of mFH reside within the amino SCR domains and surface recognition domains reside within the carboxyl termini (Cheng, Hellwage et al. 2006). The transgenic mice expressed a mutant mFH protein lacking the five

C-terminal SCR domains (FHΔ16-20) thereby creating a mutant protein that would lack surface recognition but maintain complement regulation in plasma. Wild-type mice transgenic for the mutant mouse protein were inter-crossed with FH-deficient mice (*Cfh*$^{-/-}$) to generate either homozygous (*Cfh*$^{-/-}$.FHΔ16-20) or heterozygous (*Cfh*$^{+/-}$.FHΔ16-20) FH-deficient mice transgenic for FHΔ16-20 (Pickering, de Jorge et al. 2007). Significantly higher C3 levels were observed in *Cfh*$^{-/-}$.FHΔ16-20 mice compared to *Cfh*$^{-/-}$ mice, and similarly in *Cfh*$^{+/-}$.FHΔ16-20 mice compared with *Cfh*$^{+/-}$ animals. This demonstrated that the mutant FHΔ16-20 protein could regulate plasma C3 activation *in vivo*. Furthermore, this was sufficient to prevent the accumulation of C3 along the GBM that is characteristic of FH-deficient mice. As *Cfh*$^{-/-}$.FHΔ16-20 mice aged many developed haematuria and premature mortality. In mice that were unwell there was uraemia and red cell fragmentation on peripheral blood smears. Critically, the renal pathology showed thrombotic microangiopathy and no evidence of the C3GN-like lesions typical of the FH-deficient mouse. When these mice were rendered C5-deficient (by inter-crossing with the spontaneous C5-deficient DBA2 strain that had been previously back-crossed on to the C57BL/6 genetic background) renal thrombotic microangiopathy was prevented although abnormal glomerular mesangial C3 deposition persisted (de Jorge, Macor et al. 2011). This indicated a critical role for C5 in the development of thrombotic microangiopathy in these animals and this has been recapitulated by the success of Eculizumab in aHUS. The relative contribution of C5a and MAC to renal injury is still to be ascertained.

Notably, a second transgenic line was reported that expressed FHΔ16-20 at levels of only 2% of normal wild-type FH levels. These animals did not develop thrombotic microangiopathy but did develop mild mesangial expansion in association with mesangial C3 and infrequent subendothelial electron dense deposits (Pickering, de Jorge et al. 2007). The interpretation was that for thrombotic microangiopathy to develop some degree of intact complement is required in plasma in addition to defective regulation along the renal endothelium. FH-deficient mice have no FH protein in circulation so have both defective plasma and surface regulation. However, the defective surface regulation does not result in thrombotic microangiopathy since there is no intact complement in plasma to mediate the injury.

In this respect FH-associated aHUS mutations can be thought of as deleterious because they provide inadequate surface protection whilst maintaining intact complement in plasma to mediate the endothelial injury.

1.7. Generation of a conditional FH-deficient mouse

In my thesis I wanted to explore the contribution of local FH to renal injury. To test this experimentally I wanted to be able to generate tissue-specific FH deficiency in mice. In my supervisor's laboratory, they had generated a conditional FH-deficient mouse, depicted schematically in figure 1.5. The strategy was to flank exons 2 and 3 of the mouse *cfh* gene with *loxP* sites so that this would enable excision of these exons by Cre recombinase. The Cre recombinase of the P1 bacteriophage is a site-specific recombinase that recognises a specific 34-bp consensus recognition site (termed 'locus of X-over P1' and denoted *loxP*) and recombination between the *loxP* sites results in excision of the intervening DNA sequence. Deletion of exons 2 and 3 was predicted to result in a reading frameshift leading to the generation of a stop codon in exon 4 and therefore a null allele (figure 1.6). In the original gene-targeted FH-deficient animal, deletion of exon 3 was performed. This also resulted in a reading frameshift and a predicted null allele which was subsequently demonstrated *in vivo* (Pickering, Cook et al. 2002). The targeted locus also contained a neomycin-resistant (*Neo*) positive selectable marker flanked by flippase (Flp) recombinase recognition target (*FRT*) sites. The positive selectable marker enabled selection of the desired recombination events in embryonic stem cells. The flanking *FRT* sites enabled excision of the *FRT*-flanked *Neo* cassette using Flp recombinase at a later stage if needed. The conditional FH-deficient mouse was successfully generated and was back-crossed onto the C57BL/6 genetic background prior to inter-crossing with Cre recombinase-expressing strains.

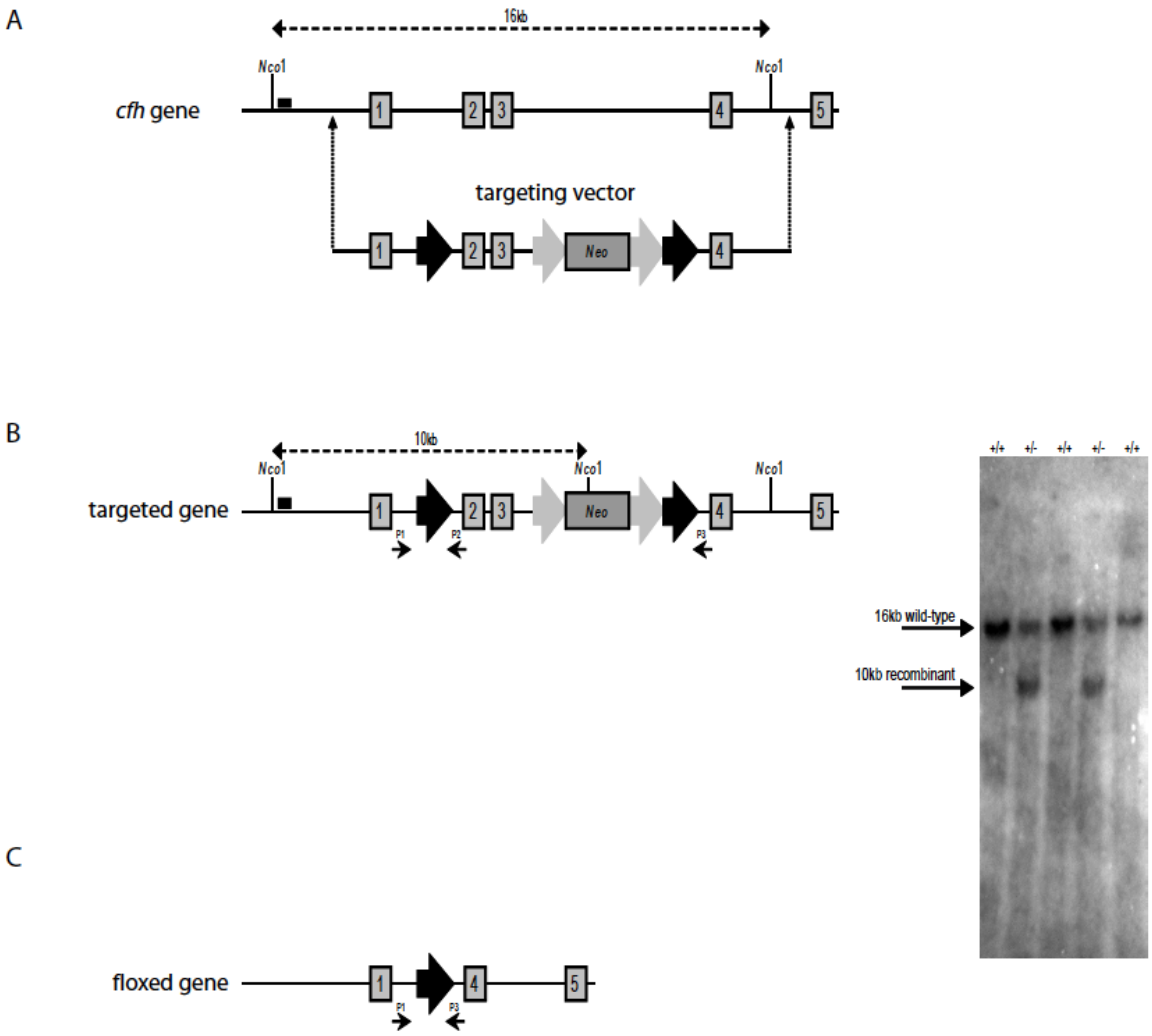


Figure 1.5 **Generation of conditional FH-deficient mouse.** (A) The targeting vector contained *loxP* sites located either side of exons 2 and 3 of the *cfh* gene and a positive selectable marker (neomycin cassette, *Neo*) flanked by *FRT* sequences (see text). (B) Following homologous recombination the targeted gene contains the construct elements without altering the exon structure of the *cfh* gene. The recombination event was detected by Southern blotting using a probe (as indicated) that differentiated between a 16-kb and 10-kb Nco1 genomic DNA fragment. (C) Predicted gene structure following Cre recombinase excision of the *loxP*-flanked DNA.

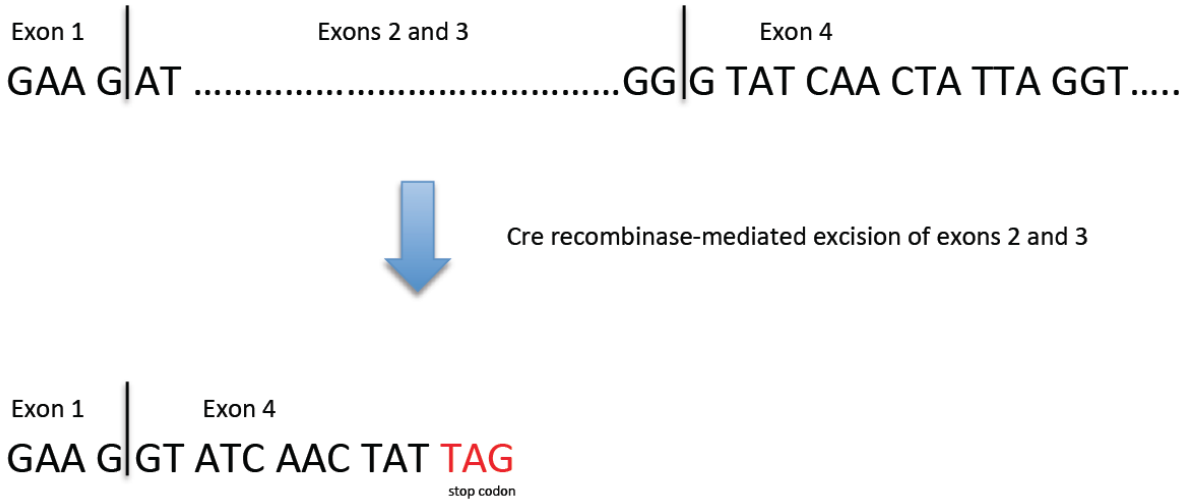


Figure 1.6 **Deletion of exons 2 and 3 of the mouse *cfh* gene.** Exon junction sequences show that exon 1 is in intron phase 1 whilst exon 3 is in intron phase 2. After excision of exons 2 and 3 there is a frameshift in the reading sequence with the generation of a stop codon (TAG) at the fourth codon within exon 4.

1.8. Aims of my project

The overarching aim of my thesis was to experimentally dissect the contributions of local and hepatic-derived FH.

My specific aims were:

1.8.1. To define the contribution of extra-hepatic FH production to plasma C3 regulation and spontaneous renal disease associated with FH deficiency.

I approached this experimentally by generating and analysing a novel hepatocyte-specific FH-deficient mouse strain. This was achieved by inter-crossing the conditional FH-deficient strain with mice expressing Cre recombinase under the control of a hepatocyte-specific promoter. I discuss these experiments in Chapter 3.

1.8.2. To define the contribution of extra-hepatic FH production to experimentally triggered renal injury.

FH-deficient mice were previously demonstrated to be hypersensitive to experimentally triggered renal injury using either the heterologous or accelerated NTN models. Based on these observations and my data from the analysis of the spontaneous phenotype of the hepatocyte-specific FH-deficient mouse, I assessed the response of this strain to accelerated NTN. I discuss these experiments in Chapter 4.

1.8.3. To understand the mechanism underlying the association between abnormalities in FHR proteins and C3 glomerulopathy.

During my thesis I characterised the recurrence of CFHR5 nephropathy in the transplant kidney and characterised an individual with C3 glomerulopathy associated with infection and heterozygous deficiency of FHR5. I discuss these experiments in Chapter 5.

2. CHAPTER TWO: MATERIALS AND METHODS

2.1. Molecular biology techniques

2.1.1. Ribonucleic acid (RNA) extraction

Mouse liver, whole mouse kidney, and human liver and kidney tissue were homogenized in liquid nitrogen and RNA extracted using RNA-Bee isolation reagent (Amsbio, Abingdon, UK). The RNAqueous®-4PCR kit (Ambion, Huntingdon, UK) and RNeasy mini kit (Qiagen, Manchester, UK) were subsequently used to extract RNA. The methodology chosen was determined by the starting material and remained the same within experiments. Human liver and kidney tissue were obtained from the tissue bank at our institution after appropriate ethical approval was given.

2.1.2. Deoxyribonucleic acid (DNA) extraction from mouse tissue

Mouse tissue was digested overnight in 400µl of lysis buffer (0.2M NaOH, 1% sodium dodecyl sulphate [SDS]) containing proteinase K at 55°C in a rotating hybridisation oven. Proteinase K was heat inactivated (75°C for 10 minutes) prior to use in polymerase chain reactions.

2.1.3. Complementary DNA (cDNA) synthesis

Whilst cDNA was initially synthesized using oligo$(dT)_{12\text{-}18}$ primers and Superscript™ II Reverse Transcriptase (RT) (Invitrogen, Paisley, UK), this was superceded by the use of random primers to ensure transcription of 5' sequence. Following denaturation at 68°C for 10 minutes, 2µg RNA was typically transcribed in the presence of buffer, deoxynucleotide triphosphate (dNTP), RT random primers, ribonuclease (RNase) inhibitor and reverse transcriptase using the Applied Biosystems High Capacity cDNA reverse transcription kit (Applied Biosystems, Warrington, UK). Thermocycling conditions were: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 seconds and 4°C to infinity.

2.1.4. Polymerase chain reaction (PCR) and quantitative PCR (qPCR)

PCR conditions were optimised for each reaction as required. Amplification of both cDNA and genomic DNA involved the use of 50-100ng DNA in each PCR reaction, accompanied by 100ng of sequence-specific oligonucleotide primers and 0.2mM of each dNTP in the presence of NH_4 buffer, $MgCl_2$ and Bio*Taq* DNA polymerase. Thermal cycling programmes typically consisted of incubation at 94°C for 2 minutes, followed by 30 cycles of: 94°C for 30 seconds (denaturation), 50-65°C for 1 minute (annealing; variable temperature depending on individual primers), 72°C for 1 minute (extension), and 72°C for a further 10 minutes.

Relative expression of FH and the FHR proteins in liver and kidney tissue was investigated using qPCR. Beta-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as housekeeping genes. Primers were designed to yield an amplicon of between 150-200 base pairs (bp) in size, and optimal annealing temperatures determined using gradient PCR. Reactions were performed with the Stratagene Brilliant II SYBR green qPCR kit (Agilent, Stockport, UK) using 100ng of cDNA for the qPCR reaction. The ABI 7900H thermal cycler (Applied Biosystems, Warrington, UK) was used to perform the reaction, and a dissociation step was added to determine the melting temperatures of the PCR products and identify the presence of any primer-dimers or non-specific products.

The primers used are listed in table 2.1.

Table 2.1.**List of primers**

Target	Primer pairs (forward + reverse primer in 5 prime to 3 prime orientation)	size (bp)
mFH	TAATAGTCGGTGGATTGGAC + CTCGGCACTTTGGTTTTT TAATAGTCGGTGGATTGGAC + CTGTCCATATCCCATTTTCAC CTGTCAGCAAGAATTATTTGGC + ACACATCGTGGCTTTTCATTGC AGGGGGAAAGAGAGAGATG + CAGCAGCTGGCCTTCTCTGCTTGTGTAAG	212 193 ** ***
FHR-A	CAGTGCCAGAACTATTATGTAC + CTGTAGCAGTAATATTCATGTGC ATATCAGTGCCAGAACTATTATG + AACTCAATATCCTCTCCTGAC	539 191
FHR-B	GTACAGAGAATGGCTGGTC + AGTGATCCTCTTGCTTTCTG CAACCTGTTTACATGCATGTG + CAGTGATCCTCTTGCTTTCTG	370 154
FHR-C	CTGTGACATGTATTAATGGCC + CACTGATAGTCAACTGATGAG	150
Beta actin Mouse Human	 CCTCTATGCCAACACAGTG + TGATCCACATCTGCTGGAAG CACCATGTACCCTGGCATTG + TGCTTGCTGATCCACATCTG	 197 170
GAPDH Mouse Human	 TGTCTCCTGCGACTTCAAC + CCAGGGTTTCTTACTCCTTG GTCTCCTCTGACTTCAACAG + CTTACTCCTTGGAGGCCATG	 176 165
FH	TGCACATGGGACTAAATTGAG + CGGATTCACACAGGAGGTG TAGGAGAAAAATGGTCTCACC + CGGATTCACACAGGAGGTG	481 172
FHR1	CATTTTCCCAGGTTCCTACAG + CTGTCTCGACAGTATATGAGC	365
FHR2	ATCTGGAAGGTGATACTGTAC + TCTGACACATTGCTCGAAATG	465
FHR3	CACACAAAATGGGTGGTCAC + CTGTCACTTCTTCCTTTTAAC	647
FHR4	GAGCCAAGAGTAATGGCATG + GTGAGAAGTGGAAGTTGGAC	569
FHR5	FP(2) + RP(3): GCCACCTCAGATACCTAATG + TTCTTCAGATACCACACATGG FP(3) + RP(3): CTCAGAATATGACAACCACAG + TTCTTCAGATACCACACATGG gFP(2) + gRP(2): TCCTCAACTCTCCAATGGTG + TCATTTCTGCAATTCACCTCG TAACAATGGAGATACCACCTC + TTCTTCAGATACCACACATGG	 363 343 266 171
mFH	loxP(FP) + loxP(RP): AGGGGGAAAGAGAGAGATG + TCCTAGTAGCAAAGCTAAACAG	 *
ALB-CRE	CRE-1(FP) + CRE-2(RP): GCGGTCTGGCAGTAAAAACTATC + GTGAAACAGCATTGCTGTCACTT	 100
IL-2	IL-2(FP) + IL-2(RP): CTAGGCCACAGAATTGAAAGATCT + GTAGGTGGAAATTCTAGCATCATCC	 324

*450-bp amplicon in wild-type *cfh* gene and 527-bp amplicon in floxed *cfh* gene

**610-bp amplicon in wild-type *cfh* gene and 318-bp amplicon in *cfh* gene after Cre recombinase-mediated excision of exons 2 and 3

***546-bp amplicon in *cfh* gene after Cre recombinase-mediated excision of exons 2 and 3

2.1.5. Agarose gel electrophoresis

Agarose was dissolved in Tris-acetate buffer (TAE) to a concentration of between 1-3% depending on the size of the DNA fragments to be visualised. TAE was prepared as a 50x stock comprised of: 2M Tris-acetate and 50mM ethylene diamine tetra-acetic acid (EDTA). Ethidium bromide was then added to a final concentration of 0.2-0.25 µg/ml, after which the mixture was poured into horizontal gel trays to set. For visualisation of PCR products, 10µl of DNA was added to 5µl of loading buffer (30% glycerol, 0.25% xylene cyanol, 0.25% bromophenol blue) and loaded into the gel alongside an appropriate size marker e.g. Hyperladder I (Bioline, London, UK). Electrophoresis was performed in 0.5x TAE buffer at a constant voltage of 120 Volts. Nucleic acids were subsequently visualised and photographed using the gel imaging system: G Box (Syngene, Cambridge, UK).

2.1.6. DNA purification from PCR mixtures and agarose gels and sequencing

Following amplification by PCR, DNA fragments were purified using the ChargeSwitch PCR clean-up kit (Life Technologies, Paisley, UK). Utilising magnetic beads with a changeable surface charge depending on the pH of the surrounding buffer, low pH conditions were used to bind DNA (from a 25µl aliquot of PCR product) to positively charged beads. Wash buffer was used to remove unbound proteins and other contaminants, and neutralisation of the surface charge by raising the pH to 8.5 resulted in elution of the purified DNA into a low salt elution buffer (elution volume 25µl).

Following electrophoresis on a 1% agarose gel and visualisation using ultraviolet light, DNA excised in an agarose band was purified using the Illustra GFX PCR DNA and gel band purification kit according to the manufacturer's instructions (GE Healthcare UK Ltd, Little Chalfont, UK).

Sequencing reactions were typically prepared using 90ng of purified DNA (at least 10ng DNA per 100bp) in a 10µl reaction containing 0.32 pmol per primer. Sequencing reactions were performed using BigDye Terminator 3.1 Cycle Sequencing Kit (Life Technologies, Paisley, UK). Both PCR and sequencing reaction products were purified using Edge Biosystems Gel Filtration plates (Life Technologies, Paisley, UK). The sequence was run on a 3730 DNA Analyzer (Life Technologies, Paisley, UK) and analyzed using the Sequencer DNA sequencing analysis software.

2.2. Mice

Mice were housed in specific pathogen-free conditions and all animal procedures were performed in accordance with institutional guidelines and under licence by the UK. Mice used in models of experimental renal disease were matched for age and sex, and aged between 10 to 12 weeks at the time of the experiment. Animals were sacrificed by terminal anaesthesia using hypnorm (fentanyl citrate and fluanisone) mixed with midazolam and distilled H_2O in a ratio of 1:1:2. Mice expressing Cre recombinase under the control of the murine albumin promoter (Alb-Cre) were purchased from the Jackson laboratories (B6.Cg-Tg[Alb-cre]21Mgn/J, strain number 003574, http://jaxmice.jax.org/strain/003574.html). Genotyping was performed by PCR on digested tissue using primers (table 2.1 and chapter 3).

2.3. Mouse phenotypic analyses

2.3.1. Preparation of mouse plasma

Mouse blood samples were collected directly into EDTA-containing Eppendorf tubes and kept on ice until centrifugation and aliquots stored at -80°C. Repeated freeze/thaw cycles were avoided.

2.3.2. Measurement of mouse plasma FH levels

Plasma FH levels were measured by enzyme-linked immunosorbent assay (ELISA) using a set of cross-reactive anti-human FH polyclonal antibodies. The capture antibody was: polyclonal sheep anti-human FH antibody (antibodies-online.com) and detection antibody was: biotinylated goat anti-human FH antibody (Quidel, San Diego, USA). FH levels in the samples were quantified relative to normal wild-type mouse plasma used in the standard curve.

The ELISA procedure was as follows: NUNC Maxisorp ELISA plates (Thermoscientific, Waltham, USA) were coated with sheep anti-human FH antibody diluted 1:1000 in 50 mM carbonate buffer (0.795g Na_2CO_3 and 1.565g $NaHCO_3$ in 500ml distilled H_2O, pH 9.6) overnight at 4°C. Following one wash with phosphate-buffered saline (PBS)-Tween, wells were blocked with 2% bovine serum albumin (BSA)/PBS (1g BSA in 50mls 1x PBS) for 2 hours at room temperature, after which two further washes with PBS-Tween were performed. Plasma samples were diluted 1:25 in 2% BSA/PBS (ascertained by prior optimisation) and incubated in duplicate at room temperature for 1 hour. A standard curve was generated on each plate using normal wild-type mouse plasma diluted in 2% BSA/PBS from a dilution of 1:12.5 to 1:256,000 using double dilutions. Biotinylated goat anti-human FH antibody was then added at a concentration of 5µg/ml for 1 hour at room temperature following four washes. Streptavidin-alkaline phosphatase (BD Biosciences, Oxford, UK) diluted 1:2000 with 2% BSA/PBS was then added for 1 hour at room temperature after repeated washing as before. Further washes were followed by the addition of p-nitrophenyl phosphate (tablets dissolved in double-distilled H_2O as per manufacturer's instructions; Sigma, Dorset, UK) and the optical densities were read at 405nm using an ELISA reader.

2.3.3. Measurement of mouse plasma C3 levels

Plasma C3 levels were measured by ELISA. The capture antibody was: polyclonal goat anti-mouse C3 antibody (MP Biomedical, Cambridge, UK) and the detection

antibody was the same antibody but conjugated to horseradish peroxidase (HRP) (MP Biomedical, Cambridge, UK). The standard curve was generated using acute phase serum containing a known quantity of C3 (Calbiochem, Hertfordshire, UK) and used as a reference to quantify samples.

The ELISA procedure was as follows: NUNC Maxisorp ELISA plates were coated with 50µl of polyclonal goat anti-mouse C3 antibody diluted 1:8000 in coating buffer (0.1M sodium bicarbonate, pH 9.5), and left at 4°C overnight. The plates were then washed once for 5 minutes with 100 µl/well of wash buffer comprising 50mM Tris base, 150mM NaCl and 0.2% Tween 20, adjusted to a pH of 7.4 using hydrochloric acid. 100µl blocking buffer (2% BSA in wash buffer) was then added to each well for 1 hour at room temperature. This was followed by two further 100µl washes with wash buffer after which samples were added to the plates. Acute phase serum was used to generate a standard curve through the use of double dilutions starting from a dilution of 1:500 down to 1:2,048,000 using blocking buffer. Plasma samples were diluted 1:12000 using blocking buffer, and as for the standard curve, were tested in duplicate and incubated for 1 hour at room temperature. Following five consecutive five-minute washes with wash buffer, 50µl of HRP-conjugated goat anti-mouse C3 antibody diluted 1:25,000 in blocking buffer, was added to each well for 1 hour at room temperature. After five further washes, 50µl of tetramethylbenzidine (TMB) substrate (prepared by mixing equal volumes of solutions A and B) was then added to each well, and the reaction was stopped thereafter by adding 20µl of stop solution. The plates were then read at 450 and 540nm.

2.3.4. Western blotting of mouse FH and C3

Mouse FH was detected using a polyclonal sheep anti-human FH antibody (antibodies-online.com) at a dilution of 1:2,000 in blocking buffer. Mouse C3 was detected using a polyclonal goat anti-mouse C3 antibody (MP Biomedical, Cambridge, UK). Detection of both antibodies was performed using HRP-conjugated monoclonal anti-goat/sheep IgG (diluted 1:20,000 in blocking buffer; Sigma-Aldrich,

Dorset, UK). Reduced samples were prepared by the addition of 2-mercaptoethanol to the sample mix and heating to 96°C for 10 minutes prior to loading into the gel. Rainbow protein marker (GE Healthcare, Amersham, UK) was used to determine protein size.

The western blotting procedure was as follows: 8% resolving and 4% stacking acrylamide gels were prepared using acrylamide:bisacrylamide solution and the respective resolving/stacking buffer dissolved in double-distilled water. 10% ammonium persulphate was added as a source of free radicals to initiate polymerisation and was stabilised by the addition of N, N, N', N'-tetramethylethylenediamine to optimise polymerisation. Gels were polymerised between two glass plates in a gel caster. The stacking gel solution was added on top of the resolving gel once it had polymerised and wells were created by the insertion of a comb prior to polymerization. Following loading onto the gel, proteins were separated by SDS-PAGE in the presence of running buffer (0.192M glycine, 0.025M Tris base, 0.1% SDS (w/v), pH 8.3) using a constant voltage of 200V across the gel for approximately 1 hour in the Bio-Rad mini protean II system (Bio-Rad, Hemel Hempstead, UK). Gels were then removed and placed in transfer buffer (0.039M glycine, 0.048M Tris base, 0.0375% SDS (w/v), pH 8.3) following removal of the stacking gel. After activation with methanol for 15 seconds, polyvinylidine fluoride (PVDF) membrane (Sigma-Aldrich, Dorset, UK) was also placed in transfer buffer. The gel and PVDF membrane remained in their respective transfer buffer at room temperature for 5 minutes, following which they were prepared for protein transfer. The gel was carefully laid onto a clean glass plate on top of which was laid a piece of PVDF membrane, with care taken to remove any air bubbles between the gel and membrane. Two pieces of 3MM paper soaked in transfer buffer were then placed on top of the membrane, followed by the outer sponge. After removal from the glass plate, two pieces of 3MM paper soaked in transfer buffer were placed on the exposed surface of the gel, on top of which was placed the remaining outer sponge. This "sandwich" was then placed into the transfer tank filled with transfer buffer, and proteins were transferred at 300mA for an hour. After transfer, the membrane was

removed and placed into blocking buffer (PBS, 0.05% Tween 20, 3% BSA) for 30 minutes at room temperature.

After incubation with the relevant antibodies for one hour, the membrane was washed three times with PBS-Tween (PBS, 0.05% Tween 20) and the appropriate HRP-conjugated secondary antibody added for 30 minutes at room temperature. Four further washes with wash buffer were followed by visualisation of the proteins using Pierce electrochemiluminescent (ECL) substrate (Thermo Scientific, IL, USA). Equal volumes of solutions 1 and 2 were mixed and then pipetted uniformly onto the membrane for 1 minute at room temperature. Excess solution was allowed to drain off and the membrane was inserted between two layers of plastic film and then exposed to Kodak BioMax MS Film (Sigma-Aldrich, Dorset, UK).

2.3.5. Plasma urea levels

Plasma urea was measured using an ultraviolet method according to the manufacturer's instructions (R-Biopharm, Glasgow, UK). Plasma samples were diluted 1:40 using 1x PBS and a standard preparation of urea of known concentration was included as an internal control.

2.3.6. Albuminuria

Mice were placed in metabolic cages for 24 hours to facilitate urine collection for quantification of albuminuria. Radial immunodiffusion using a rabbit anti-mouse albumin antibody (AbD Serotec, Oxford, UK) was used to quantify albuminuria, with purified mouse albumin (Sigma-Aldrich, Dorset, UK) used as a standard. 10ml aliquots of 1.2% agarose were prepared by dissolving 1.2g agarose in 100ml 1x PBS, and were equilibrated at 55°C prior to the addition of 150µl of rabbit anti-mouse albumin antibody. Following mixing, the gel was poured onto a 3-inch glass plate and allowed to set at 4°C for 30 minutes. Wells of a known volume were then formed in the gel and arranged in a 7x4 matrix. A standard curve was generated using double dilutions of purified mouse albumin in 1x PBS, starting from a concentration of 10mg/ml down to 0.078mg/ml. Analysis of proteinuria using urine reagent sticks

determined whether urine samples were added neat to the wells or at a dilution of 1:10. Urine samples with 3+ or 4+ proteinuria by urine dipstick were diluted 1:10 in 1x PBS, whilst those with ≤ 2+ proteinuria were added neat. 5µl of urine or standard was added to each well, and the gel was then left in a wet box overnight at 4°C. 1x PBS was then used to wash the gel three times after which the gel was dried on gel bond. Pre-wetted 3MM paper was then placed on top of the gel followed by a stack of towels and a weight for an hour at room temperature. The gel bond (with the transferred gel) was dried and placed in Coomassie blue for approximately 30 minutes until stained. Gels were destained using destain solution: 1500mls methanol, 500mls glacial acetic acid and 3000mls distilled water. The diameter of each ring was measured using a graduated magnifying lens and the concentration of albumin in each sample calculated by reference to the standard curve.

2.3.7. Urinalysis

Proteinuria and haematuria were assessed by adding urine to Hema-Combistix urine reagent strips (Siemens, Frimley, UK). For haematuria the strips detected: negative, trace, 1+, 2+ and 3+. For proteinuria the strips detected: negative, trace, 1+ (0.3 g/l), 2+ (1 g/l), 3+ (3 g/l) and 4+ (≥20 g/l).

2.3.8. Haematocrit

Haematocrit was measured by centrifuging EDTA-treated blood in ammonium-heparin capillary tubes (VWR International Ltd, Lutterworth, UK) at 13,000rpm for 5 minutes after which haematocrit was calculated by measuring the packed cell height to total height ratio.

2.3.9. Red cell fragments

4ml of EDTA-treated blood was used to make a peripheral blood smear on glass slides. Once dry, the smear was stained using the Diff Quick staining kit (Polysciences, Warrington, USA) and mounted with DePex mounting medium (VWR

International Ltd, Lutterworth, UK). Red cell fragments were quantified as the number per 200 red blood cells counted at high power (x40).

2.4. Histological studies

2.4.1. Light microscopy

Kidneys were fixed in Bouin's solution (Sigma-Aldrich, Dorset, UK) for 2-4 hours and then transferred to 70% ethanol. Sections were embedded in paraffin and stained with periodic acid-Schiff (PAS) reagent and examined by light microscopy. Sections were scored for glomerular thrombosis and glomerular neutrophil infiltration. Analyses were all done blinded to the sample identity with 20 glomeruli scored per section. Glomerular thrombosis was scored according to the amount of PAS-positive material per glomerular cross-section as follows: grade 0, no PAS-positive material; grade 1, 0-25%; grade 2, 25-50%; grade 3, 50-75%; grade 4, 75-100%. Glomerular neutrophils were scored according to the number of neutrophils per glomerular cross-section. Glomerular histology was graded as follows: grade 0, normal; grade 1, segmental hypercellularity in 10–25% of the glomeruli; grade 2, hypercellularity involving >50% of the glomerular tuft in 25–50% of glomeruli; grade 3, hypercellularity involving >50% of the glomerular tuft in 50–75% of glomeruli; grade 4, glomerular hypercellularity in >75% or crescents in >25% of glomeruli.

2.4.2. Immunostaining for mouse C3, mouse IgG and sheep IgG

Kidneys were placed in 1x PBS on collection and then transferred into 15 x 15 x 5mm plastic moulds (Dispomould, CellPath Ltd, Newtown, UK) containing OCT embedding matrix (CellPath Ltd, Powys, UK). They were then snap frozen in isopentane pre-cooled over liquid nitrogen and stored at -80°C until cut on a cryostat (Bright Instruments, Huntingdon, UK). Frozen sections were cut at a thickness of 5µm, dried in acetone (immersed for 15 minutes and allowed to air dry) and stored at -80°C until use. Kidney sections were stained for murine C3, murine IgG and sheep

IgG by direct immunofluorescence. Slides were defrosted for 10 minutes and the section then encircled using a PAP pen (Invitrogen, Paisley, UK).

For mouse C3 and mouse IgG staining, sections were blocked using 60µl of 20% normal goat serum (diluted in 1x PBS) for 30 minutes at room temperature. For C3 staining: 60µl of fluorescein-isothiocyanate (FITC)-labelled polyclonal goat anti-mouse C3 (MP Biomedical, Cambridge, UK) diluted 1:100 in 1x PBS was applied for 1 hour at room temperature. For mouse IgG staining: a FITC-conjugated polyclonal goat anti-mouse IgG Fc γ-chain specific antibody (Sigma-Aldrich, Dorset, UK) diluted 1:200 in 1x PBS was used. For sheep IgG, sections were incubated with 60µl of FITC-conjugated monoclonal mouse anti-goat/sheep IgG (Sigma-Aldrich, Dorset, UK) diluted 1:100 in 1x PBS for 1 hour at room temperature without a preceding blocking step. Sections were then washed three times in 1x PBS and mounted using Vectashield Hard Set mounting medium (Vector Laboratories, Peterborough, UK). Sections were kept covered in the dark at 4°C and left overnight for the mounting medium to set.

2.4.2.1. Immunostaining for mouse C3d

Sections were blocked using 60µl of 20% normal goat serum (diluted in 1x PBS) for 30 minutes at room temperature. After a brief wash in 1x PBS they underwent the first step of biotin blocking using 1 drop of avidin for 10 minutes at room temperature (Dako, Ely, UK). A 5-minute wash with 1x PBS was then followed by a second blocking step at room temperature using 1 drop of biotin for 10 minutes. Following a further 5-minute wash, a biotinylated goat anti-mouse C3d antibody (R&D, Minneapolis, USA) was added (diluted 1:10 in 1x PBS) for 1 hour at room temperature. The sections were then incubated with 1:100 streptavidin-phycoerythrin (BD Biosciences, Oxford, UK) for 30 minutes at room temperature after three washes had been performed. The sections were mounted using Vectashield Hard Set mounting medium (Vector Laboratories, Peterborough, UK) after three final 5-minute washes with 1x PBS. For dual staining (mouse C3d and mouse C3) the

procedure was followed by addition of FITC-labelled polyclonal goat anti-mouse C3 (MP Biomedical, Cambridge, UK).

2.4.2.2. Immunostaining for mouse FH

Sections were blocked using 60µl of 20% normal goat serum (diluted in 1x PBS) for 30 minutes at room temperature. For mouse FH a cross reactive anti-human FH antibody was used: goat anti-human FH antibody (Quidel, San Diego, USA) diluted 1:100 in 1x PBS was applied to the section for 1 hour at room temperature. Three washes in 1x PBS were followed by incubation with FITC-conjugated mouse anti-goat/sheep IgG (Sigma-Aldrich, Dorset, UK) diluted 1:100 in 1x PBS for an hour at room temperature. Sections were mounted as described above. For dual staining (mouse FH and mouse C3): goat anti-human FH antibody (Quidel, San Diego, USA) diluted 1:100 in 1x PBS was applied to the section for one hour at room temperature. Three washes in 1x PBS were followed by application of a Texas Red-conjugated rabbit anti-goat IgG (Abcam, Cambridge, USA) diluted 1:200 in 1x PBS for an hour at room temperature. Following three washes with 1x PBS, FITC-labelled polyclonal goat anti-mouse C3 (MP Biomedical, Cambridge, UK) diluted 1:100 in 1x PBS was applied for 1 hour at room temperature as described above. Sections were mounted as described above.

2.4.2.3. Quantitative immunofluorescence analysis

Quantitative immunofluorescence analyses were performed on sections stained at the same time to prevent variability due to fluorochrome decay and without knowledge of the sample identity as previously described (Robson, Cook et al. 2001). Sections were visualised using an Olympus fluorescent microscope with digital camera (Olympus, Southend, UK) and images analysed using Image-Pro Plus software (Media Cybernetics, Silver Spring, USA). Ten glomeruli were examined per section with the mean fluorescence intensity expressed in arbitrary fluorescence units (AFU).

2.4.2.4. Electron microscopy

1mm^3 samples of renal cortex were fixed in 3% glutaraldehyde overnight and post-fixed in 2% aqueous osmium tetroxide prior to embedding in Spurr's resin. Ultra-thin sections were stained with 1% aqueous uranyl acetate and Reynold's lead citrate.

2.5. Serum nephrotoxic nephritis

Nephrotoxic nephritis (NTN) was induced by the intravenous injection of 200µl of sheep nephrotoxic serum (NTS), a sheep IgG serum fraction containing anti-mouse GBM antibodies. The NTS had been previously prepared in my supervisor's laboratory. Five days prior to administration of the NTS, mice were pre-immunised with an intra-peritoneal injection of 200µg of sheep IgG (Sigma-Aldrich, Dorset, UK) in complete Freund's adjuvant (CFA) (Sigma-Aldrich, Dorset, UK). The sheep IgG in CFA was prepared as an emulsion containing 4mls of 1x PBS, 5mls of CFA and 10mg of sheep IgG. The final concentration of sheep IgG in the emulsion was 200 micrograms per 200 microlitres (the injection volume). Prior to immunisation and the administration of NTS, all mice were weighed and urinalysis recorded. Thereafter, urinalysis was recorded daily and on the penultimate day of the experimental period, when all mice were placed in metabolic cages overnight in order that urine could be collected for formal quantification of albuminuria. The duration of the experimental period was variable depending on the strain of mice used (see chapter 4).

2.6. Analysis of FHR5

2.6.1. Quantification of FHR5 by ELISA

An ELISA was used to measure serum FHR5 levels. The capture antibody was: rabbit anti-human FHR5 (Abnova, Taipei, Taiwan). The detection antibody was: mouse anti-human FHR5 (Abnova, Taipei, Taiwan). A standard curve generated using recombinant FHR5 (R&D, Minneapolis, USA) was used to calculate serum FHR5 concentrations. NUNC maxisorp ELISA plates were coated with 50µl of rabbit anti-human FHR5 antibody diluted 1:500 in capture buffer (0.1M $NaHCO_3$, pH 9.5)

overnight at 4°C. Following washing with PBS-Tween (0.2%), 1% BSA (diluted in wash buffer) was used as blocking buffer with 100µl added to all wells for 1 hour at room temperature. After washing twice with PBS-Tween, samples as prepared below were added to the plate. Recombinant FHR5 diluted in blocking buffer was used to generate a standard curve through the use of double dilutions. As for the standard curve, serum samples diluted in blocking buffer were added in duplicate (50µl/well) and incubated at room temperature for 1 hour. Following four washes, 50µl of mouse anti-human FHR5 antibody (diluted 1:1000 in blocking buffer) was added to each well for 1 hour at room temperature. This was followed by incubation with HRP-conjugated polyclonal rabbit anti-mouse immunoglobulin antibody (Dako, Ely, UK) diluted 1:1000 in blocking buffer for 30 minutes at room temperature after four washes had been completed. TMB substrate (comprising equal volumes of solutions A and B) was then added to each well for 5-10 minutes. The reaction was subsequently stopped with stop solution and the plate read at 450 and 540nm.

2.6.2. Western blotting of FHR5

FHR5 was detected using a rabbit anti-human FHR5 (Abnova, Taipei, Taiwan) and HRP-conjugated polyclonal swine anti-rabbit immunoglobulin antibody (Dako, Ely, UK). Following separation of non-reduced serum samples by SDS-PAGE, western blotting and detection were performed as described above using Pierce electrochemiluminescent (ECL) substrate and Kodak BioMax MS Film.

2.7. List of antibodies

The following table provides the details of the antibodies used in the thesis:

Table 2.2 **List of antibodies**

Antibody description	Supplier:	Cat. No.
FITC-conjugated polyclonal goat anti-mouse C3 (IgG fraction)	MP Biomedical, Cambridge, UK	55500
Polyclonal goat anti-mouse C3d (antigen affinity purified)	R&D, Minneapolis, USA	AF2655
FITC-conjugated polyclonal goat anti-mouse IgG (Fc specific, affinity isolated antibody, buffered aqueous solution)	Sigma-Aldrich, Dorset, UK	F5387
FITC-conjugated monoclonal mouse anti-goat/sheep IgG (purified immunoglobulin, buffered aqueous solution)	Sigma-Aldrich, Dorset, UK	F4891
Texas Red-conjugated polyclonal rabbit anti-goat IgG (IgG fraction)	Abcam, Cambridge, USA	A6739
Polyclonal goat anti-human FH (whole anti-serum)	Quidel, San Diego, USA	A312
Polyclonal mouse anti-human FHR5 (MaxPab® antibody*, buffered aqueous solution)	Abnova, Taipei, Taiwan	H00081494-B01P
Polyclonal rabbit anti-human FHR5 (MaxPab® antibody*, buffered aqueous solution)	Abnova, Taipei, Taiwan	H00081494-D01P
Horseradish peroxidase-conjugated polyclonal rabbit anti-mouse immunoglobulins	Dako, Ely, UK	P0260
Horseradish peroxidase-conjugated polyclonal swine anti-rabbit immunoglobulins	Dako, Ely, UK	P0217

Cat. No. = Supplier's catalogue number, *polyclonal antibodies generated from annotated sequence-verified full-length proteins expressed in mammalian cell lysates

2.8. Statistical analysis

Non-parametric data was represented as the median with the range of values given in parentheses. Two groups were analysed using the Mann-Whitney test whilst one-way analysis of variance with Bonferroni's multiple comparison test was used to analyse three or more groups. Data were analyzed using GraphPad Prism version 3.0 for Windows (GraphPad Software, San Diego, USA). P value of <0.05 was considered significant.

3. CHAPTER THREE: GENERATION OF HEPATOCYTE-SPECIFIC FACTOR H-DEFICIENT MICE

3.1. Introduction

Mice with complete FH deficiency (*Cfh*$^{-/-}$) have marked depletion of plasma C3 secondary to uncontrolled activation of the AP and develop spontaneous membranoproliferative glomerulonephritis (MPGN)(Pickering, Cook et al. 2002). Light microscopy in these animals showed mesangial matrix expansion and hypercellularity with capillary wall thickening and a double contour appearance to the GBM. Immunofluorescence revealed linear capillary wall C3 and C9 deposition. The absence of disease in mice with concurrent FB deficiency demonstrated that the renal disease was dependent on C3 activation. Capillary wall C3 deposition in *Cfh*$^{-/-}$ mice appears deleterious even in the absence of TP activation. One-year old mice with combined FH and C5 deficiency (*Cfh*$^{-/-}$.*C5*$^{-/-}$) had comparable albuminuria to *Cfh*$^{-/-}$ mice, indicating that chronic C3 deposition along the GBM could disrupt the glomerular permeability barrier in the absence of C5 activation. Furthermore, capillary wall double contours and subendothelial electron dense deposits were present in *Cfh*$^{-/-}$.*C5*$^{-/-}$ mice demonstrating that MPGN can develop in this setting in the absence of C5 (Pickering, Warren et al. 2006). The appearance of marked capillary wall C3 deposition in *Cfh*$^{-/-}$*C3*$^{-/-}$ kidneys transplanted into *Cfh*$^{-/-}$ hosts also indicates that the GBM C3 seen in *Cfh*$^{-/-}$ mice is derived from the circulation (Rose, Paixao-Cavalcante et al. 2008).

Despite the presence of uncontrolled C3 activation, mice with FI deficiency (*Cfi*$^{-/-}$) do not develop either spontaneous C3 deposition along the GBM or MPGN (Rose, Paixao-Cavalcante et al. 2008). These animals develop spontaneous mesangial expansion, often with hyalinosis, and have abnormal C3 staining within the mesangium. In contrast to *Cfh*$^{-/-}$ mice, the *Cfi*$^{-/-}$ animals do not develop GBM C3 accumulation. Furthermore, the absence of MPGN in mice with combined FH and FI deficiency (*Cfh*$^{-/-}$*Cfi*$^{-/-}$) and the finding of similar glomerular C3 staining to FI-deficient animals alone, illustrates the absolute requirement for FI for the GBM C3 deposition and MPGN observed in FH deficiency (Rose, Paixao-Cavalcante et al. 2008). C3 predominantly circulates as C3b in *Cfh*$^{-/-}$*Cfi*$^{-/-}$ and *Cfi*$^{-/-}$ mice since the proteolytic cleavage of C3b is FI dependent. When serum containing FI is administered to *Cfh*$^{-/-}$ *Cfi*$^{-/-}$ animals, cleavage of C3b is seen. Unexpectedly, this is accompanied by the loss of mesangial C3 staining and the appearance of C3 along the GBM. One

interpretation of this data is that in the setting of uncontrolled C3 activation, metabolites of C3b (iC3b and/or C3d) target the GBM, whilst C3b localises to the mesangial areas.

Mice with heterozygous FH deficiency have modestly reduced plasma C3 levels compared to wild-type mice. However, they do not develop capillary wall C3 deposition or MPGN (Pickering, Cook et al. 2002). Consistent with this, pigs with heterozygous FH deficiency also do not develop spontaneous MPGN (Hogasen, Jansen et al. 1995).

The liver is the major source of FH, but extra-hepatic synthesis has been reported in human umbilical vein endothelial cells, fibroblasts, mesangial cells and podocytes. The significance of this extra-hepatic FH production is unknown. Studies in human liver transplantation suggest that extra-hepatic C3 production accounts for between 5.6-11.4% of the circulating plasma C3 pool (Naughton, Botto et al. 1996). C6-sufficient rats receiving C6-deficient liver transplants demonstrated that 30-40% of systemic C6 in rats is extra-hepatic in origin (Brauer, Baldwin et al. 1994).

To investigate the biological significance of extra-hepatic FH production, I generated mice with hepatocyte-specific FH deficiency (hepatocyte-*Cfh*$^{-/-}$). My aim was to firstly measure circulating FH levels and determine if these were sufficient to maintain plasma C3 regulation and prevent the development of GBM C3 deposition.

In mice with functioning *loxP*-flanked target loci, hepatocyte-specific gene disruption has been achieved *in vivo* by selectively expressing Cre recombinase in hepatocytes using the albumin promoter/enhancer sequence (Postic, Shiota et al. 1999). In my supervisor's laboratory, a mutant animal containing *loxP* sites flanking exons 2 and 3 of the *Cfh* gene (*Cfh*$^{loxP/loxP}$) was generated. Following Cre recombinase-mediated excision of exons 2 and 3, generation of a null *Cfh* allele was predicted as a similar strategy had been used in the original *Cfh*$^{-/-}$ strain (Pickering, Cook et al. 2002). To generate hepatocyte-*Cfh*$^{-/-}$ mice, my plan was to inter-cross *Cfh*$^{loxP/loxP}$ mice with a strain expressing Cre recombinase under the control of the albumin promoter (Alb-Cre). In this chapter, I present the data showing the successful generation of a hepatocyte-*Cfh*$^{-/-}$ strain.

3.2. Inter-crossing conditional FH-deficient animals with mice expressing *Cre* recombinase under the control of the mouse albumin promoter

Hepatocyte-*Cfh*$^{-/-}$ mice were generated by inter-crossing *Cfh*$^{loxP/loxP}$ animals with mice expressing Cre recombinase under the control of the murine albumin promoter (Alb-Cre). Offspring were screened for the presence of the floxed *cfh* allele (figure 3.1A) and the Cre recombinase transgene (Alb-Cre, figure 3.1B). Hepatocyte-*Cfh*$^{-/-}$ animals were viable and fertile under specific pathogen-free conditions. Hepatocyte deletion of exons 2 and 3 of the *cfh* gene was confirmed by amplifying the floxed *cfh* locus from cDNA synthesised from hepatic RNA isolated from hepatocyte-*Cfh*$^{-/-}$ animals. Cre recombinase-mediated excision of exons 2 and 3 in these animals resulted in a smaller 318-bp amplicon not present in the Alb-Cre animals (figure 3.2). Data demonstrating that this Cre-mediated recombination was hepatocyte-specific is shown in the appendix. I next expanded the hepatocyte-*Cfh*$^{-/-}$ strain for phenotypic analysis.

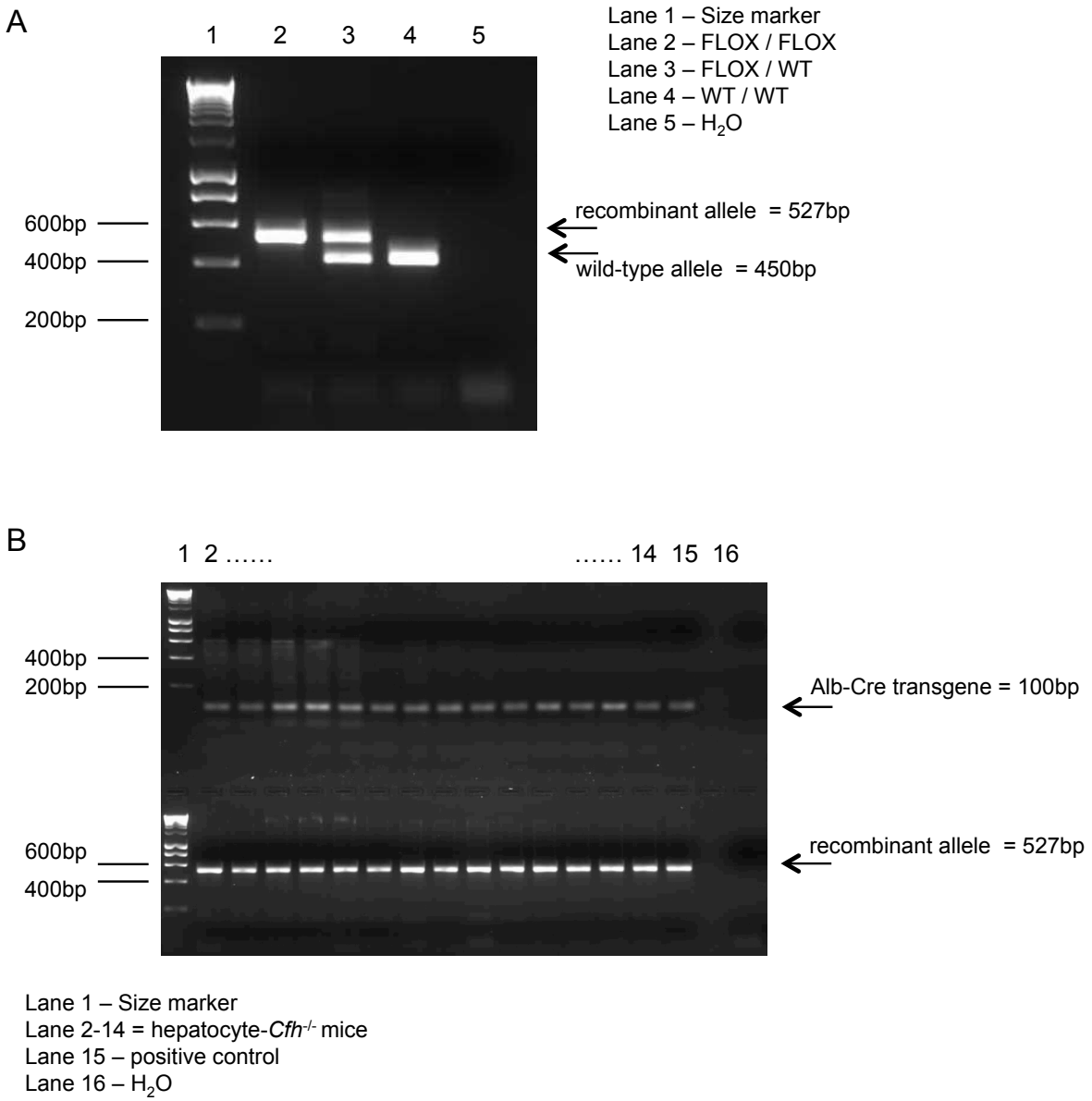


Figure 3.1 **Genotyping PCR in hepatocyte-*Cfh*$^{-/-}$ mice**. (A) The presence of the floxed *cfh* allele results in a 527-bp amplicon, whereas these primers amplify a 450-bp amplicon in the wild-type *cfh* locus. (B) The presence of the Alb-Cre transgene was also determined by PCR and these primers amplify a 100-bp amplicon when the transgene is present. In this gel, DNA from mice 1-14 generates this amplicon together with a single 527-bp amplicon using the primers in (A). These mice are therefore all hepatocyte-*Cfh*$^{-/-}$ mice.

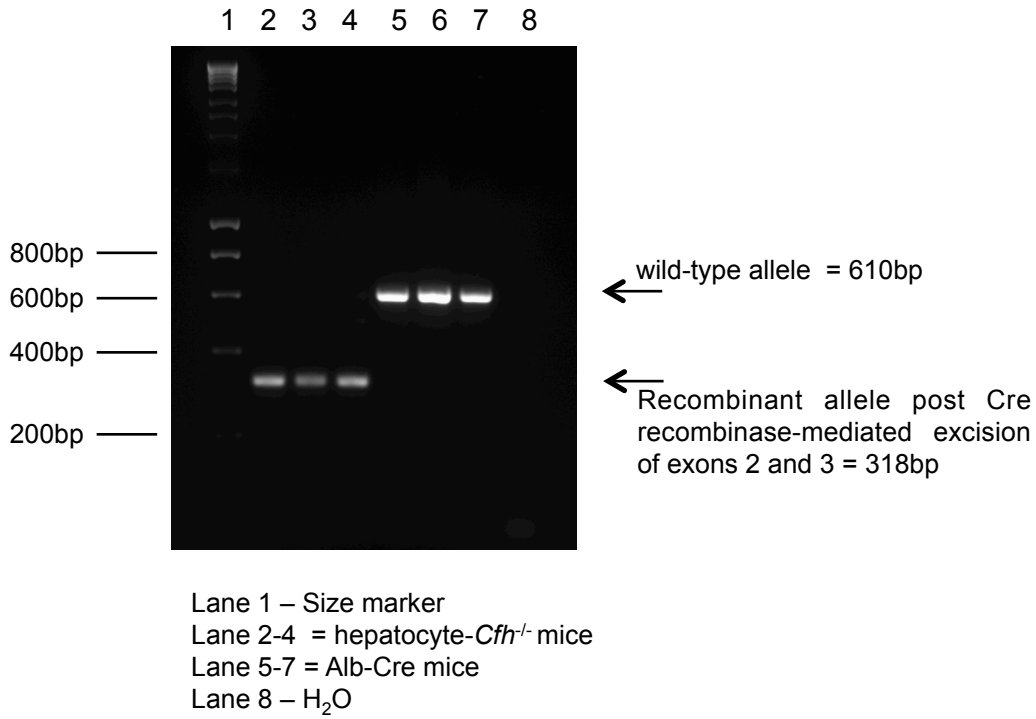


Figure 3.2 **PCR on cDNA generated from RNA from livers of hepatocyte-*Cfh*$^{-/-}$ (lanes 2-4) and Alb-Cre mice (lanes 5-7)**. The PCR used primers that flanked exons 2 and 3 and resulted in a 610-bp amplicon using cDNA from wild-type mice. After Cre recombinase-mediated excision of exons 2 and 3 the amplicon is reduced to 318bp. The smaller amplicon is seen in all hepatocyte-*Cfh*$^{-/-}$ mice (lanes 2-4).

3.3. Spontaneous complement profile in hepatocyte-specific FH-deficient mice

3.3.1. Plasma FH

The critical role played by FH in regulating complement activation within plasma is evident from the phenotype seen in complete FH deficiency, where uncontrolled activation leads to secondary depletion of complement components such as C3 (Pickering, Cook et al. 2002). To date, the contribution made by extra-hepatic FH to the total FH pool has not been described. I therefore sought to answer this by measuring plasma FH levels in 8-12 week old hepatocyte-*Cfh*$^{-/-}$ mice by ELISA (figure 3.3) and western blotting (figure 3.4).

Using an ELISA, plasma FH levels were significantly reduced in hepatocyte-*Cfh*$^{-/-}$ mice (median 19% of wild-type levels, range 14-29%, n=7) compared to *Cfh*$^{loxP/loxP}$ animals (median 59.5% of wild-type levels, range 37-103%, n=8, $p<0.001$, Bonferroni multiple comparison test). This was evident from a semi-quantitative assessment by western blotting for plasma FH using samples from these animals (figure 3.4).

To conclude that extra-hepatic FH was responsible for approximately 19% of the plasma FH pool (derived from the FH levels in the hepatocyte-*Cfh*$^{-/-}$ mice), I would need to be certain that not only was there (i) no hepatocyte-specific FH production remaining in the hepatocyte-*Cfh*$^{-/-}$ mice (i.e. no 'leakiness'), but also (ii) that production of FH from the floxed *cfh* allele was equivalent to that from wild-type *cfh* allele. Unexpectedly, *Cfh*$^{loxP/loxP}$ animals had significantly lower plasma FH levels (median 59.5% of wild-type, range 37-103%, n=8) than Alb-Cre mice (median 93% of wild-type, range 71-137%, n=6, $p<0.01$, Bonferroni multiple comparison test). This suggested to me that the production of FH from the floxed *cfh* allele was not normal.

To investigate this further I assessed plasma C3 levels in all groups of mice to determine if, as expected plasma C3 levels would be reduced in the hepatocyte-*Cfh*$^{-/-}$ mice in proportion to the reduction in plasma FH. I also compared C3 levels between Alb-Cre and *Cfh*$^{loxP/loxP}$ animals to determine if the lower plasma FH levels in the latter group were correlated with a fall in plasma C3.

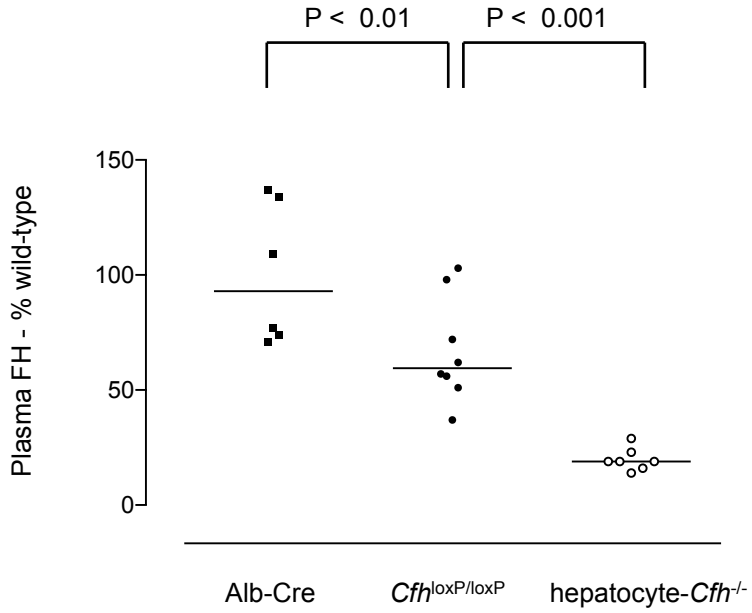


Figure 3.3 Plasma FH levels in 12-week old Alb-Cre (n=6), *Cfh*$^{loxP/loxP}$ (n=8) and hepatocyte-*Cfh*$^{-/-}$ (n=7) mice. Horizontal bars denote median values.

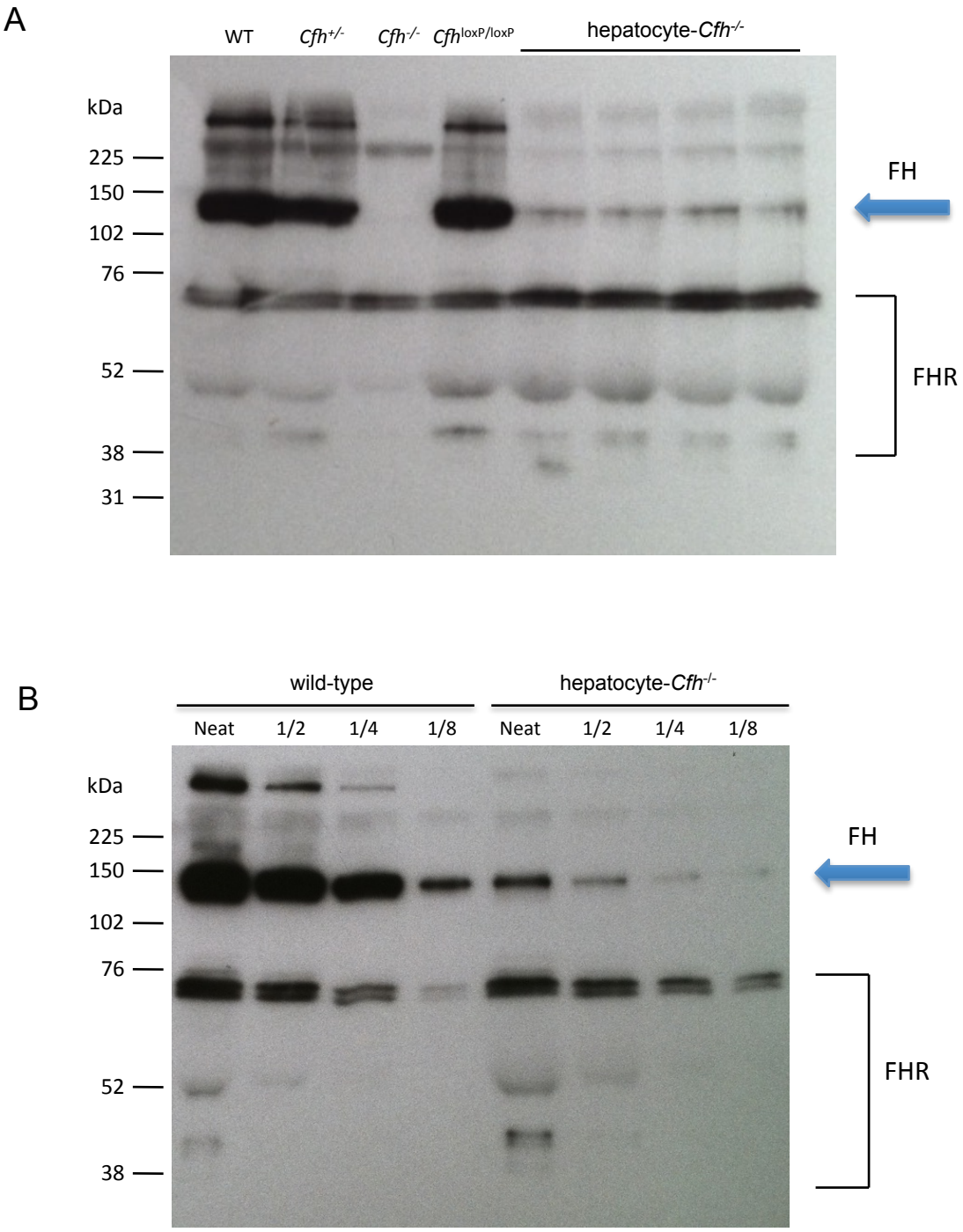


Figure 3.4 **Plasma FH western blot.** In (A) plasma FH in hepatocyte-*Cfh*$^{-/-}$ plasma is compared with wild-type (WT), heterozygous (*Cfh*$^{+/-}$) and homozygous (*Cfh*$^{-/-}$) FH-deficient mice and *Cfh*$^{loxP/loxP}$ samples. FH is indicated by arrow. Lower bands represent factor H-related proteins (FHR). In (B) serial plasma dilutions from WT and hepatocyte-*Cfh*$^{-/-}$ plasma confirm that plasma FH is significantly reduced in the hepatocyte-*Cfh*$^{-/-}$ strain.

3.3.2. Plasma C3

The contribution of extra-hepatic FH to fluid-phase C3 regulation is also unknown. I therefore measured plasma C3 levels in hepatocyte-*Cfh*$^{-/-}$ mice by ELISA and compared these with the levels in *Cfh*$^{-/-}$, *Cfh*$^{loxP/loxP}$ and Alb-Cre animals. Consistent with the low plasma FH levels, hepatocyte-*Cfh*$^{-/-}$ mice had a secondary reduction in circulating C3 levels (median 44.3µg/ml, range 26.0-81.2, n=7). These were significantly lower than in *Cfh*$^{loxP/loxP}$ animals (median 188.2µg/ml, range 97.6-247.0, n=8, $p<0.001$, Bonferroni multiple comparison test) and higher than that seen in *Cfh*$^{-/-}$ mice (median 26.1µg/ml, range 21.2-64.7, n=13, figure 3.5).

Importantly, the plasma C3 concentration in the *Cfh*$^{loxP/loxP}$ animals was significantly lower (median 188.2µg/ml, range 97.6-247.0, n=8) than in Alb-Cre mice (median 277.4µg/ml, range 176.4-396.8, n=6, $p<0.001$, Bonferroni multiple comparison test). This confirmed my suspicion that the floxed *cfh* allele had impaired production of FH and that this resulted in sub-optimal plasma C3 regulation. These data meant that I could not draw firm conclusions on the contribution of hepatic and extra-hepatic FH synthesis in the hepatocyte-*Cfh*$^{-/-}$ mice.

I next determined if the reduced FH levels were associated with spontaneous renal disease in not only the hepatocyte-*Cfh*$^{-/-}$ mice but also the *Cfh*$^{loxP/loxP}$ strain.

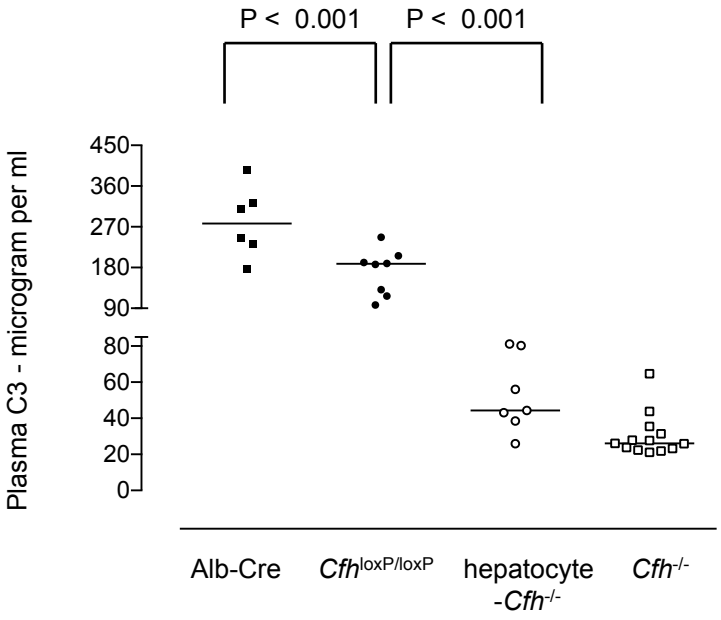


Figure 3.5 Plasma C3 levels in 12-week old Alb-Cre (n=6), *Cfh*$^{loxP/loxP}$ (n=8), hepatocyte-*Cfh*$^{-/-}$ (n=7) and *Cfh*$^{-/-}$ (n=13) mice. Horizontal bars denote median values.

3.4. Spontaneous renal phenotype in hepatocyte-specific FH-deficient mice

The uncontrolled complement activation in *Cfh*$^{-/-}$ mice is associated with increased albuminuria compared to wild-type controls and results in spontaneous MPGN. I wanted to investigate whether extra-hepatic FH production influenced this phenotype. Specifically, I hypothesised that extra-hepatic FH would ameliorate the spontaneous renal phenotype seen in *Cfh*$^{-/-}$ mice.

To investigate this experimentally I generated cohorts of mice, which were monitored for a period of 9 months. The cohorts comprised:

Strain	Male	Female	Total
hepatocyte-*Cfh*$^{-/-}$	12	13	25
Cfh$^{-/-}$	8	15	23
Cfh$^{loxP/loxP}$	4	5	9
Alb-Cre	2	6	8

Two deaths occurred during the 9-month observation period. One was a small hepatocyte-*Cfh*$^{-/-}$ mouse aged 5 weeks. The cause of death is not known although renal histology was normal. One Alb-Cre mouse died at 17 weeks with apparent bowel perforation. Renal histology was normal. All other animals remained well at 9 months.

At the end of the 9-month period I examined renal function and histology in all surviving mice. I chose this time period since spontaneous renal disease (MPGN) has previously been shown to be fully penetrant histologically at this age (Pickering, Cook et al. 2002).

3.4.1. Renal function and haematological parameters

Median urea levels were normal (<20mmol/l) in all mice with the exception of one animal in the hepatocyte-*Cfh*$^{-/-}$ cohort (urea = 26.5mmol/l) and two animals in the *Cfh*$^{-/-}$ cohort (20.5 and 21.7mmol/l, figure 3.6). The median and range of values for each cohort were: hepatocyte-*Cfh*$^{-/-}$ cohort = 12.0mmol/l (range 7.4-26.5, n=25); *Cfh*$^{-/-}$ cohort = 12.4mmol/l (range 6.0-21.7, n=23); *Cfh*$^{loxP/loxP}$ cohort = 7.2mmol/l (range 3.1-13.7, n=9) and Alb-Cre cohort = 10.9mmol/l (range 6.0-19.7, n=8).

Albuminuria was only detectable in one of the 25 hepatocyte-*Cfh*$^{-/-}$ mice at a concentration of 2.1mg/ml. Albuminuria was absent from the remaining hepatocyte-*Cfh*$^{-/-}$ mice and all other mice in the *Cfh*$^{-/-}$, *Cfh*$^{loxP/loxP}$ and Alb-Cre cohorts.

Since FH dysfunction in mice has been associated with renal thrombotic microangiopathy it was important to monitor for haematological abnormalities in the cohorts. I measured haematocrit at sacrifice and analysed blood smears for any evidence of red cell fragmentation. The haematocrit in hepatocyte-*Cfh*$^{-/-}$ mice (median 35.5%, range 32-44, n=24) was not significantly different from that in *Cfh*$^{-/-}$ (median 37%, range 33-42, n=23), *Cfh*$^{loxP/loxP}$ (median 35%, range 33-41, n=9) and Alb-Cre mice (median 39%, range 35-41, n=8, figure 3.7A). Furthermore, there was no evidence of red cell fragments on blood smears (figure 3.7B). I concluded that there was no haematological evidence of thrombotic microangiopathy in the hepatocyte-*Cfh*$^{-/-}$ mice.

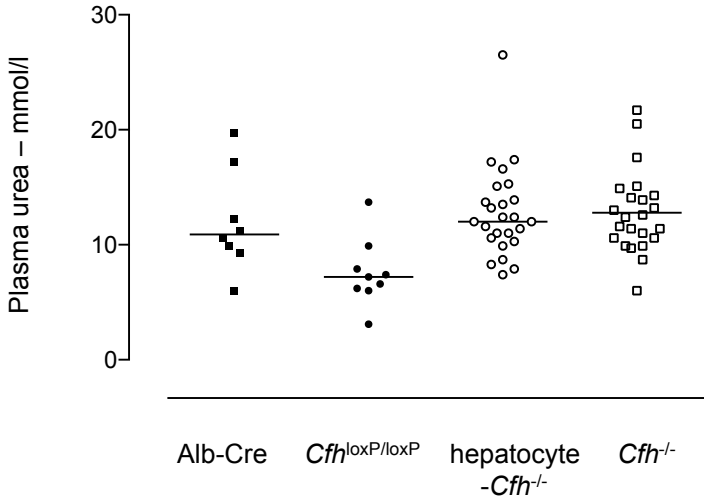
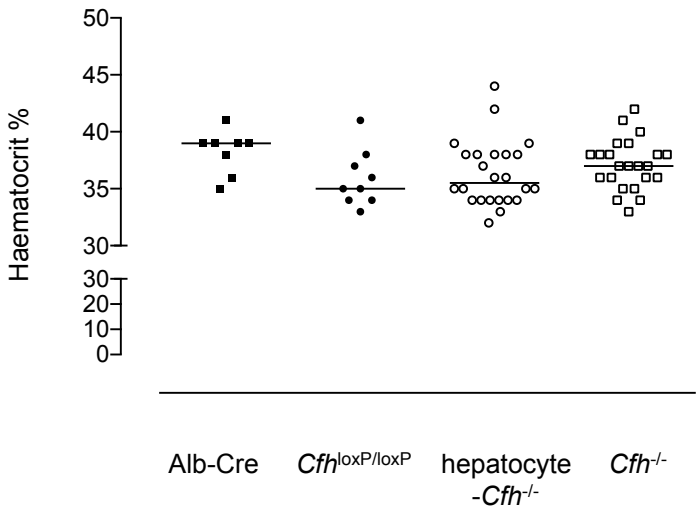


Figure 3.6 **Plasma urea in 9-month old Alb-Cre, *Cfh*$^{loxP/loxP}$, hepatocyte-*Cfh*$^{-/-}$ and *Cfh*$^{-/-}$ mice.** Levels were normal (<20 mmol/l) in all mice with the exception of a single hepatocyte-*Cfh*$^{-/-}$ and two *Cfh*$^{-/-}$ mice. Horizontal bars denote median values.

A



B

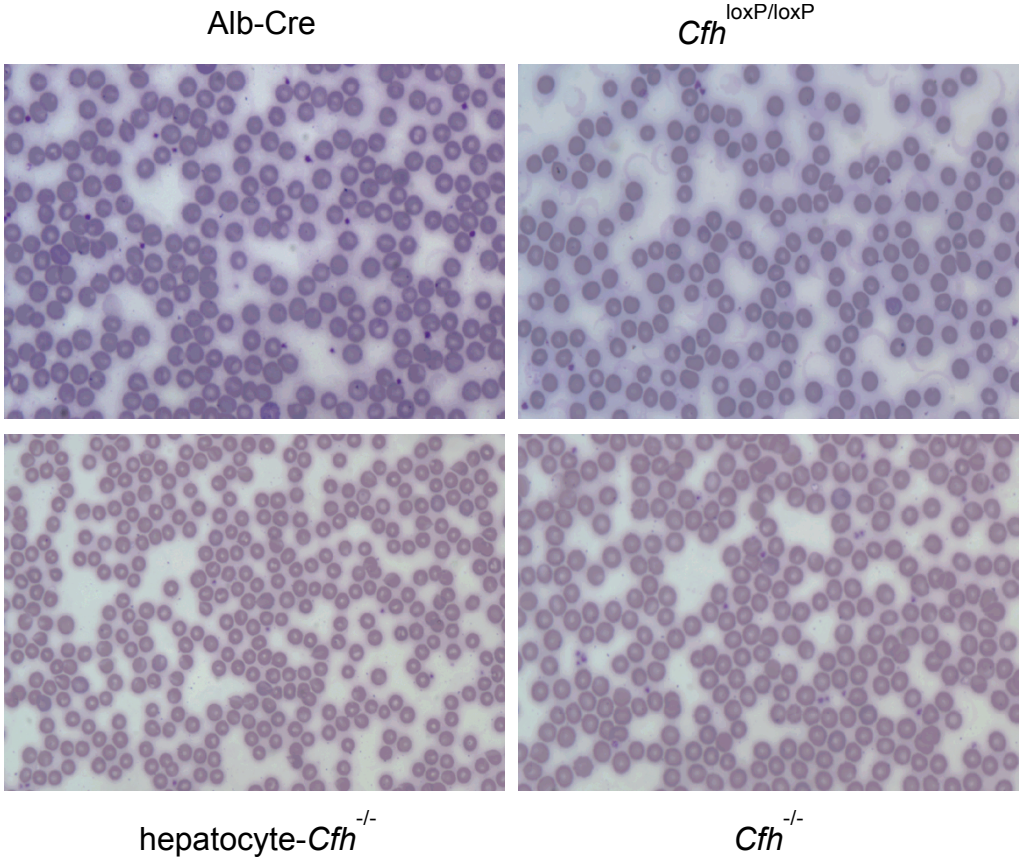


Figure 3.7 **Haematocrit (A) and red cell fragments (B) in Alb-Cre, *Cfh*$^{loxP/loxP}$, hepatocyte-*Cfh*$^{-/-}$ and *Cfh*$^{-/-}$ mice.** Horizontal bars denote median values. Representative images of blood smears in Alb-Cre, *Cfh*$^{loxP/loxP}$, hepatocyte-*Cfh*$^{-/-}$ and *Cfh*$^{-/-}$ mice.

3.4.2. Renal histology – Light microscopy

Glomerular hypercellularity was seen in hepatocyte-*Cfh*$^{-/-}$ mice to a comparable extent to *Cfh*$^{-/-}$ animals (figure 3.8A). Glomerular cellularity was graded as follows: grade 0, normal; grade 1, segmental hypercellularity in 10–25% of the glomeruli; grade 2, hypercellularity involving >50% of the glomerular tuft in 25–50% of glomeruli; grade 3, hypercellularity involving >50% of the glomerular tuft in 50–75% of glomeruli; grade 4, glomerular hypercellularity in >75% or crescents in >25% of glomeruli.

There was evidence of capillary wall thickening in only a small number of animals in these two groups: 1 out of 25 of the hepatocyte-*Cfh*$^{-/-}$ cohort and 2 out of 23 *Cfh*$^{-/-}$ animals.

Consistent with the normal appearance of the peripheral blood film and normal haematocrits, there was no evidence of glomerular thrombosis in hepatocyte-*Cfh*$^{-/-}$ mice (figure 3.8B).

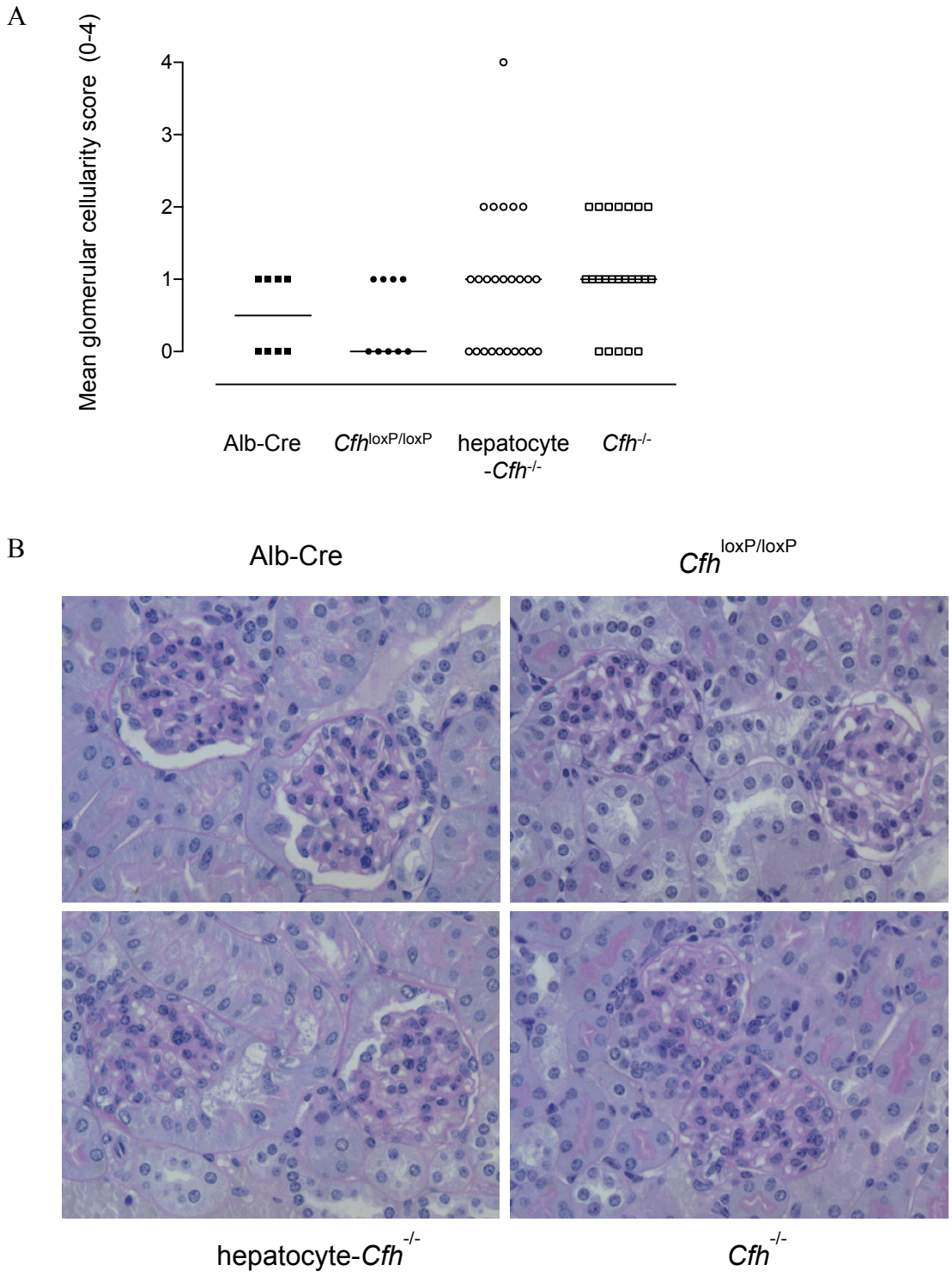


Figure 3.8 **(A) Glomerular cellularity in 9-month old Alb-Cre, *Cfh*$^{loxP/loxP}$, hepatocyte-*Cfh*$^{-/-}$ and *Cfh*$^{-/-}$ mice.** Horizontal bars denote median values. (B) Representative images of glomerular appearances in Alb-Cre, *Cfh*$^{loxP/loxP}$, hepatocyte-*Cfh*$^{-/-}$ and *Cfh*$^{-/-}$ mice. Original magnification x40.

3.4.3. Renal histology – Immunofluorescence studies for C3 and IgG

Spontaneous renal disease in *Cfh*$^{-/-}$ mice is triggered by the deposition of C3 along the GBM producing a characteristic linear C3 staining pattern (Pickering, Cook et al. 2002). Consistently, in my cohort all *Cfh*$^{-/-}$ mice had glomerular linear C3 staining using a polyclonal anti-mouse C3 antibody (figure 3.9). Abnormal glomerular C3 deposition was also observed in hepatocyte-*Cfh*$^{-/-}$ mice (figure 3.9). However, this was distinct from that seen in *Cfh*$^{-/-}$ mice. In hepatocyte-*Cfh*$^{-/-}$ mice glomerular C3 was granular in nature and deposited within the mesangium. When I quantified mesangial and capillary wall C3 staining in the *Cfh*$^{-/-}$ and hepatocyte-*Cfh*$^{-/-}$ mice, using a graded score of 0-3 (corresponding to absent, mild, moderate and marked staining) in a blinded fashion, this distinction was clear (figure 3.10A and B).

Tubulointerstitial C3 staining is normally present in wild-type mice. It is dependent on an intact AP in plasma (Quigg, Lim et al. 1998, Thurman, Ljubanovic et al. 2003) and has been shown to be absent in *Cfh*$^{-/-}$ mice as these animals have secondary plasma C3 depletion (Pickering, Cook et al. 2002). This finding was recapitulated in my *Cfh*$^{-/-}$ cohort. In contrast the hepatocyte-*Cfh*$^{-/-}$ mice had detectable tubulointerstitial C3 staining. However, this was reduced compared to the normal staining intensity seen in the Alb-cre mice (figure 3.9). I concluded that this was due to the partial rather than total AP dysregulation present in the hepatocyte-*Cfh*$^{-/-}$ mice.

These data showed that, although the plasma FH levels in the hepatocyte-*Cfh*$^{-/-}$ mice were low, this was sufficient to (i) completely alter the glomerular C3 staining pattern seen in complete FH deficiency and (ii) partially maintain tubulointerstitial C3 staining.

Notably, renal C3 immunostaining was normal in both the glomerular and tubulointerstitial compartments in the *Cfh*$^{loxP/loxP}$ mice (figure 3.9). This is consistent with the normal C3 immunostaining seen in heterozygous FH-deficient mice (Pickering, Cook et al. 2002). Therefore, although the impaired production of FH from the floxed *cfh* allele resulted in a reduction in plasma C3 levels it did not alter spontaneous renal C3 deposition.

Mesangial IgG is commonly seen in older mice and I could detect small amounts of mesangial IgG in all of the cohorts (figure 3.9). When I performed quantitative immunofluorescence scoring in hepatocyte-*Cfh*$^{-/-}$ and *Cfh*$^{-/-}$ mice there was significantly more IgG in the *Cfh*$^{-/-}$ group: median fluorescent score 554.1 AFU (range 284.1-1246, n=12) compared to 354.6 AFU (range 166.1-567, n=12, P=0.0386, Mann-Whitney test, figure 3.11).

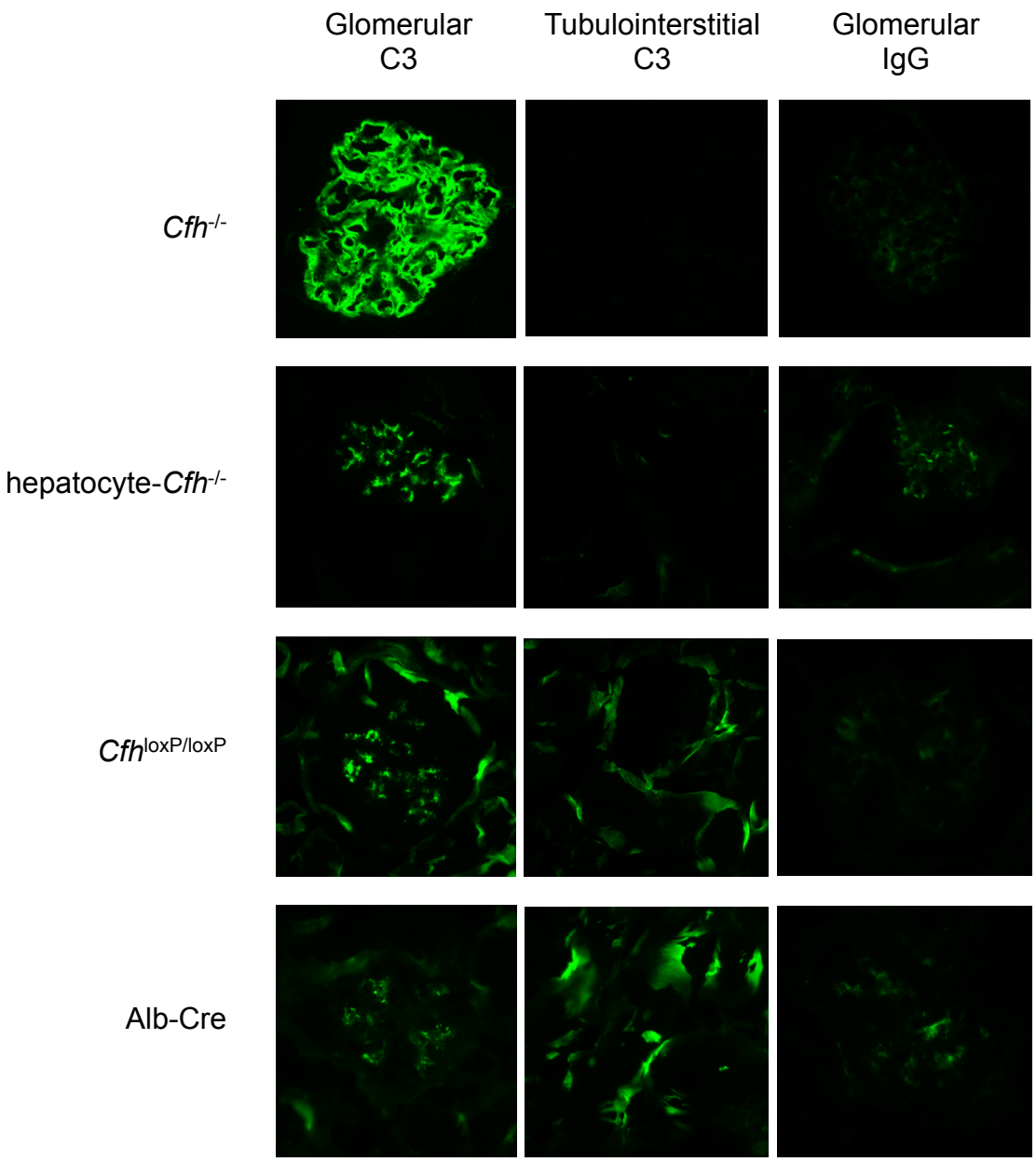


Figure 3.9 **Immunostaining for C3 and IgG in 9-month old Alb-Cre, *Cfh*$^{loxP/loxP}$, hepatocyte-*Cfh*$^{-/-}$ and *Cfh*$^{-/-}$ mice.** The characteristic linear capillary wall C3 deposition seen in *Cfh*$^{-/-}$ mice is replaced by a mesangial staining pattern in hepatocyte-*Cfh*$^{-/-}$ mice. Furthermore, tubulointerstitial C3 deposition typically absent in *Cfh*$^{-/-}$ mice is present in hepatocyte-*Cfh*$^{-/-}$ mice, but at a reduced intensity compared to *Cfh*$^{loxP/loxP}$ and Alb-Cre animals.

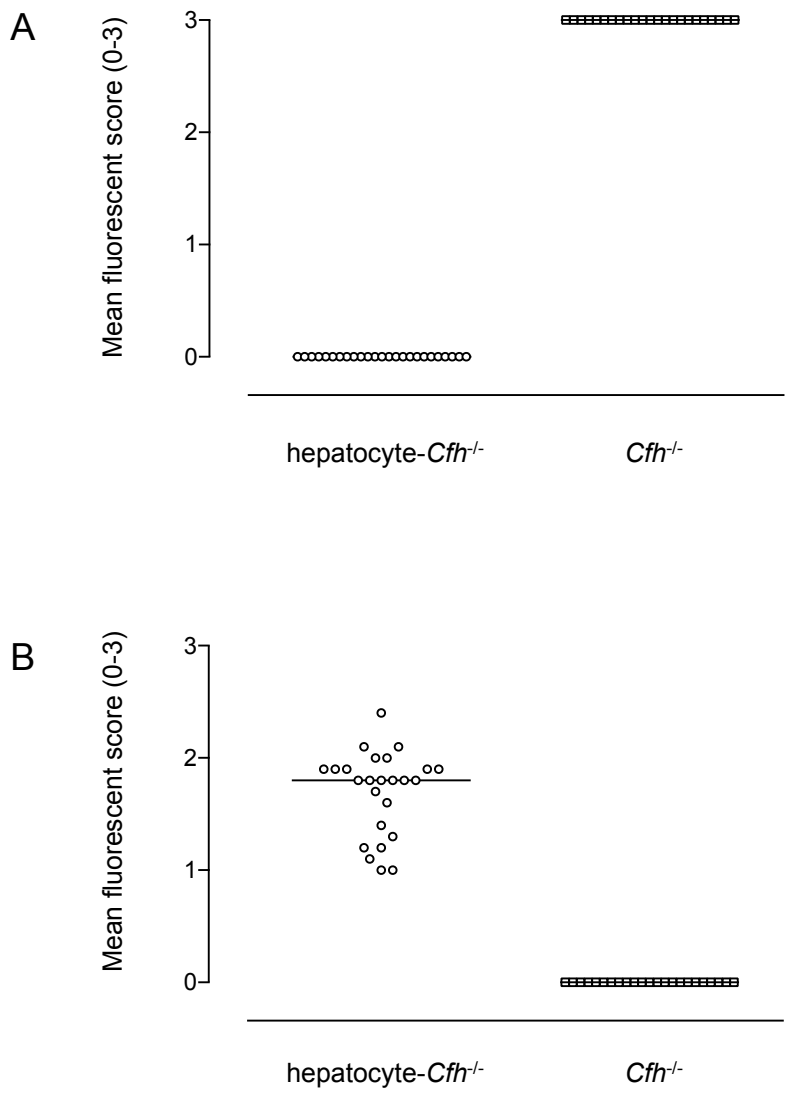


Figure 3.10 **Glomerular capillary wall (A) and mesangial (B) C3 scores in hepatocyte-*Cfh*$^{-/-}$ and *Cfh*$^{-/-}$ mice.** Horizontal bars denote median values.

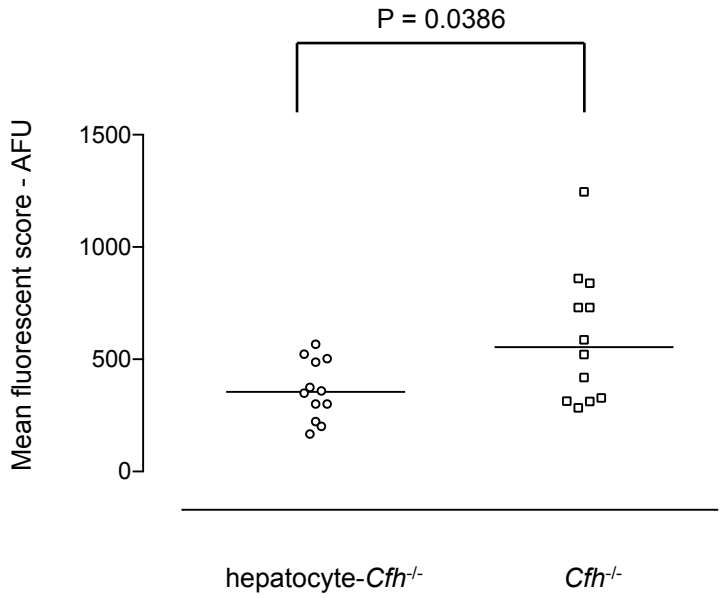


Figure 3.11 **Quantitative immunofluorescence staining for IgG in hepatocyte-*Cfh*$^{-/-}$ and *Cfh*$^{-/-}$ mice.** This group comprised 12 mice from each cohort. Horizontal bars denote median values. AFU – arbitrary fluorescence units.

3.4.4. Renal histology – Immunofluorescence studies for C3d and mouse FHR proteins

To determine the nature of the glomerular C3 present in the hepatocyte-*Cfh*$^{-/-}$ mice, I performed C3 immunostaining with a polyclonal goat anti-mouse C3d antibody and compared this with the reactivity seen with the goat polyclonal anti-mouse C3 antibody used above. The anti-mouse C3d antibody does not detect C3b deposits in the mesangium of mice that are FI-deficient demonstrating that it does not react with tissue-bound C3b (Paixao-Cavalcante, Hanson et al. 2009). It does recognise the linear C3 glomerular deposits in *Cfh*$^{-/-}$ mice (Paixao-Cavalcante, Hanson et al. 2009), indicating that these contain iC3b and/or C3d. Consistent with this published data, when I performed dual staining with the anti-C3 and anti-C3d antibodies in sections from *Cfh*$^{-/-}$ mice, linear glomerular staining was seen with both and this staining co-localised (figure 3.12). I next used this approach in the glomerular sections from the hepatocyte-*Cfh*$^{-/-}$ mice (figure 3.12). This demonstrated very weak reactivity within the mesangium when the anti-C3d antibody was used indicating that the mesangial C3 in these mice did not contain much iC3b or C3d. When the anti-C3 antibody was used there was clear mesangial reactivity that did not co-localise with the anti-C3d antibody. The mesangial C3 could be intact C3, C3b or C3c. I considered that C3c was the most likely candidate but this would require demonstration with mono-specific anti-mouse C3 antibodies.

Previous data showed that the glomerular C3 in *Cfh*$^{-/-}$ mice reacted with a polyclonal goat anti-human FH antibody (Paixao-Cavalcante, Hanson et al. 2009). Since these mice completely lack mFH it was concluded that the anti-human FH antibody was interacting with mouse FHR proteins that had bound *in vivo* to the GBM-bound C3. When I performed dual staining using this anti-human FH antibody and the anti-C3 antibody in *Cfh*$^{-/-}$ mice, I reproduced these observations (figure 3.13). When I performed the procedure on hepatocyte-*Cfh*$^{-/-}$ mice there were areas of C3 reactivity that did and did not co-localise with the anti-human FH antibody (figure 3.13). It is difficult to draw conclusions from these data since although human FHR proteins interact with C3b, iC3b and C3d (Goicoechea de Jorge, Caesar et al. 2013) little is known about the interactions of mouse FHR proteins. I speculate that the areas that

were reactive with the anti-C3 but not the anti-human FH or anti-C3d antibody are most likely to represent C3c.

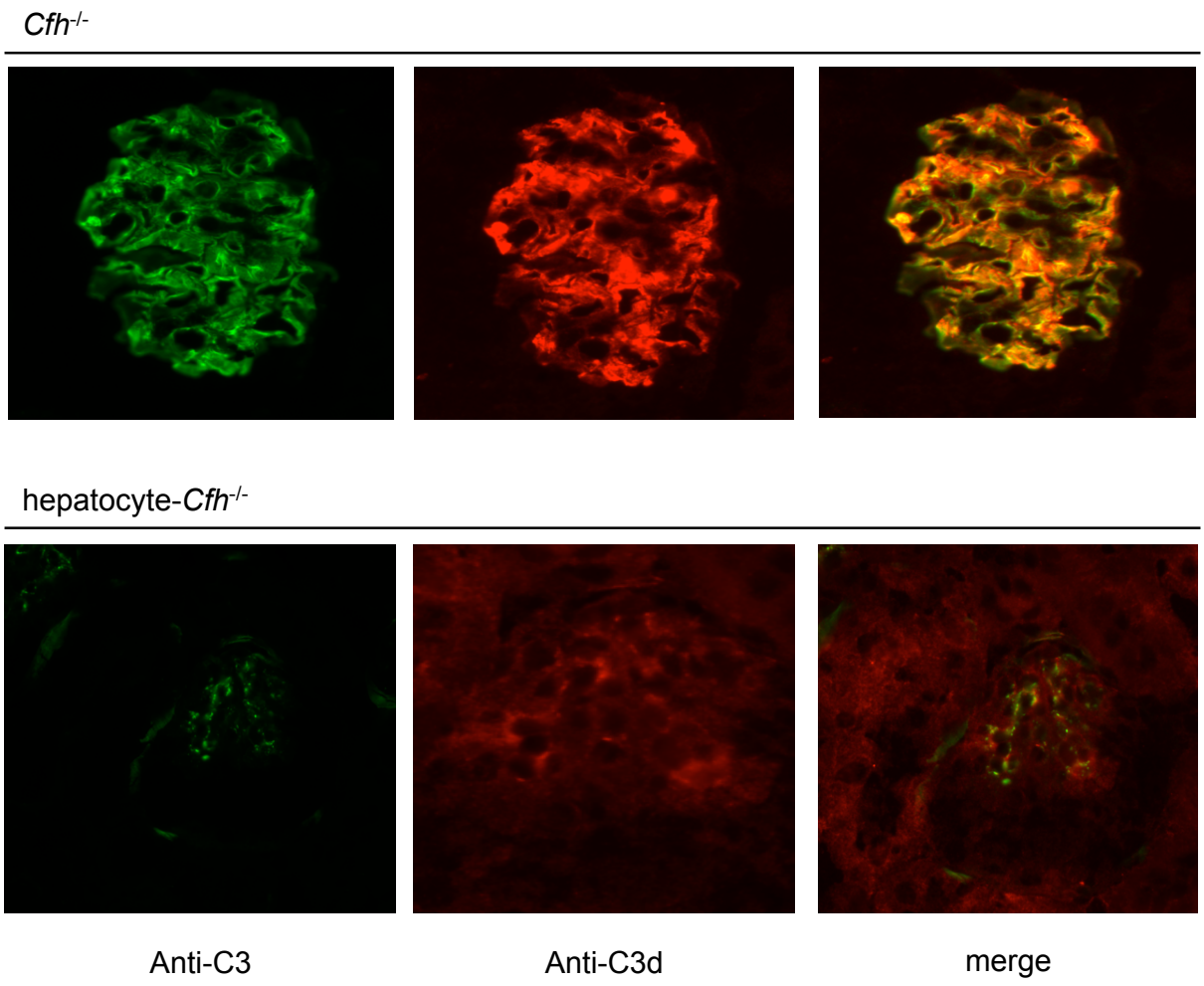


Figure 3.12 **Dual staining using anti-mouse C3 (green) and anti-mouse C3d (red) antibodies in *Cfh*$^{-/-}$ and hepatocyte-*Cfh*$^{-/-}$ mice.** Linear C3 deposits that co-localise can be seen in the *Cfh*$^{-/-}$ mice. Mesangial C3 deposits that do and do not co-localise can be seen in hepatocyte-*Cfh*$^{-/-}$ mice.

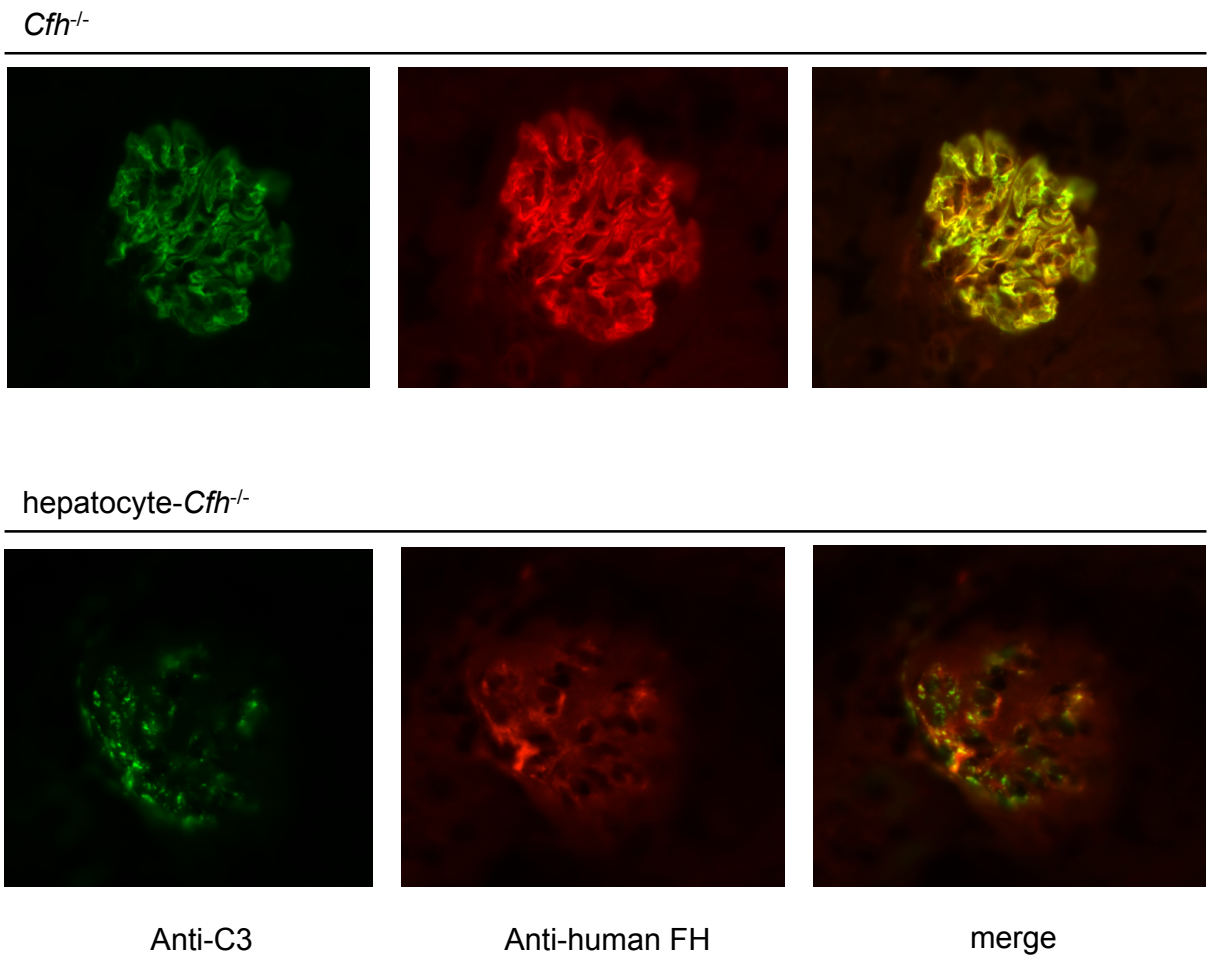
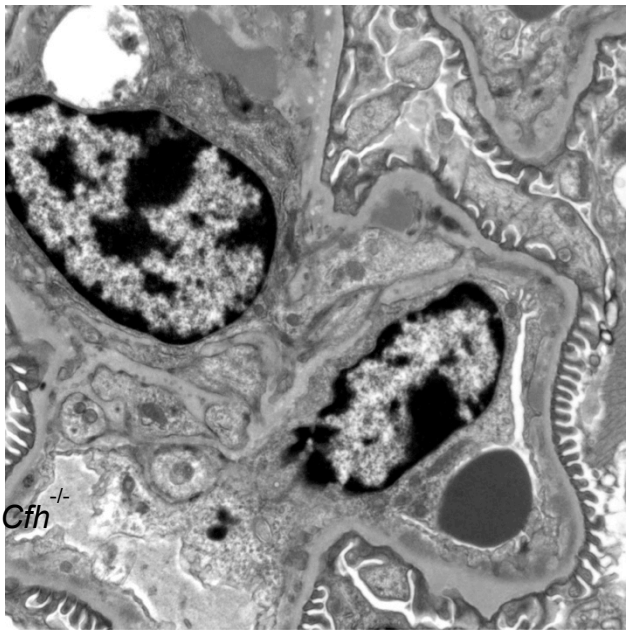


Figure 3.13 **Dual staining using anti-mouse C3 (green) and anti-human FH (red) antibodies in *Cfh*$^{-/-}$ and hepatocyte-*Cfh*$^{-/-}$ mice.** Linear C3 deposits that co-localise can be seen in the *Cfh*$^{-/-}$ mice. Mesangial C3 deposits that do and do not co-localise can be seen in hepatocyte-*Cfh*$^{-/-}$ mice.

3.4.5. Renal histology - Electron microscopy

I was able to obtain electron microscopy images of renal tissue from 4 of the 9-month old hepatocyte-*Cfh*$^{-/-}$ mice. The electron microscopy was performed by Dr. Jill Moss, Consultant Histopathologist, Charing Cross Hospital, London. In the hepatocyte-*Cfh*$^{-/-}$ mice examined the appearances were within normal limits. Specifically, there was no evidence of electron dense deposits in the GBM and only a single electron dense deposit was seen within the mesangium of one of the glomeruli examined.



hepatocyte-*Cfh*$^{-/-}$

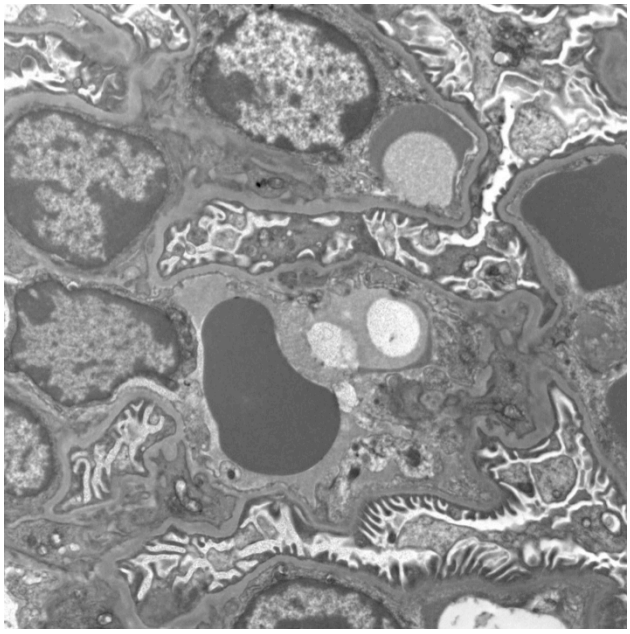


Figure 3.14 **Electron microscopy images.** Subendothelial electron dense GBM deposits are seen in 9-month old *Cfh*$^{-/-}$ mice (A) whereas the appearances were normal in age-matched hepatocyte-*Cfh*$^{-/-}$ mice (B).

3.5. Discussion

Mice with hepatocyte-specific FH deficiency (hepatocyte-*Cfh*$^{-/-}$ mice) were established by inter-crossing conditional FH-deficient animals with mice expressing Cre recombinase under the control of the murine albumin promoter. Hepatocyte-*Cfh*$^{-/-}$ mice had median plasma FH levels of 19% of wild-type levels. This resulted in a secondary reduction in plasma C3 levels as a result of impaired regulation of the AP. The hepatocyte-*Cfh*$^{-/-}$ mice did not develop uraemia, albuminuria, anaemia or red cell fragmentation at the age of 36 weeks. There was mild glomerular hypercellularity comparable to that seen in aged-matched *Cfh*$^{-/-}$ mice. Notably, the pattern of glomerular C3 staining was different in the hepatocyte-*Cfh*$^{-/-}$ animals compared to both control strains (Alb-Cre and *Cfh*$^{loxP/loxP}$) and the mice with complete FH deficiency (*Cfh*$^{-/-}$ mice). Glomerular C3 in the hepatocyte-*Cfh*$^{-/-}$ mice was granular in pattern and mesangial in distribution. This staining pattern was not seen in the control strains and was clearly different to the linear pattern seen in the *Cfh*$^{-/-}$ mice. In addition, hepatocyte-*Cfh*$^{-/-}$ mice did have tubulointerstitial C3 staining, which is absent in *Cfh*$^{-/-}$ mice. Tubulointerstitial C3 deposition has previously been shown to be dependent on intact circulating C3 and the ability to activate the AP (Alexander, Wang et al. 2007, Lenderink, Liegel et al. 2007, Paixao-Cavalcante, Hanson et al. 2009). Therefore, the fact that tubulointerstitial staining was present but reduced when compared to wild-type mice was consistent with partial regulation of plasma C3 activation through the AP. My data, taken together, demonstrate that plasma FH levels of approximately 19% of wild-type were sufficient to significantly alter the glomerular C3 phenotype described in *Cfh*$^{-/-}$ mice (Pickering, Cook et al. 2002).

The mesangial C3 deposition seen in hepatocyte-*Cfh*$^{-/-}$ mice was similar to that seen in *Cfh*$^{-/-}$ mice following the administration of purified mouse (mFH) or human FH (Paixao-Cavalcante, Hanson et al. 2009, Fakhouri, de Jorge et al. 2010). Twenty-four hours after intra-peritoneal injection of mFH, plasma C3 levels in *Cfh*$^{-/-}$ mice rose in association with the appearance of intact circulating C3 and a reduction in GBM C3 deposition. Notably, these changes were accompanied by the appearance of both mesangial and tubulointerstitial C3 staining. Administration of human FH to *Cfh*$^{-/-}$ mice similarly led to a rise in plasma C3 levels comparable to wild-type levels, together with the appearance of mesangial and tubulointerstitial C3 staining in

association with a reduction in GBM C3 deposition (Fakhouri, de Jorge et al. 2010). Repeated FH injections led to normalisation of glomerular C3 reactivity with barely detectable mesangial staining and nothing along capillary walls.

Using the polyclonal anti-C3 and anti-C3d antibodies used above, mesangial C3 detected by the anti-C3 antibody following injection of mFH to *Cfh*$^{-/-}$ mice showed no reactivity with the anti-C3d antibody (Paixao-Cavalcante, Hanson et al. 2009). Similar observations were made in *Cfh*$^{-/-}$ mice given a single dose of human FH (Fakhouri, de Jorge et al. 2010). This anti-C3d antibody has been shown to react with C3dg but not with C3b (Leung, Yun et al. 2009). Based on these observations, it was concluded that the mesangial C3 seen in *Cfh*$^{-/-}$ mice after either mFH or human FH could represent C3b, iC3b or C3c but not C3dg. Recently, the mesangial C3 seen in *Cfh*$^{-/-}$ mice after administration of FH was shown to not only co-localise with properdin but also to be a properdin-dependent phenomenon (Ruseva, Vernon et al. 2013). This suggests that the mesangial C3 is either a result of properdin-dependent surface activation and/or C3 fragments combined with properdin.

Since my data showed that the mesangial C3 in hepatocyte-*Cfh*$^{-/-}$ mice did not react with the anti-C3d antibody I considered that the mesangial C3 could also represent C3b, iC3b or C3c. Depletion of properdin either genetically or by use of an anti-properdin antibody would enable the properdin-dependent nature of mesangial C3 deposition in hepatocyte-specific FH deficiency to be determined. In addition, there was no clear co-localisation between the anti-C3 and anti-human FH antibodies suggesting that the mesangial C3 did not contain either mFH or mouse FHR proteins. Clearly, to definitively characterise the nature of the mesangial C3 I would need to develop monospecific antibodies to each C3 fragment.

In mice with FI deficiency spontaneous mesangial C3 deposits are seen that are reactive with the anti-C3 but not the anti-C3d antibody (Rose, Paixao-Cavalcante et al. 2008). In this situation the absence of FI means that proteolytic conversion of C3b to downstream fragments cannot occur. In this situation the mesangial C3 is C3b but its nature is very different to the mesangial C3 seen in either the hepatocyte-*Cfh*$^{-/-}$ mice or in *Cfh*$^{-/-}$ mice reconstituted with FH. In the FI-deficient mice the mesangial C3b is much more marked and associated with electron dense deposits within the

mesangial matrix and PAS-reactive material within the mesangium on light microscopy. Whether or not this mesangial C3b in FI-deficiency is properdin-dependent is not known.

What is the relevance of this mesangial C3 to human glomerular disease associated with C3 dysregulation? Paramesangial deposits composed solely of C3c have been reported in DDD (the original report used the descriptor: MPGN type II) specifically during episodes of hypocomplementaemia (West, Witte et al. 2001). It was hypothesised that these derived from plasma C3c that had been released following the FI-mediated proteolytic cleavage of iC3b to C3dg. Certainly, this mechanism could be operating in the hepatocyte-*Cfh*$^{-/-}$ mice continuously as they have chronic AP activation with hypocomplementaemia due to sub-total FH deficiency.

A critical observation was that both plasma FH and C3 levels were reduced in the *Cfh*$^{loxP/loxP}$ mice. This indicated that the targeted locus had impaired expression of FH. Impaired expression of targeted genes due to the introduction of *loxP* sites and/or selectable markers has been reported. For example, in a study in which the murine glucokinase gene was conditionally targeted with three *loxP* sites and a neomycin resistance gene, expression of the targeted gene was reduced by 28% compared to the wild-type gene (Postic, Shiota et al. 1999). The mechanisms through which these exogenous genetic elements impair gene expression have been established in some cases. For example, the insertion of a neomycin resistance gene within an intron of a floxed gene was shown to generate cryptic splice sites (Jacks, Shih et al. 1994, Carmeliet, Ferreira et al. 1996, Wassarman, Lewandoski et al. 1997) and these may result in the generation of a hypomorphic allele (Meyers, Lewandoski et al. 1998). Targeting cassettes containing neomycin resistance genes may affect the expression of not only the target gene but also genes in close proximity. For example, mice with a targeted deletion within the murine β-globin locus control region were found to have up to a 5-fold reduction in expression of all genes in the locus (Fiering, Epner et al. 1995). Importantly, after removal of the neomycin resistance gene within the targeted locus, expression of all affected genes returned to normal.

When the construct was designed it was anticipated that the neomycin resistance gene could interfere with FH expression. To address this, FRT sites were placed either side of the neomycin resistance gene. This would enable the neomycin resistance gene to be excised by Flp recombinase. In view of my data showing that FH expression is impaired, my supervisor is currently inter-crossing the *Cfh*$^{loxP/loxP}$ mice with mice expressing Flp recombinase. The plan will be to examine plasma FH and C3 levels after removal of the neomycin resistance gene to determine if the neomycin resistance gene was responsible for the impaired FH expression of the targeted allele. Clearly, with the knowledge that there is impaired FH expression from the targeted allele, I am unable to derive firm conclusions with respect to the contribution of extra-hepatocyte FH to the total FH pool.

Despite this limitation, the spontaneous phenotype of the hepatocyte-*Cfh*$^{-/-}$ strain was informative in that it demonstrated that sub-total FH deficiency (in this case approximately 20% of normal FH levels) was sufficient to completely prevent the spontaneous renal phenotype associated with complete FH deficiency. However, the hepatocyte-*Cfh*$^{-/-}$ strain did develop spontaneous mesangial C3 deposition and this type of lesion has been reported as a subset of human C3 glomerulopathy (Servais, Fremeaux-Bacchi et al. 2007). It is now appreciated that many patients with C3 glomerulopathy develop renal impairment in association with an environmental trigger, such as infection. The hypothesis is that the inability to regulate the AP predisposes not only to spontaneous renal disease but also to exaggerated renal injury during any intercurrent event that triggers complement activation within the kidney. To model this, I next examined the effect of serum nephrotoxic nephritis in the hepatocyte-*Cfh*$^{-/-}$ strain.

3.6. Conclusions

3.6.1. Conditional FH-deficient mice had significantly reduced basal plasma FH and C3 levels compared to wild-type control mice indicating that expression of FH by the targeted locus was impaired.

3.6.2. Mice with hepatocyte-specific FH deficiency have been successfully established by inter-crossing conditional FH-deficient mice with mice expressing Cre recombinase under the control of a hepatocyte-specific promoter.

3.6.3. Hepatocyte-*Cfh*$^{-/-}$ mice have low plasma FH and C3 levels and develop spontaneous mesangial C3 deposition without any evidence of membranoproliferative glomerulonephritis or thrombotic microangiopathy at 36 weeks of age.

4. CHAPTER FOUR: RESPONSE OF HEPATOCYTE-SPECIFIC FACTOR H-DEFICIENT MICE TO EXPERIMENTAL NEPHRITIS

4.1. Introduction

Renal antibody deposition is considered a key event in the aetiology of immune-mediated glomerulonephritis. Nephrotoxic nephritis (NTN) is a widely used model of glomerulonephritis that utilises anti-GBM antibodies (Hammer and Dixon 1963). Following injection of the anti-GBM antibody preparation (termed nephrotoxic serum) renal injury develops in two phases. The initial phase of renal injury is a consequence of inflammation secondary to the binding of the anti-GBM antibody along the GBM. Since the anti-GBM antibody is raised in a different species (typically rabbit or, as in my experiments, sheep) to the experimental animal (mouse or rat) this phase is referred to as the heterologous phase. Subsequently, the experimental animal (e.g. mouse) develops an immune response to the anti-GBM antibody. This results in the formation of immune complexes along the GBM, which comprise the heterologous anti-GBM antibody and the host IgG. This results in a second phase of renal injury, termed the autologous phase. The autologous phase can be triggered immediately after injecting anti-GBM antibody by using animals that have been pre-immunised with immunoglobulin from the species used to generate the anti-GBM antibody. In my experiments I used a sheep anti-mouse GBM antibody. To generate accelerated NTN I immunised mice with sheep IgG in adjuvant 5 days prior to injection of the anti-GBM antibody.

Recruitment of neutrophils to glomeruli is important in the heterologous stage since neutrophil depletion is protective (Cochrane, Unanue et al. 1965). Complement activation is pivotal to neutrophil recruitment since these cells do not accumulate in glomeruli following either complement depletion or the use of non-complement fixing anti-GBM antibody (Cochrane, Unanue et al. 1965). Fc receptors are critical for the development of injury in NTN since mice with genetic deletion of the Fc receptor gamma chain are completely protected against renal injury (Park, Ueda et al. 1998, Suzuki, Shirato et al. 1998). The lack of renal injury in CD40-deficient mice, that are unable to produce IgG, IgA or IgE responses due to defective immunoglobulin class switching, indicated that antigen-specific IgM is insufficient to trigger disease (Wakayama, Hasegawa et al. 2000). Whilst this would suggest a dominant role for antibody in the effector phase, other studies have shown antibody-independent renal injury. Most notably, μ-chain deficient mice that have intact cell-mediated immunity

but no mature B cells, develop crescent formation, proteinuria and renal dysfunction comparable to wild-type animals (Li, Holdsworth et al. 1997). The genetic background of mice influences the phenotype possibly through differences in T cell responses to antigen. C57BL/6 mice (those used in my experiments) develop primarily a Th1 response, whilst a Th2 response is characteristic in BALB/c mice. Complement depletion (using cobra venom factor) had no effect on disease in C57BL/6 mice, but led to a marked attenuation in BALB/c mice where disease is characterised by significant glomerular IgG and complement deposition (Huang, Holdsworth et al. 1997).

Consistent with both injurious and protective functions, complement has been shown to exacerbate and protect from renal injury in NTN. In C3-deficent mice, disease is milder in the heterologous phase but more severe in the autologous phase (Sheerin, Springall et al. 2001). The latter may relate to the inability of C3-deficient mice to physiologically remove immune complexes. Defective clearance of immune deposits and apoptotic cells from glomeruli was proposed as the explanation for the observation that C1q-deficient mice developed more severe injury during NTN (Robson, Cook et al. 2001).

The inability to regulate complement activation is associated with increased renal injury during NTN. FH-deficient mice are more susceptible to both heterologous (Pickering, Warren et al. 2006) and autologous NTN (Pickering, Cook et al. 2002). This injurious phenotype was completely abolished when AP activation was prevented in this strain by introducing FB deficiency (Pickering, Cook et al. 2002). In this chapter, I present data from the study of hepatocyte-*Cfh*$^{-/-}$ mice during accelerated NTN. My hypothesis was: sub-total FH deficiency enhances renal injury during experimental NTN because of impaired regulation of complement activation within the kidney.

4.2. Overview of NTN experiments

My aim was to compare the response of hepatocyte-*Cfh*$^{-/-}$ mice to wild-type mice and I performed two experiments in which I compared these two groups. In a further two experiments I also included *Cfh*$^{loxP/loxP}$ animals and used either wild-type or Alb-Cre mice as controls. The experiments are summarised as follows:

NTN experiment	Wild-type	Alb-Cre	hepatocyte-*Cfh*$^{-/-}$	*Cfh*$^{loxP/loxP}$
1 - female mice	N=10	-	N=7	-
2 - male mice	-	N=5	N=6	N=5
3 - male mice	N=7	-	N=3	-
4 - female mice	N=9	-	N=14	N=12

The outcome of these experiments was that it was clear that the hepatocyte-*Cfh*$^{-/-}$ mice had an exaggerated response to the NTN compared to the response of wild-type mice. Furthermore, the *Cfh*$^{loxP/loxP}$ animals also displayed a hypersensitive response to NTN. In this chapter, I present all of the experimental data relating to NTN experiment 4 since this is representative of the dataset as a whole. In the appendix I summarise the data from NTN experiments 1 – 3. The small number of hepatocyte-*Cfh*$^{-/-}$ animals (n=3) in NTN experiment 3 was due to a genotyping error.

4.3. Survival

In my initial experiments it was evident that the hepatocyte-*Cfh*$^{-/-}$ mice became unwell within days of administration of the nephrotoxic serum (NTS). This is in marked contrast to what is seen in wild-type mice in this model. Wild-type mice are typically sacrificed 10-12 days after administration of the NTS and it is uncommon for these animals to show any signs of disease other than abnormal urinalysis. The rapid adverse reaction of hepatocyte-*Cfh*$^{-/-}$ mice necessitated culling of the animals at day 3 after administration of the NTS. In NTN experiment 4, two out of the 14 hepatocyte-*Cfh*$^{-/-}$ mice died 3 days after receiving NTS. There were no deaths amongst the 12 *Cfh*$^{loxP/loxP}$ and 9 wild-type animals.

4.4. Renal function: urinalysis

Within 24 hours of receiving NTS, all hepatocyte-*Cfh*$^{-/-}$ mice had maximal haematuria (defined as 3+ using the reagent strips) whereas haematuria was absent in all wild-type mice and variably present in the *Cfh*$^{loxP/loxP}$ mice (figure 4.1a). On day 3 post administration of the NTS the median haematuria in the *Cfh*$^{loxP/loxP}$ mice had risen whereas no significant haematuria was present in the wild-type mice (figure 4.1b). In contrast, proteinuria did not differ statistically between the groups on either day one or day three (figure 4.2). In view of the clinical condition of the hepatocyte-*Cfh*$^{-/-}$ mice (ruffled coat and anasarca), I did not place the animals in metabolic cages to collect a timed urine sample.

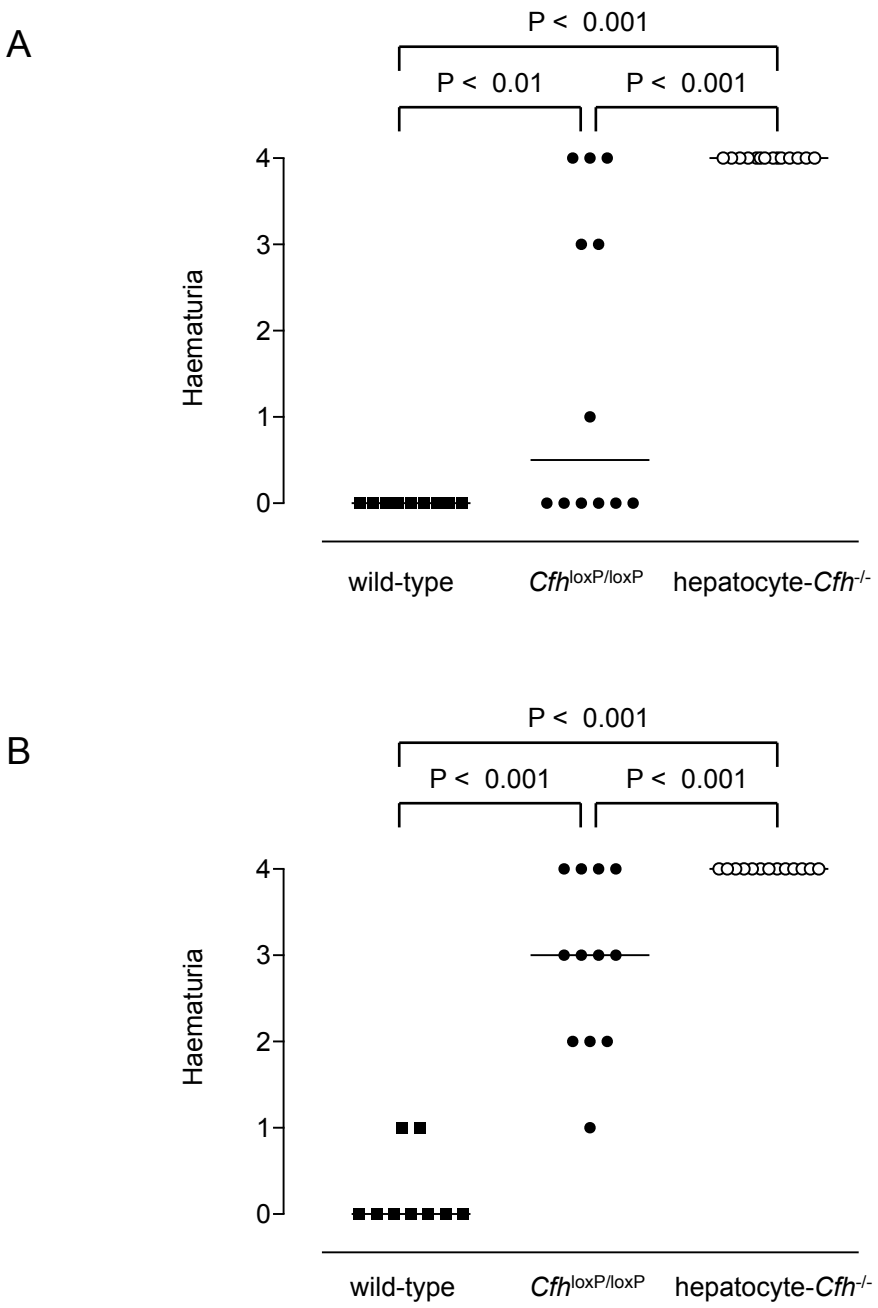


Figure 4.1 **Haematuria in wild-type, *Cfh*$^{loxP/loxP}$ and hepatocyte-*Cfh*$^{-/-}$ mice at day 1 (A) and 3 (B) following NTS injection.** Haematuria was already maximal by dipstick analysis within 24 hours of receiving NTS in hepatocyte-*Cfh*$^{-/-}$ mice. Haematuria grading: 0 = negative, 1 = trace, 2 = 1+, 3 = 2+ and 4 = 3+. P values calculated using Bonferroni multiple comparison test. Horizontal bars denote median values.

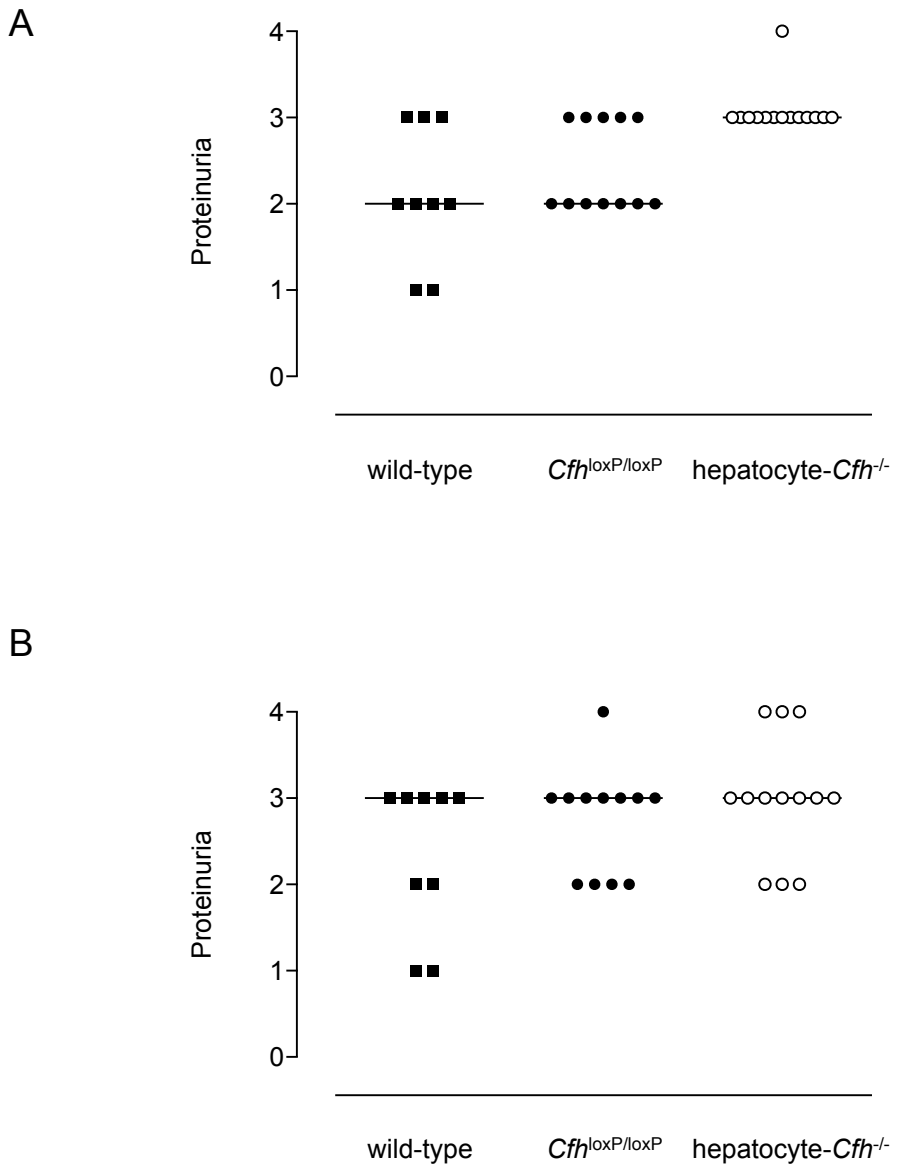


Figure 4.2 **Proteinuria in wild-type, *Cfh*$^{loxP/loxP}$ and hepatocyte-*Cfh*$^{-/-}$ mice at day 1 (A) and 3 (B) following NTS injection.** Proteinuria was not statistically different between the groups at either time point. Proteinuria grading: negative or trace = 0, 1+ (0.3g/l) = 1, 2+ (1g/l) = 2, 3+ (3g/l) = 3 and 4+ (≥20g/l) = 4. Horizontal bars denote median values.

4.5. Renal function: serum urea

In keeping with more severe haematuria, hepatocyte-*Cfh*$^{-/-}$ mice had significantly higher urea levels (median 121.9mmol/l, range 86.3-172.0, n=12) than both *Cfh*$^{loxP/loxP}$ (median 32.6mmol/l, range 10.8-62.5, n=12, p<0.001, Bonferroni multiple comparison test) and wild-type mice (median 22.1mmol/l, range 8.3-30.6, n=9, p<0.001, Bonferroni multiple comparison test, figure 4.3). Urea levels in *Cfh*$^{loxP/loxP}$ mice were higher than in wild-type mice but this did not reach statistical significance.

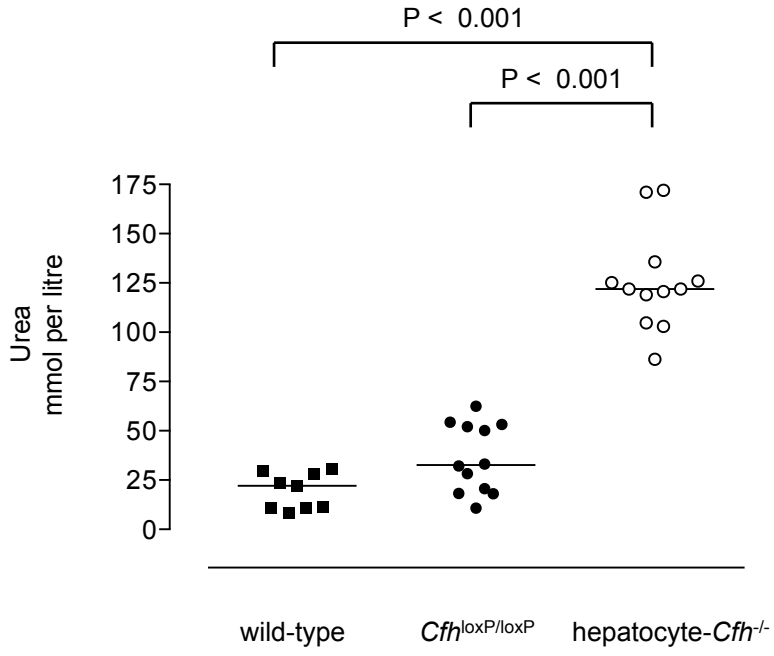


Figure 4.3 **Serum urea in wild-type, *Cfh*$^{loxP/loxP}$ and hepatocyte-*Cfh*$^{-/-}$ mice at day 3 following NTS injection.** P values calculated using Bonferroni multiple comparison test. Horizontal bars denote median values.

4.6. Haematological parameters

Hepatocyte-*Cfh*$^{-/-}$ mice had significantly lower haematocrits (median 22.5%, range 16-26, n=12) than wild-type mice (median 35%, range 30-40, n=9, $p<0.001$, Bonferroni multiple comparison test, figure 4.4a).

Red cell fragments were also present on peripheral blood film examination in hepatocyte-*Cfh*$^{-/-}$ mice (median 20 per 200 red blood cells (RBCs) counted, range 10-37, n=12, versus median 3 per 200 RBCs in wild-type mice, range 1-10, n=9, $p<0.001$, Bonferroni multiple comparison test, figure 4.4b).

Blood film examination in hepatocyte-*Cfh*$^{-/-}$ mice was also notable for the paucity of platelets (figure 4.5).

The reduced haematocrit, red cell fragmentation and thrombocytopenia were consistent with the presence of a microangiopathic haemolytic anaemia in the hepatocyte-*Cfh*$^{-/-}$ mice.

Consistent with the reduced basal expression of the floxed *Cfh* allele in *Cfh*$^{loxP/loxP}$ mice, induction of NTN in these animals gave rise to a haematological phenotype intermediate to that of wild-type and hepatocyte-*Cfh*$^{-/-}$ mice. Haematocrit in *Cfh*$^{loxP/loxP}$ mice (median 24%, range 16-32, n=12) was significantly lower than in wild-type mice (median 35%, range 30-40, n=9, $p<0.001$, Bonferroni multiple comparison test, figure 4.4a), whilst red cell fragments were significantly greater (median 17.5 per 200 RBCs, range 6-43, n=12 versus median 3 per 200 RBCs, range 1-10, n=9, $p<0.001$, Bonferroni multiple comparison test, figure 4.4b).

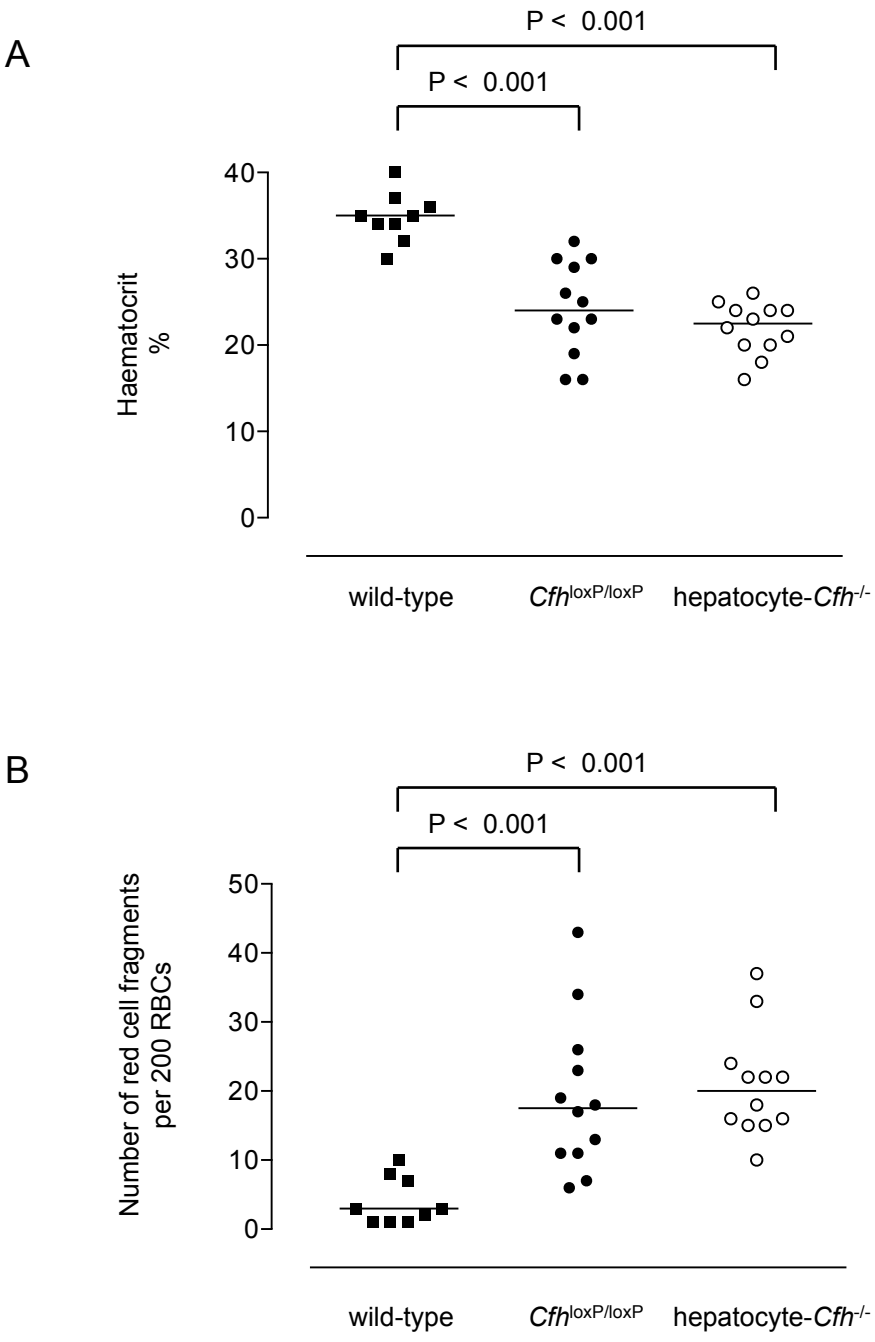
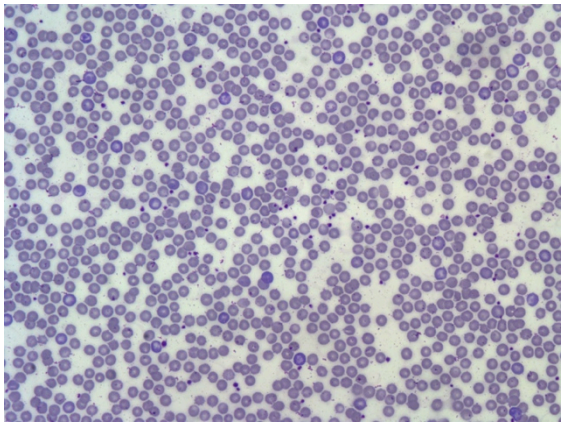
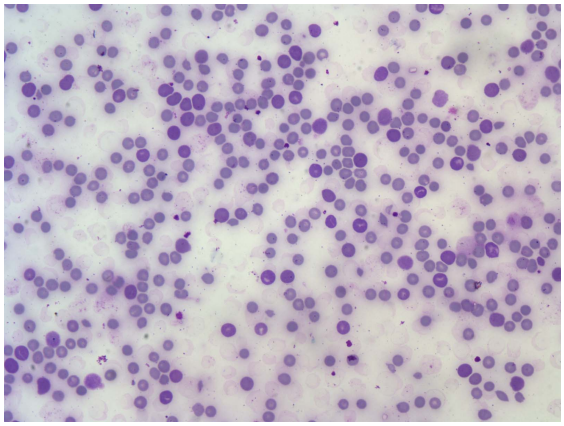


Figure 4.4 Haematocrit (A) and red cell fragments (B) in wild-type, *Cfh*$^{loxP/loxP}$ and hepatocyte-*Cfh*$^{-/-}$ mice on day 3 following NTS injection. Hepatocyte-*Cfh*$^{-/-}$ mice developed a microangiopathic haemolytic anaemia characterised by a low haematocrit and red cell fragmentation. *Cfh*$^{loxP/loxP}$ animals displayed an intermediate phenotype. P values calculated using Bonferroni multiple comparison test. Horizontal bars denote median values.

wild-type



Cfh$^{loxP/loxP}$



hepatocyte-*Cfh*$^{-/-}$

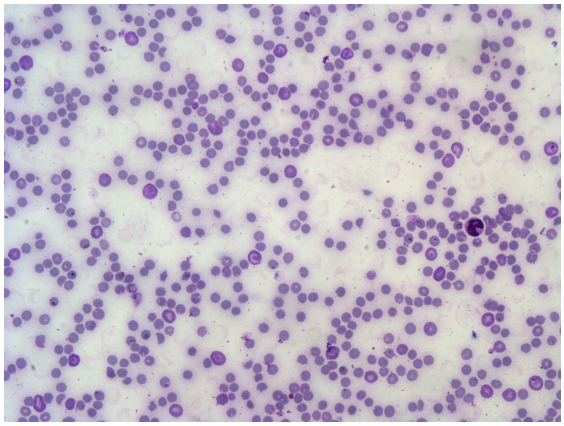


Figure 4.5 **Peripheral blood film examination in wild-type, *Cfh*$^{loxP/loxP}$ and hepatocyte-*Cfh*$^{-/-}$ mice 3 days following NTS injection.** Red cell fragments and thrombocytopaenia were typically seen in hepatocyte-*Cfh*$^{-/-}$ and to a lesser extent in *Cfh*$^{loxP/loxP}$ mice.

4.7. Renal histology

I first noted macroscopic evidence of more severe renal injury in the sacrificed hepatocyte-*Cfh*$^{-/-}$ animals. Kidneys from these animals at this time point had haemorrhagic areas over their surface. In contrast, kidneys from *Cfh*$^{loxP/loxP}$ and wild-type animals were macroscopically normal.

4.7.1. Glomerular light microscopy

Severe glomerular thrombosis was evident in all of the hepatocyte-*Cfh*$^{-/-}$ animals at day 3 following NTS injection (median 3.2, range 2.9-3.8, n=14, figure 4.6 and figure 4.7). This was in stark contrast to the almost complete absence of these changes in wild-type animals at this time point (median 0.1, range 0-1, n=9, $p<0.001$, Bonferroni multiple comparison test).

Consistent with other haematological and biochemical parameters after induction of NTN, *Cfh*$^{loxP/loxP}$ mice exhibited glomerular thrombosis that was intermediate between hepatocyte-*Cfh*$^{-/-}$ and wild-type animals (median 1.9, range 0.6-3.1, n=12, $p<0.001$ vs. either hepatocyte-*Cfh*$^{-/-}$ or wild-type animals, Bonferroni multiple comparison test, figure 4.6 and figure 4.7).

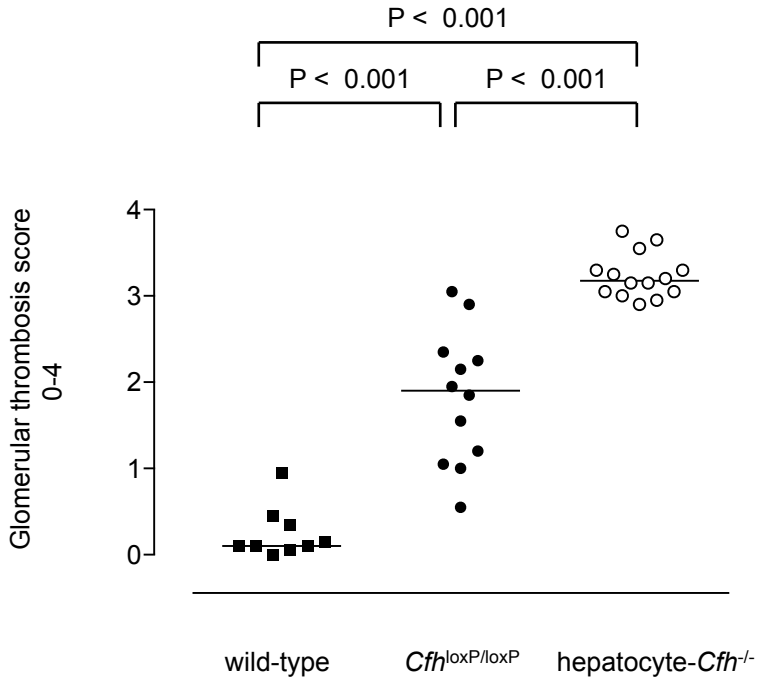
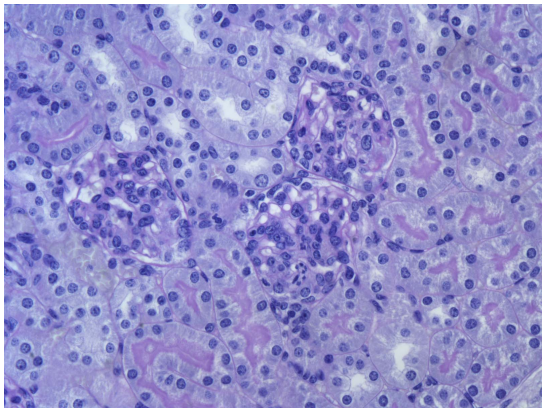
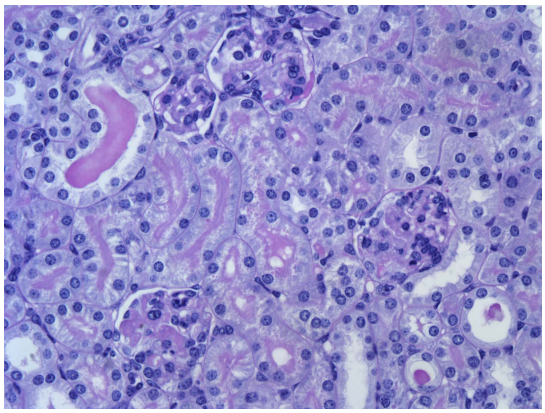


Figure 4.6 **Glomerular thrombosis in wild-type, *Cfh*$^{loxP/loxP}$ and hepatocyte-*Cfh*$^{-/-}$ mice at day 3 following NTS injection.** Marked glomerular thrombosis typical of haemolytic uraemic syndrome was observed in all hepatocyte-*Cfh*$^{-/-}$ mice. P values calculated using Bonferroni multiple comparison test. Horizontal bars denote median values.

wild-type



Cfh$^{loxP/loxP}$



hepatocyte-*Cfh*$^{-/-}$

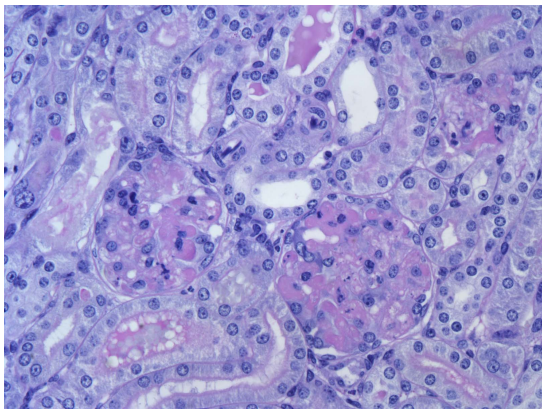


Figure 4.7 **Representative images of renal sections in wild-type, *Cfh*$^{loxP/loxP}$ and hepatocyte-*Cfh*$^{-/-}$ mice at day 3 following NTS injection.** Marked glomerular thrombosis typical of haemolytic uraemic syndrome was observed in all hepatocyte-*Cfh*$^{-/-}$ mice. Renal tissue is PAS-stained and original magnification x40.

4.7.2. Glomerular immunofluorescence for mouse C3

Hepatocyte-*Cfh*$^{-/-}$ mice have abnormal spontaneous mesangial C3 accumulation but during NTN glomerular C3 deposition altered. At the time of sacrifice C3 staining was evident within thrombosed glomerular capillary loops. When I quantified glomerular C3 immunofluorescence there were increased scores in hepatocyte-*Cfh*$^{-/-}$ (median score 2.4, range 2-3, n=12) compared to wild-type animals (median score 0, range 0-0.5, n=9, $p<0.001$, Bonferroni multiple comparison test, figure 4.8).

Glomerular C3 deposition in *Cfh*$^{loxP/loxP}$ mice (median 0.6, range 0.1-2.0, n=11) was increased compared to wild-type animals ($p<0.01$, Bonferroni multiple comparison test) but less than that seen in the hepatocyte-*Cfh*$^{-/-}$ mice ($p<0.001$, Bonferroni multiple comparison test, figure 4.8).

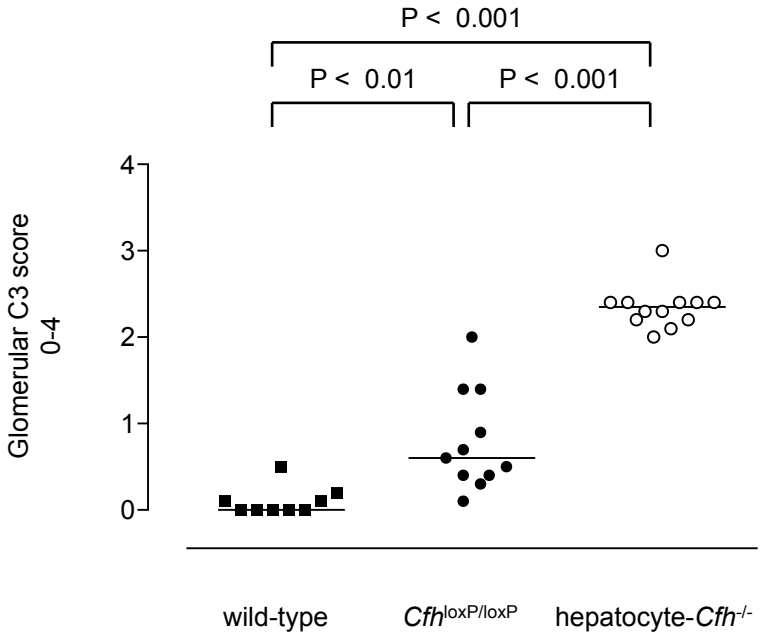


Figure 4.8 **Glomerular C3 immunofluorescence studies in wild-type, *Cfh*$^{loxP/loxP}$ and hepatocyte-*Cfh*$^{-/-}$ mice at day 3 following NTS injection**. P values calculated using Bonferroni multiple comparison test. Horizontal bars denote median values.

4.7.3. Glomerular immunofluorescence for mouse IgG

There were no significant differences in glomerular mouse IgG deposition between the hepatocyte-*Cfh*$^{-/-}$ (median 308.3 AFU, range 143.5-625.4, n=12) and wild-type animals (median 418.8 AFU, range 317.7-786.3, n=9, figure 4.9). Hepatocyte-*Cfh*$^{-/-}$ mice had less glomerular mouse IgG detected than *Cfh*$^{loxP/loxP}$ animals (median 503.8 AFU, range 228.3-1113.0, n=11, $p<0.05$, Bonferroni multiple comparison test) despite having more severe disease.

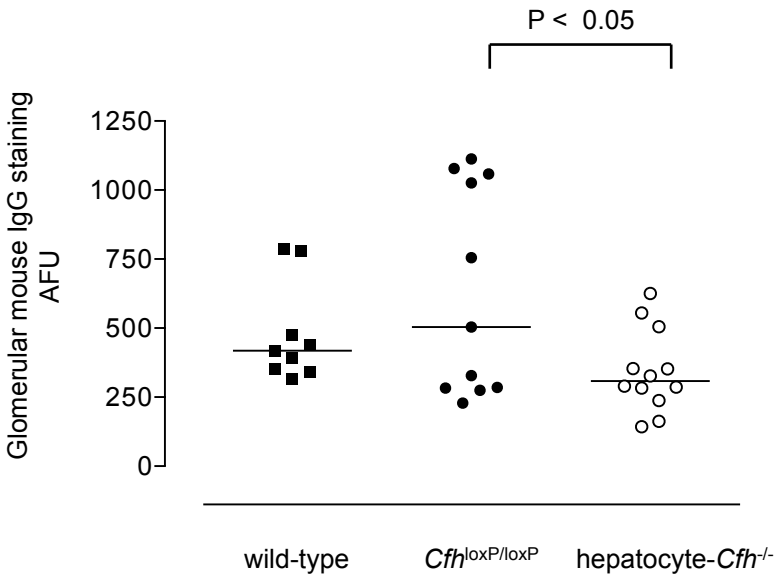


Figure 4.9 **Glomerular mouse IgG immunofluorescence studies in wild-type, *Cfh*$^{loxP/loxP}$ and hepatocyte-*Cfh*$^{-/-}$ mice at day 3 following NTS injection**. P values calculated using Bonferroni multiple comparison test. Horizontal bars denote median values. AFU – arbitrary fluorescence units.

4.7.4. Glomerular immunofluorescence for sheep IgG

To determine whether the sheep anti-mouse GBM antibody had deposited to a comparable extent in the three groups, I quantified glomerular sheep IgG immunofluorescence staining (figure 4.10).

Glomerular sheep IgG deposition in hepatocyte-*Cfh*$^{-/-}$ mice (median 591.7 AFU, range 457.8-834.2, n=12) was significantly lower than that seen in wild-type mice (median 953.9 AFU, range 722.7-1256.0, n=9, $p<0.001$, Bonferroni multiple comparison test). Interestingly, whilst this might be predicted to give rise to less disease (since there was ostensibly less antibody within the glomeruli) the converse was found. My interpretation was that the extensive thrombosis and damaged glomerular architecture precluded an accurate quantitative assessment of the deposited sheep anti-GBM antibody in these animals. In contrast, where the glomerular appearance was almost normal by light microscopy in the wild-type animals at this time point, this approach was valid (figure 4.11).

Like wild-type animals, the glomerular sheep IgG deposition in the *Cfh*$^{loxP/loxP}$ animals (median 762.3 AFU, range 626.9-954.1, n=12) was significantly greater than that seen in the hepatocyte-*Cfh*$^{-/-}$ mice ($p<0.01$, Bonferroni multiple comparison test). Consistent with my interpretation that severe glomerular thrombosis was a confounding factor in this analysis, glomerular sheep IgG deposition in *Cfh*$^{loxP/loxP}$ animals was intermediate between hepatocyte-*Cfh*$^{-/-}$ and wild-type mice.

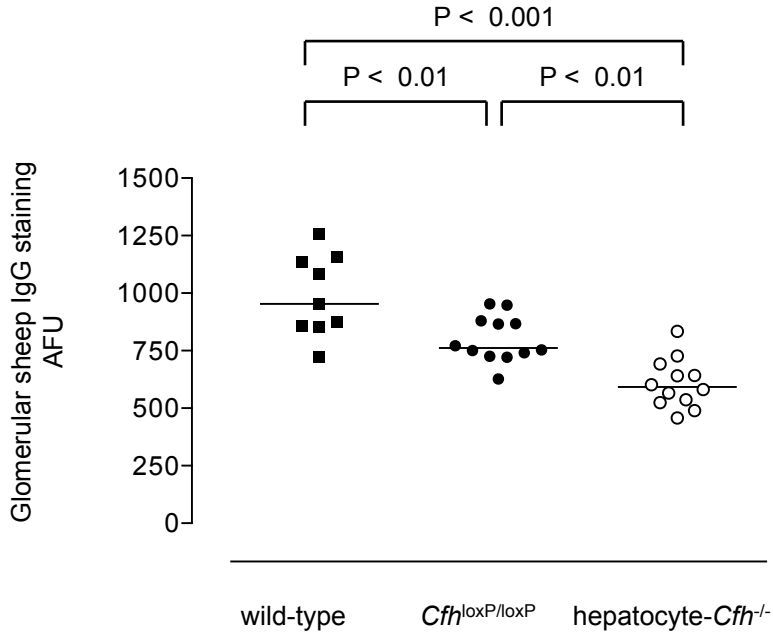


Figure 4.10 **Glomerular sheep IgG immunofluorescence studies in wild-type, *Cfh*$^{loxP/loxP}$ and hepatocyte-*Cfh*$^{-/-}$ mice at day 3 following NTS injection**. P values calculated using Bonferroni multiple comparison test. Horizontal bars denote median values. AFU – arbitrary fluorescence units.

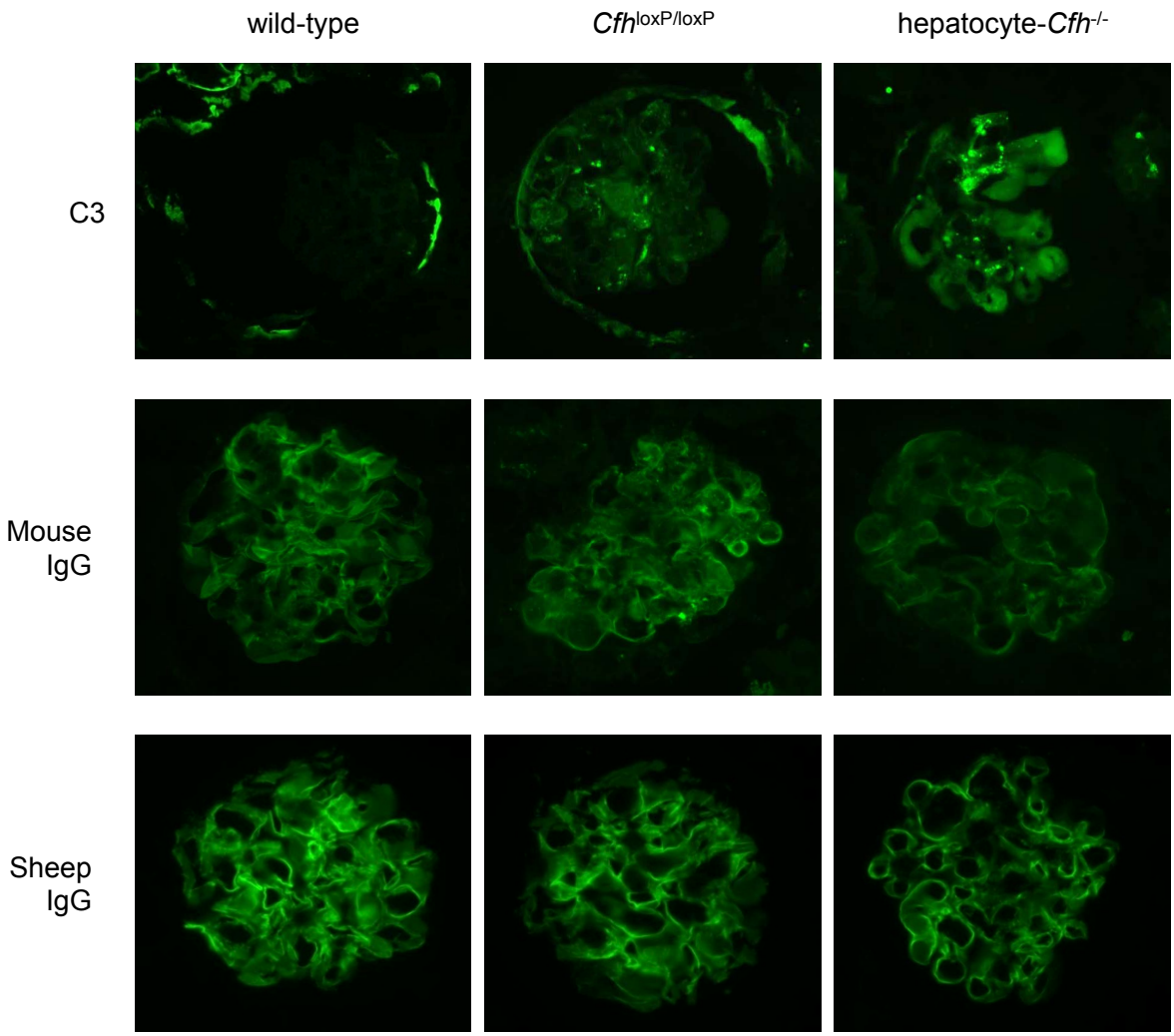


Figure 4.11 **Representative immunofluorescence staining images for C3, mouse IgG and sheep IgG in wild-type, *Cfh*$^{loxP/loxP}$ and hepatocyte-*Cfh*$^{-/-}$ mice at day 3 following NTS injection**. Glomerular C3 deposition was significantly greater in hepatocyte-*Cfh*$^{-/-}$ mice compared to wild-type animals.

4.8. Immune response in hepatocyte-specific FH-deficient mice

In studies by my colleague, Dr. Marieta Ruseva, the immune response to sheep IgG administered intra-peritoneally in complete Freund's adjuvant was assessed in hepatocyte-*Cfh*$^{-/-}$, *Cfh*$^{loxP/loxP}$ and wild-type animals. These data showed that there was no significant difference in the immune response between the experimental groups (figure 4.12). From her data, I concluded that the hypersensitive response of the hepatocyte-*Cfh*$^{-/-}$ animals was not due to an aberrant immune response to the injected sheep IgG.

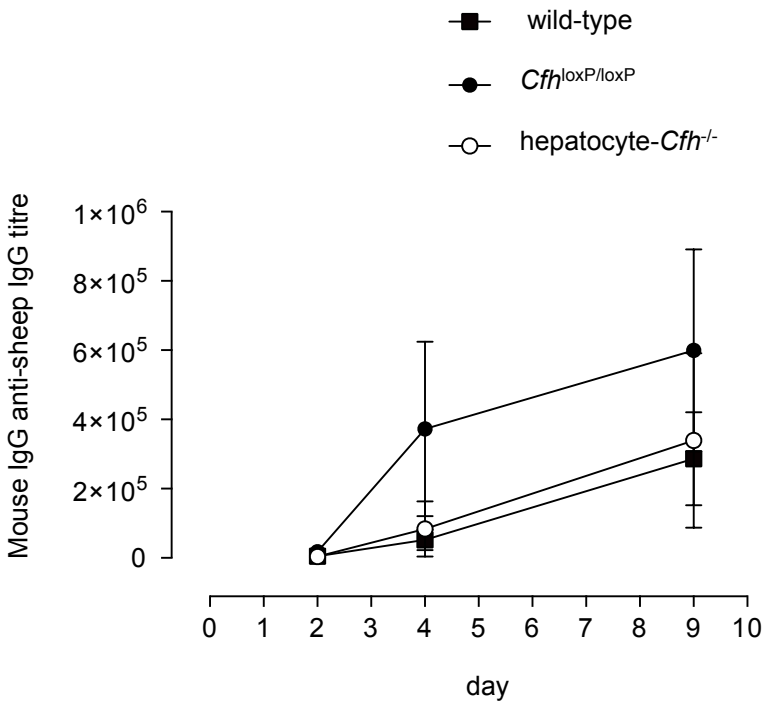


Figure 4.12 **Immune response to sheep IgG in complete Freund's adjuvant in the absence of NTN in wild-type, *Cfh*$^{loxP/loxP}$ and hepatocyte-*Cfh*$^{-/-}$ mice (courtesy of Dr. Marieta Ruseva)**. Sheep IgG in adjuvant was administered intra-peritoneally at day 0 and serum samples collected at days 2, 4 and 9. No significant differences in the mouse IgG anti-sheep IgG titres were seen between the groups at any of the time points.

4.9. Discussion

Since hepatocyte-*Cfh*$^{-/-}$ mice had sub-total FH deficiency, I hypothesised that these animals would be more susceptible to experimental renal injury induced by NTN as a consequence of an inability to regulate complement within the kidney. In this chapter I have presented data demonstrating that these mice were extremely sensitive to renal injury during NTN. Within 24 hours of receiving heterologous sheep anti-mouse GBM antibody, all hepatocyte-*Cfh*$^{-/-}$ mice developed haematuria and at day three had glomerular thrombosis, reduced haematocrit, and red cell fragmentation on peripheral blood films. My interpretation was that they had developed a phenotype consistent with haemolytic uraemic syndrome (HUS) during NTN.

It is interesting that the spontaneous phenotype and the phenotype observed during NTN were so different. These animals developed spontaneous accumulation of C3 within the mesangium but no evidence of either renal impairment or spontaneous thrombotic microangiopathy by the age of 36 weeks. These are features of C3 glomerulopathy. During NTN, there was rapid development of HUS. In my introduction I reviewed the association between FH dysfunction and the renal disorders: C3 glomerulopathy and HUS. Hepatocyte-*Cfh*$^{-/-}$ mice provide some insight into these associations since they illustrate how the same genetic defect can predispose to both spontaneous C3 glomerulopathy and environmentally triggered HUS. It is evident that there is phenotypic variation among individuals with the same complement mutation. For example, in CFHR5 nephropathy the spectrum of renal disease varies from microscopic haematuria to end-stage renal failure (Athanasiou, Voskarides et al. 2011). Similarly in atypical HUS, incomplete penetrance in the setting of complement mutations is common (Caprioli, Castelletti et al. 2003). It is also established that most episodes of atypical HUS are preceded by a triggering event such as infection, drugs and pregnancy (Caprioli, Castelletti et al. 2003). Furthermore, environmental events can aggravate established disease. For example, upper respiratory tract infection is well recognised as an exacerbating factor in IgA nephropathy, where it manifests clinically as synpharyngitic macroscopic haematuria. It is now known that this phenomenon is frequently seen in patients with C3 glomerulopathy. For example, in CFHR5 nephropathy synpharyngitic macroscopic haematuria is reported (Gale, de Jorge et al. 2010,

Vernon, Goicoechea de Jorge et al. 2012). It is relevant to note that the hepatocyte-*Cfh*$^{-/-}$ mice were housed in specific pathogen-free conditions so this is an artificial environment and I do not know how the renal phenotype might change in the setting of infection in these mice.

In mouse models of FH-associated renal injury, renal thrombotic microangiopathy (the pathological hallmark of HUS) is dependent on (1) the ability to regulate both C3 and C5 in plasma and (2) the presence of defective surface regulation by FH. Mice with complete FH deficiency clearly have impaired surface regulation since there is a complete absence of FH. However, the absence of FH results in complete consumption of both C3 and C5 through uncontrolled AP activation. Hence they do not develop spontaneous HUS (Pickering, Cook et al. 2002). When FH-deficient mice were engineered to transgenically express a FH protein that could regulate C3 but not mediate surface regulation, spontaneous HUS developed (Pickering, de Jorge et al. 2007). The important role of C5 was illustrated by the observation that HUS did not develop in these transgenic animals that were additionally rendered C5-deficient (de Jorge, Macor et al. 2011). These observations demonstrate that, in the setting of impaired surface regulation by FH, the development of HUS is influenced by the presence of functionally intact C3 and C5, with C5 playing a critical role. I have shown that the hepatocyte-*Cfh*$^{-/-}$ mice have sub-total FH deficiency, which is able to maintain plasma C3 levels at approximately 20% of normal. In my thesis I have not examined C5 activity but Dr. Marieta Ruseva in my supervisor's laboratory has subsequently developed C5 assays and shown that C5 is present in the hepatocyte-*Cfh*$^{-/-}$ mice (unpublished data). Extrapolating from these data and the published observations, hepatocyte-*Cfh*$^{-/-}$ mice were susceptible during NTN due to a combination of (1) the *inability* of the sub-total FH deficiency to adequately regulate complement within the kidney together with (2) the *ability* of the sub-total FH deficiency to preserve sufficient C3 and C5 in plasma. I would predict that C5 activation is the key effector mechanism during NTN in these mice and this is presently being tested in my supervisor's laboratory.

It was also important and clear from my data that the response of the *Cfh*$^{loxP/loxP}$ mice during NTN was not equivalent to wild-type animals. This strain developed glomerular thrombosis that was significantly greater than that seen in wild-type mice

but significantly less than that seen in the hepatocyte-*Cfh*$^{-/-}$ mice. The finding that *Cfh*$^{loxP/loxP}$ mice developed greater injury compared to wild-type mice was not completely unexpected since my data had shown that these animals had reduced FH levels. Their NTN phenotype indicated to me that this reduction in FH was sufficient to impair complement regulation within the kidney during NTN although not to the same extent as that seen in hepatocyte-*Cfh*$^{-/-}$ mice.

4.10. Conclusions

4.10.1. Conditional FH-deficient mice develop greater renal injury during NTN compared to wild-type mice consistent with impaired expression of FH by the targeted locus.

4.10.2. Mice with hepatocyte-specific FH deficiency develop greater renal injury during NTN compared to both wild-type and conditional FH-deficient mice.

5. CHAPTER FIVE: THE ROLE OF FACTOR H-RELATED PROTEINS IN C3 GLOMERULOPATHY

5.1. Introduction

In this chapter I present data relating to the biological role of the factor H-related (FHR) proteins. I will first summarise the key points relating to the disease associations of the FHR protein family and the recent data that have revealed that some of these proteins antagonise the action of FH, a process termed FH deregulation.

As discussed in my introduction, there are five FHR proteins termed FHR1, FHR2, FHR3, FHR4 and FHR5. These are encoded by separate but related genes located immediately downstream of the *CFH* gene. The presence of large genomic repeat regions at this locus predisposes to recombination events, which can result in gene deletion, exon loss or hybrid genes. Genetic variability within the *CFH-CFHR* locus is increasingly recognised as influencing disease susceptibility. Polymorphic deletion of the *CFHR3* and *CFHR1* genes (Δ*CFHR3-1*) is associated with a reduction in the risk of age-related macular degeneration (Hughes, Orr et al. 2006) and IgA nephropathy (Gharavi, Kiryluk et al. 2011) but increased susceptibility to systemic lupus erythematosus (Zhao, Wu et al. 2011). The deletion is also associated with the development of autoantibodies to FH in atypical HUS (Skerka and Zipfel 2008). Abnormal rearrangements within this locus include mutant FHR proteins and hybrid genes, which are associated with atypical HUS (Venables, Strain et al. 2006, Francis, McNicholas et al. 2012) and C3 glomerulopathy (Gale, de Jorge et al. 2010, Malik, Lavin et al. 2012, Tortajada, Yebenes et al. 2013) respectively. These are shown schematically in figure 5.1.

Some *in vitro* studies claimed that some of the FHR proteins have intrinsic complement regulatory activity. FHR1 was reported to inhibit the C5 convertase (Heinen, Hartmann et al. 2009), FHR5 inhibited the C3 convertase (McRae, Duthy et al. 2005) and FHR3, FHR4 and FHR5 have been reported to have cofactor activity for the FI-mediated cleavage of C3b (Hellwage, Jokiranta et al. 1999, McRae, Cowan et al. 2001, Fritsche, Lauer et al. 2010, Hebecker, Okemefuna et al. 2010). More recently, and since I completed my thesis work, data has emerged that has demonstrated that FHR1, FHR2 and FHR5 have no intrinsic complement regulatory activity but are able to compete with FH for binding to C3b. Under physiological

conditions neither FHR1 nor FHR5 inhibit or interact with C5 or possess FI cofactor activity (Goicoechea de Jorge, Caesar et al. 2013, Tortajada, Yebenes et al. 2013). FHR1, FHR2 and FHR5 compete with FH for interaction with C3b and, unlike FH, when these FHR proteins interact with C3b further C3b amplification can occur. FHR4 also promotes complement activation by facilitating C3 convertase formation when C3b is bound to ligand (Hebecker and Jozsi 2012). The current paradigm is that these proteins prevent the ability of FH to inactivate C3b (Goicoechea de Jorge, Caesar et al. 2013, Tortajada, Yebenes et al. 2013). This FH antagonism has implications for how we should now view the mechanistic basis of the association of abnormal FHR proteins in renal disease.

The liver is the presumed major site of synthesis of the FHR proteins, although a comprehensive description of the cell types responsible for their synthesis is yet to be defined. FHR5 was first identified as a component of glomerular complement deposits (Murphy, Georgiou et al. 2002). FHR1 has also been detected in the kidney in the lining of larger arteries and in afferent and efferent arterioles (Heinen, Hartmann et al. 2009). FHR1 is expressed in the retina (although at levels >250,000-fold lower than in the liver (Hageman, Hancox et al. 2006)), whilst FHR1-5 have all been detected in middle ear effusion fluid in children with otitis media (Narkio-Makela, Hellwage et al. 2001). Defining the ability of tissues like the kidney and retina to synthesise FHR proteins is likely to be important in understanding their biological role. For example, up-regulation of mRNA expression of a glomerular epithelial cell rat factor H-related protein, reportedly similar to human FHR5, has been described in a rodent model of complement activation (Ren, Doshi et al. 2002). The ability of locally produced complement components to exert functionally significant effects is established for C3 (Springall, Sheerin et al. 2001, Pratt, Basheer et al. 2002, Farrar, Zhou et al. 2006).

The data in this chapter is in three parts. The first two parts describe clinical studies I performed in two informative patients with C3 glomerulopathy and the third outlines work that I performed to determine if there was significant renal synthesis of the FHR proteins. The first study demonstrates that renal-derived FHR5 could not prevent the recurrence of CFHR5 nephropathy. The second describes the association between

C3 glomerulopathy and haploinsufficiency of FHR5. In the third section I sought to characterise the expression of the human FHR proteins in liver and kidney tissue.

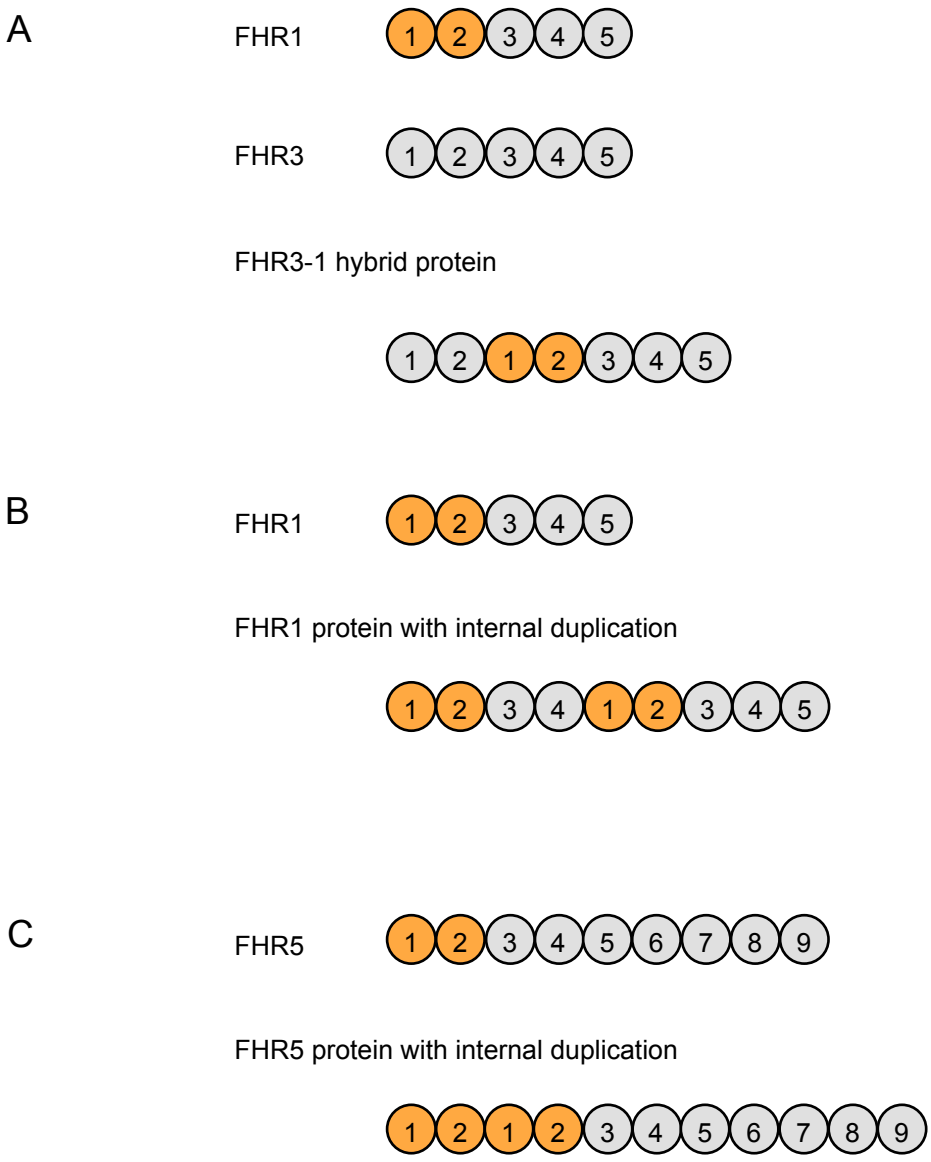


Figure 5.1 **Schematic depiction of abnormal FHR proteins associated with C3 glomerulopathy.** (A) hybrid FHR3-1 protein; (B) internal duplication in FHR1 and (C) an internal duplication in FHR5. The respective normal protein(s) are shown above the abnormal protein. The coloured domains 1 and 2 depict those that mediate the obligate dimerization amongst FHR1, FHR2 and FHR5.

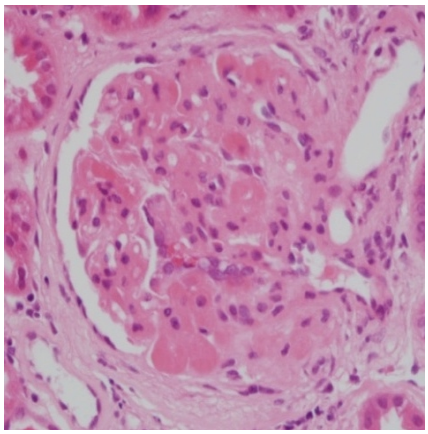
5.2. Recurrence of familial C3 glomerulonephritis in a renal transplant

In this section I present my work characterising the first known case of recurrence of CFHR5 nephropathy in an unrelated deceased donor renal transplant (Vernon, Gale et al. 2011). CFHR5 nephropathy was the term used to describe C3 glomerulonephritis (C3GN) associated with an internal duplication of exons 2 and 3 of the *CFHR5* gene (Gale, de Jorge et al. 2010). It is a familial renal disease characterised by persistent microscopic haematuria, synpharyngitic macroscopic haematuria and progressive renal failure in individuals of Cypriot origin. The natural course of the disease appears more aggressive in males who possess a higher risk of progressing to end-stage renal failure (Athanasiou, Voskarides et al. 2011). The pathological features of C3GN include membranoproliferative or mesangial proliferative changes within affected glomeruli.

5.2.1. Outline of the case

The index case was a 47-year old British male with Cypriot ancestry who reached end-stage renal failure after presenting 11 years earlier with persistent microscopic haematuria, synpharyngitic macroscopic haematuria and renal dysfunction. Retrospective review of two renal biopsies performed during his progression to end-stage renal disease demonstrated features consistent with C3GN. Capillary wall C3 accompanied an increase in mesangial cellularity and matrix, along with subendothelial, mesangial and more rarely, subepithelial deposits (figure 5.2). The second biopsy also revealed capillary wall thickening with double contours and mesangial cell interposition. Staining for immunoglobulins including IgA was negative.

B



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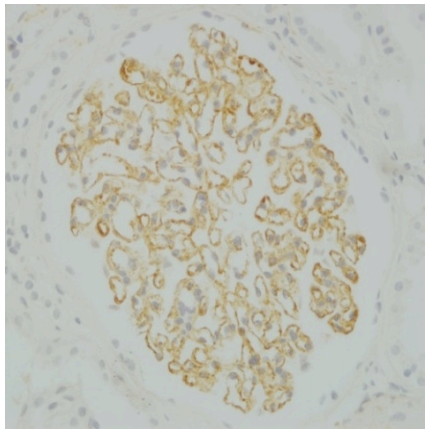


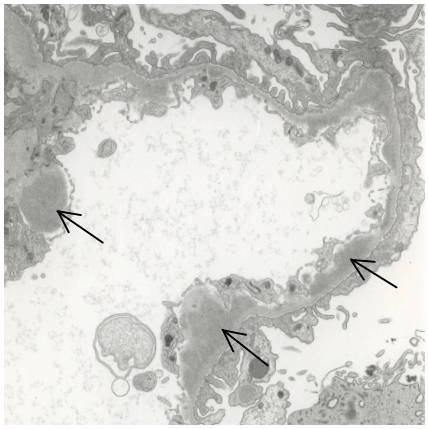


Figure 5.2 **Native renal biopsy.** (A) Light microscopic appearance showing segmental glomerulosclerosis and hyalinosis and increased mesangial matrix (hematoxylin and eosin). (B) Immunoperoxidase staining showing granular capillary wall C3 deposition. C3 was detected using a polyclonal rabbit anti-human C3c antibody (Dako, Ely, UK). (C) Electron micrograph showing multiple subendothelial electron dense deposits (denoted by arrows).

5.2.2. Diagnosis of CFHR5 nephropathy

To establish the diagnosis of CFHR5 nephropathy two approaches are commonly adopted: (i) screening of genomic DNA for the presence of the break point within the *CFHR5* gene and (ii) screening of plasma for the presence of the abnormal FHR5 protein.

Detection of the *CFHR5* gene break point is readily done by performing PCR on genomic DNA using primers that amplify both the wild-type and mutant loci (Gale, de Jorge et al. 2010). The nature of the break point that results in the internal duplication of exons 2 and 3 of the *CFHR5* gene means that this approach is unable to distinguish between individuals carrying the break point in heterozygosis or homozygosis.

However, since both the wild-type and mutant plasma proteins are secreted and differ significantly in size, the presence of the mutation and whether or not it is present in heterozygosis (both mutant and wild-type protein) or homozygosis (mutant protein alone) can be determined by analysing serum FHR5 by western blotting. The wild-type protein is comprised of 9 SCR domains and the mutant protein composed of 11 SCR domains. Using western blotting of serum from the affected individual, I was able to demonstrate the presence of both the mutant and wild-type FHR5 proteins using a polyclonal anti-FHR5 antibody. This confirmed the diagnosis of CFHR5 nephropathy and that the mutation was present in heterozygosis (figure 5.3).

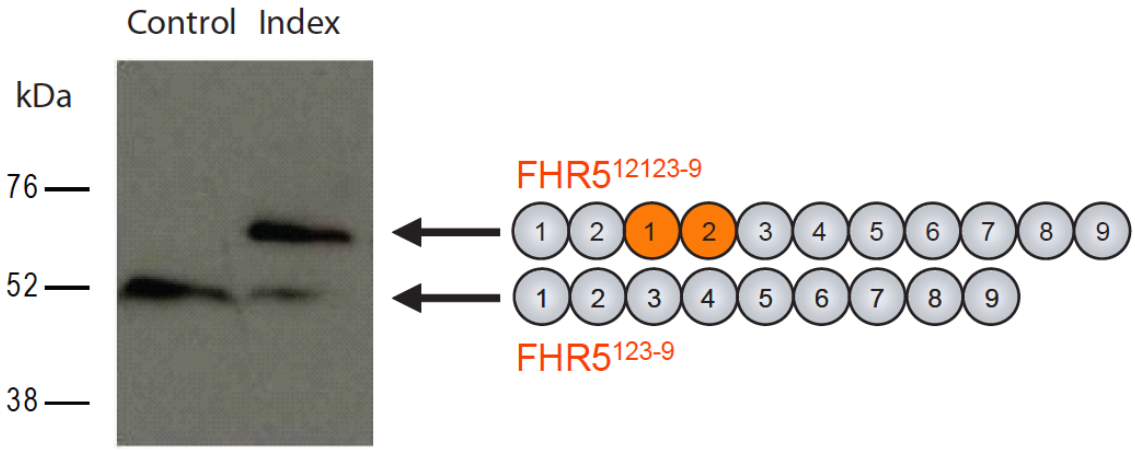


Figure 5.3 **Western blot of serum for FHR5 from the affected individual.** Both the wild-type FHR5 protein and the higher molecular weight mutant FHR5 protein are seen. The schematic depicts the short consensus repeat (SCR) domain structures of the wild-type and mutant proteins. The wild-type contains 9 SCR domains and the mutant contains 11 domains since domains 1 and 2 are duplicated. SCR – short consensus repeat.

5.2.3. Renal transplantation

At the age of 53, he received an unrelated deceased donor renal transplant (2,1,1 mismatch), which proceeded without complication. The donor had normal renal function and there were no recipient class I or II human leucocyte antigen antibodies at the time of transplantation. Immunosuppression comprised Alemtuzumab induction and peri-operative corticosteroids with Tacrolimus monotherapy continued on discharge.

Whilst his creatinine initially fell, failure to fall below 186µmol/l in the presence of microscopic haematuria and a single episode of macroscopic haematuria, led to the decision to perform a renal transplant biopsy (figure 5.4). At the time of biopsy, serum C3 was 0.73g/L (normal range 0.7-1.7g/L), FH 320mg/L (207% of normal control) and FI 32mg/L (177% of normal control).

Only 46 days post transplantation, capillary wall C3 and C9 deposition was found accompanying an increase in mesangial matrix with mesangial and subendothelial deposits. C4d staining was negative.

A second biopsy, undertaken 3 months later after a further decline in graft function, confirmed recurrence of his original disease. In this biopsy sample I had access to frozen tissue. This enabled me to determine if there was evidence of TP activation since it had been shown that a monoclonal mouse anti-human C5b-9 antibody (Dako, Ely, UK) reliably detected C5b-9 in frozen tissue sections. I applied an immunofluorescence staining approach using a two-step procedure in which I first applied the mouse anti-human C5b-9 antibody and then applied a fluorescein isothiocyanate-labelled goat anti-mouse IgG (Sigma, Dorset, UK). Using this approach I was able to detect C5b-9 staining in the second transplant kidney biopsy (figure 5.5).

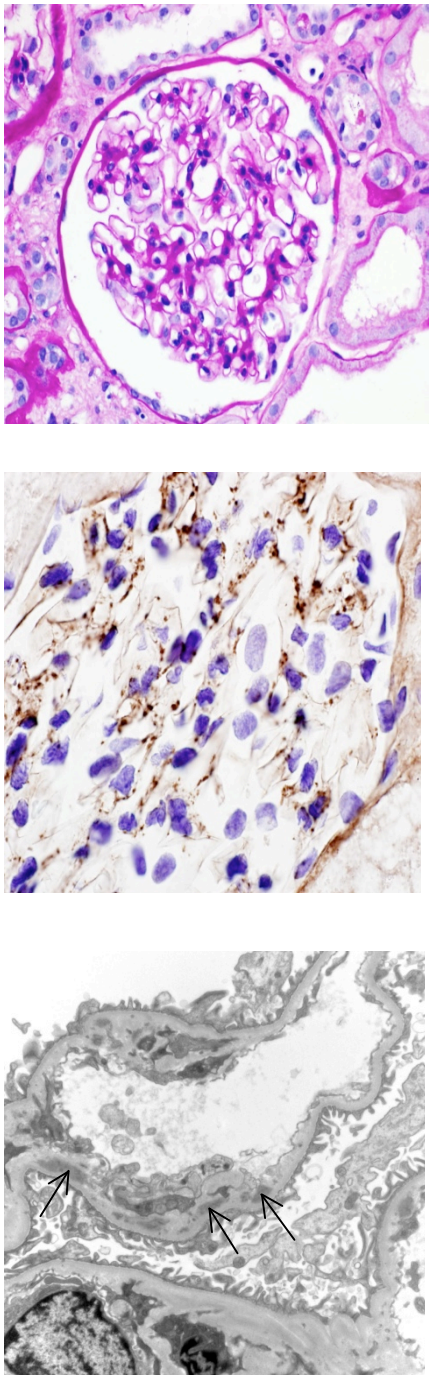


Figure 5.4 **Renal transplant biopsy.** (A) Light microscopy showing a normal glomerulus (periodic acid-Schiff). (B) Immunoperoxidase staining showing granular capillary wall and mesangial C3 deposition. C3 was detected using a polyclonal rabbit anti-human C3c antibody (Dako, Ely, UK). (C) Electron micrograph showing subendothelial deposits (denoted by arrows) with new basement membrane beneath.

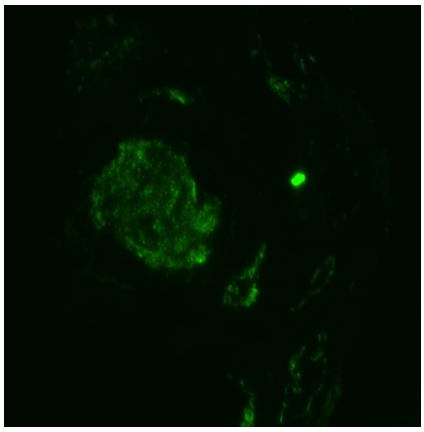


Figure 5.5 **Renal transplant biopsy staining for C5b-9.** C5b-9 was detected using a monoclonal mouse anti-human C5b-9 antibody and a FITC-labelled goat anti-mouse IgG. Diffuse glomerular staining for C5b-9 is evident.

5.2.4. Summary

In summary this was the first characterised case of recurrence of CFHR5 nephropathy in a renal transplant. The points that this study revealed were (i) the recurrence of abnormal complement deposition along the GBM was rapid i.e. 46 days post transplantation and (ii) if there is any biologically relevant synthesis of FHR5 within the kidney, this renal-derived FHR5 could not prevent the recurrence of CFHR5 nephropathy.

5.3. Persistent glomerulonephritis following streptococcal infection in an individual with heterozygous FHR5 deficiency

In this section I present my work characterising the complement profile of a patient who developed persistent glomerulonephritis following a streptococcal infection (Vernon, Goicoechea de Jorge et al. 2012). The patient was a 12-year old female with persistent kidney disease and hypocomplementaemia 5 years after streptococcal infection and I identified a heterozygous mutation in the *CFHR5* gene. This single nucleotide insertion in exon 4 of *CFHR5* resulted in a premature stop codon and was associated with reduced serum FHR5 levels and C3 glomerulopathy on renal biopsy.

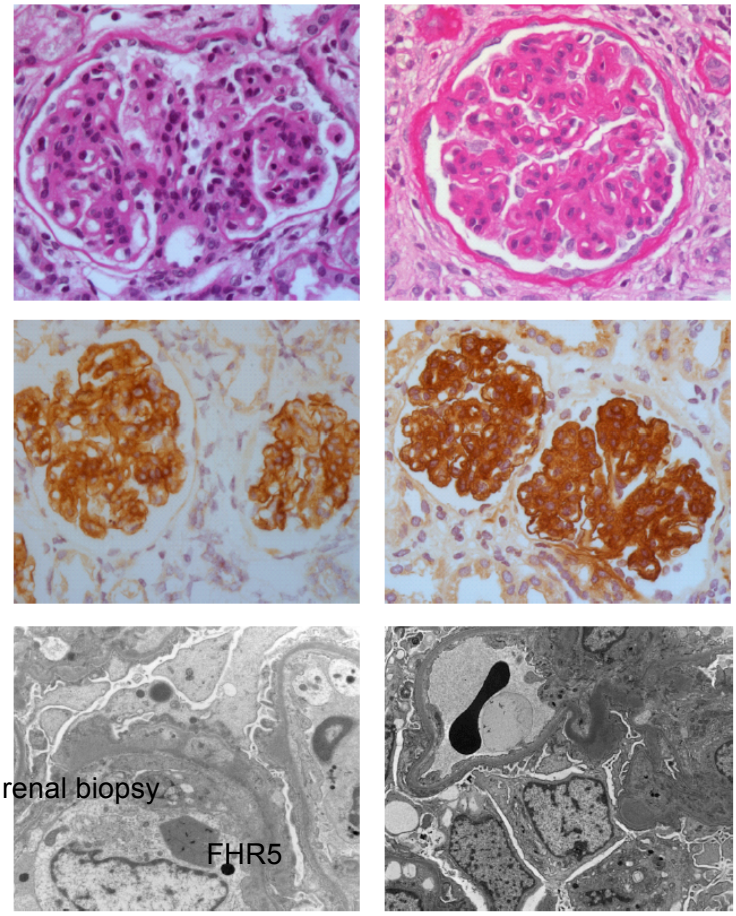
5.3.1. Outline of the case

A 7-year old Caucasian female presented with a 10-day history of sore throat, cough, fever and lethargy accompanied by abdominal pain, vomiting and dark-coloured urine. Previously fit and well, clinical examination and investigation revealed periorbital oedema, abnormal urinalysis (blood [3+] and protein [3+]; protein/creatinine ratio 590mg/mmol) and renal impairment (serum urea 17.9mmol/l and creatinine 87μmol/l). Whilst serum C4 was normal (0.29g/L [reference range 0.11-0.43g/L]), C3 was low (0.36g/L [reference range 0.75-1.65g/L]), and anti-streptolysin O (>200U/mL) and anti-DNase antibody (360U/mL) titres were both elevated. Renal ultrasound showed loss of corticomedullary differentiation. Treatment with phenoxymethylpenicillin led to clinical improvement but microscopic haematuria and proteinuria persisted.

Following a further episode of macroscopic haematuria coinciding with an upper respiratory tract infection, she underwent a renal biopsy nine months after the initial presentation on account of persistent haematuria, proteinuria and low C3 levels (figure 5.6). Mesangial hypercellularity, segmental endocapillary hypercellularity and capillary wall double contours were accompanied by marked granular capillary wall and mesangial C3, C9 and FHR5 deposition. Immunoglobulins and C1q were absent. Electron microscopy revealed a thickened GBM with segmental duplication and mesangial cell interposition. Intramembranous electron dense deposits (EDD) were seen with occasional subendothelial and hump-like subepithelial deposits.

These findings are consistent with C3 glomerulopathy with a membranoproliferative pattern of glomerulonephritis and were also seen on a second biopsy performed 20 months after the original illness. Persistent membranoproliferative glomerulonephritis and tubulointerstitial scarring affecting 40% of the cortex were observed with both intramembranous and mesangial deposits (figure 5.6).

Angiotensin receptor blockade and glucocorticoid therapy led to a reduction in proteinuria, but serum C3 levels remained low throughout. Her clinical course is summarised in figure 5.7. She has not developed ocular drusen or lipodystrophy.



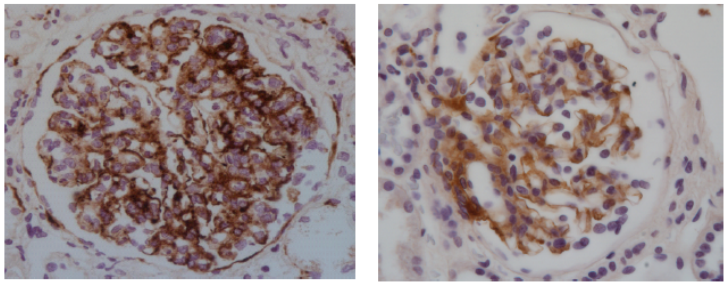


Figure 5.6 **Kidney biopsies performed at 9 and 20 months post presentation**. Light microscopy (first row) shows mesangial hypercellularity, segmental endocapillary hypercellularity, and capillary wall double contours with marked capillary wall C3, C9, and FHR5 seen on immunoperoxidase staining (second [C3] and fourth [C9, FHR5] rows). On electron microscopy (third row), there were intramembranous and occasional subendothelial electron dense deposits. Rare hump-like subepithelial deposits were seen in the first biopsy specimen.

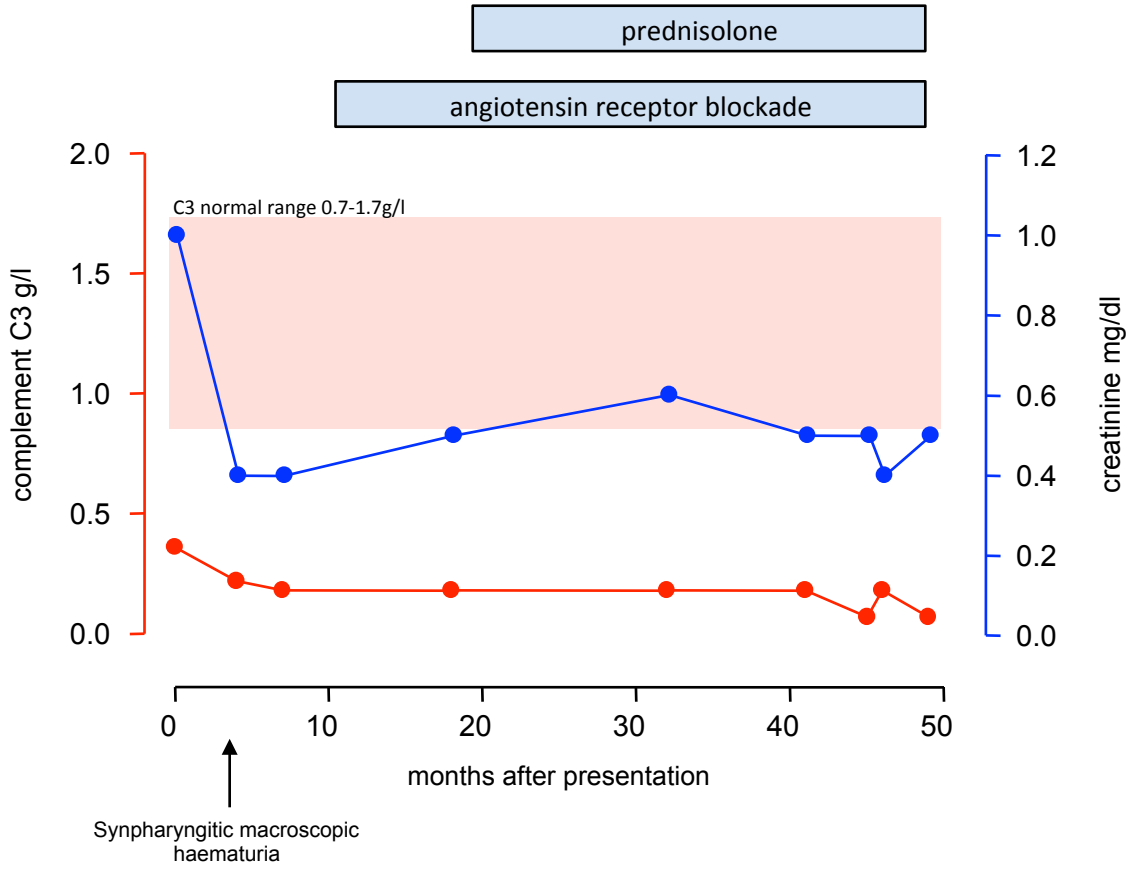


Figure 5.7 **Serum complement C3 and creatinine levels versus time**. Serum C3 levels remained profoundly depressed throughout the illness.

5.3.2. Assessment of serum factor triggering C3 activation

It was clear that this individual had chronic low levels of C3 so it was important to exclude the presence of C3 nephritic factor and autoantibodies to FH. Both these were excluded by assays used in clinical practice and performed by our clinical immunology department.

So my next approach was to exclude any other serum factor that might be causing complement C3 consumption. To achieve this I tested whether or not exogenous C3 was consumed when added to the serum of the index case, as would occur in sera that contained C3 nephritic factor. In my assay I added 0.5% purified C3 (Merck Sharpe and Dohme, Hertfordshire, UK) to serum from the index case and compared its haemolytic activity at 2 and 4 hours with that of C3-deficient human serum reconstituted with purified C3 in an identical fashion. Haemolytic activity at 2 (60% vs 57%) and 4 hours (43% vs 47%) did not differ between the test and control sera, indicating that there was no evidence of accelerated serum C3 conversion in serum of the index case.

5.3.3. Complement genetic investigations

In parallel my supervisor's laboratory organized genetic screening of complement genes to exclude mutations that have been associated with C3 glomerulopathy. There were no mutations in the exons and splice sites of the genes encoding FH, FI, CD46, FB and C3. In view of the characterization of CFHR5 nephropathy the laboratory performed sequence analysis of the *CFHR5* gene. This was done by Mrs. Kirsten Rose and revealed a single heterozygous nucleotide duplication in exon 4 (c.485dupA) which generates a reading frame shift at amino acid 163 and a premature stop codon at amino acid position 197 (p.Glu163Argfs*34, figure 5.8). This variant was not detected by sequencing of 198 ethnically matched DNA samples (obtained from the UK Blood Services Collection of Common Controls) and was not present in the databases.

The healthy mother (I-2) and sister (II-1), but not the 2 other siblings examined (II-2 and II-4), were heterozygous for this sequence variant (figure 5.9).

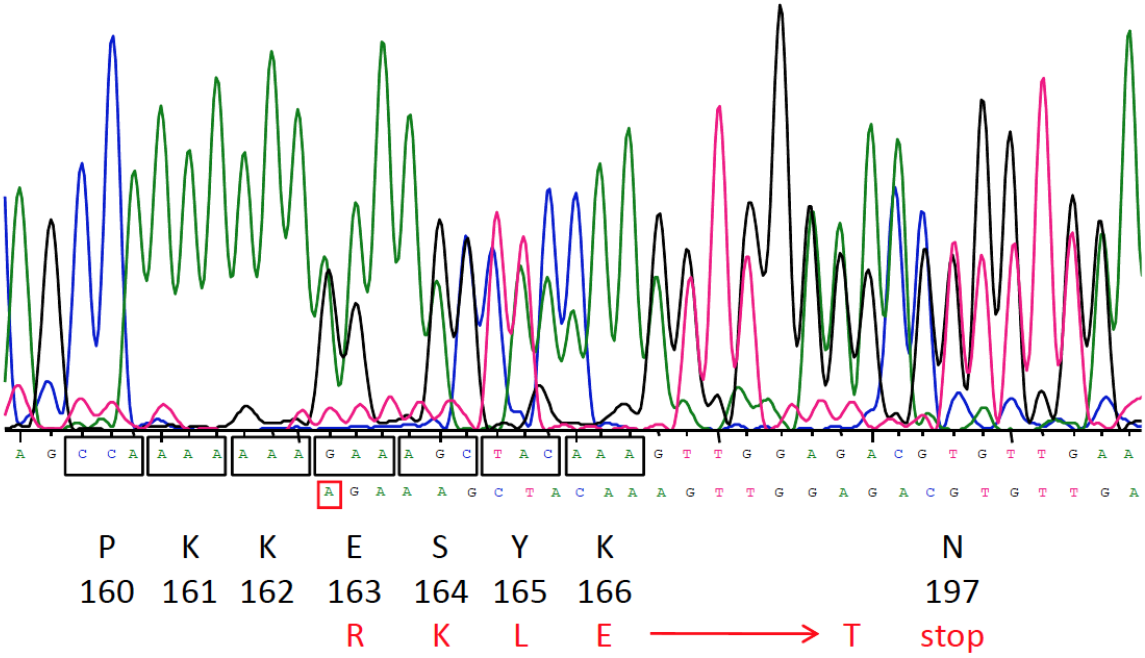


Figure 5.8 **Sequence chromatogram from the index case**. Sequence depicts *CFHR5* exon 4 sequence demonstrating duplicated sequence from the nucleotide c485. This results in a reading frame shift altering the amino acids (denoted in red) and generating a stop codon at amino acid position 197. Nucleotides: G – guanine, A – adenine, T – thymine, C – cytosine. Amino acids: P – phenylalanine, K – lysine, E – glutamic acid, S – serine, Y – tyrosine, N – asparagine, R – arginine, L – leucine, T - threonine. Numbering – A of ATG = 1, Methionine = 1.

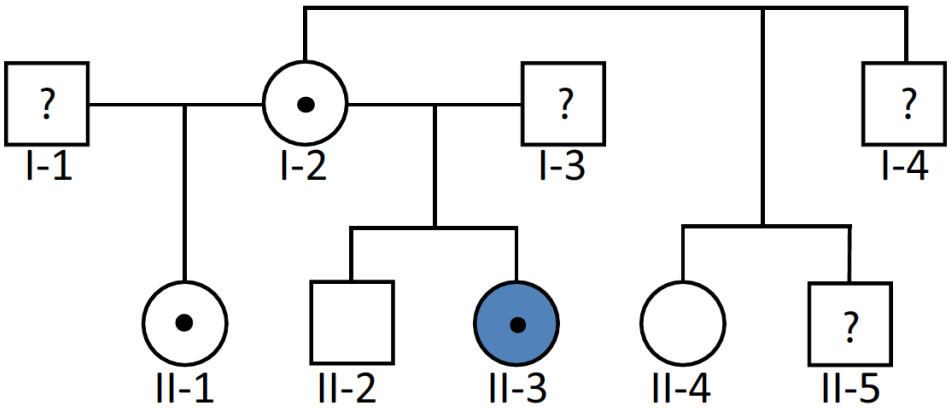


Figure 5.9 **Family pedigree**. II-3 (filled blue circle) denotes the index patient. A central dot within the symbol (circle and square denoting female and male individuals, respectively) indicates the sequence variant is present, empty symbols denote absence of the sequence variant, and symbols with a question mark indicate genetic status unknown.

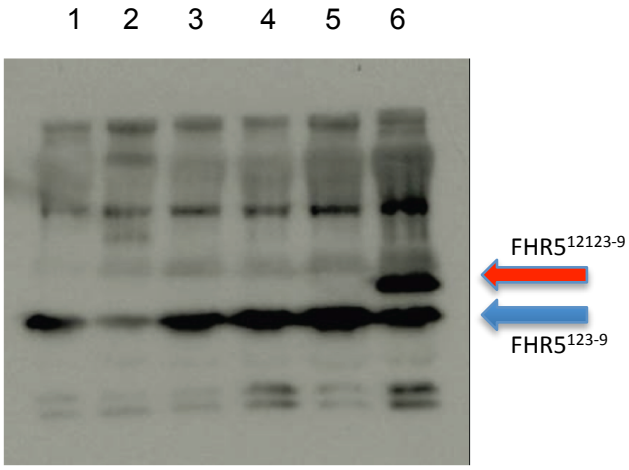
5.3.4. Assessment of FHR5

To further assess FHR5 in this pedigree I first performed serum western blotting for FHR5 to determine if there was any evidence of internal duplication that would result in abnormally large protein. This was important, as Sanger sequencing does not detect exon duplications such as that seen in CFHR5 nephropathy. In this pedigree there was no evidence of an aberrant FHR5 protein on western blotting of sera (figure 5.10).

I next measured serum FHR5 levels using an ELISA (see methods) and this demonstrated that, as was suspected from the FHR5 western blotting, serum FHR5 was reduced in the index case but not in the two other members of the family with the *CFHR5* mutation. These values and the complement parameters performed by the clinical immunology laboratory are summarized in table 5.1.

To determine the normal range for serum FHR5 I performed the ELISA using sera from 13 healthy individuals (figure 5.11). This showed a median level of 5.5µg/ml, range 3.4 - 10.1 (n=13). This range clearly needs to be confirmed in a much larger cohort but is close to the range reported in a previous publication which quoted a range of 4-6µg/ml but did not provide details of the assay used or numbers analysed (McRae, Duthy et al. 2005).

Based on my data it was clear that the mutation alone could not account for the low serum FHR5 level in the index case (since levels were not similarly reduced in unaffected relatives with the mutation). I hypothesised that this could be explained by the fact that serum FHR5 was sequestered by complement present in the kidney of the index case. Consistent with this was the observation that FHR5 could be detected within glomeruli on renal biopsy (figure 5.6). Additionally, I was able to measure FHR5 levels in 23 patients with biopsy-proven C3 glomerulonephritis in whom *CFHR5* mutations had been excluded. These were provided by Dr. Fremeaux-Bacchi (Paris, France). The median level in these patients, 4.3µg/ml (range 1.2 – 7.4, n=23), was significantly lower than that of my control cohort (P=0.02, Mann-Whitney test, figure 5.11).



LANES:

1 – normal human sera
2 – index case (II-3)
3 – II-1
4 – I-2
5 – II-2
6 – Patient with CFHR5 nephropathy

Figure 5.10 **Western blot for FHR5 using sera from pedigree members**. No evidence of an aberrant FHR5 protein was seen. Lane 6 contains a positive serum control for aberrant protein which is the FHR5$^{12123\text{-}9}$ protein associated with CFHR5 nephropathy. Equal amounts of sera were loaded per lane and it was evident that the levels of FHR5 in the index case were low.

Table 5.1 Complement profile in the index case and other pedigree members where available.

Pedigree number	I-2	II-1	II-2	II-4	II-3
CFHR5 mutation	YES*	YES*	NO	NO	YES*
C3 g/l (0.7-1.7)	1.43	1.03	1.08	1.14	0.07
C4 g/l (0.16-0.54)	0.24	0.17	0.21	0.16	0.22
Factor H %NHP**	181	132	132	147	148
Factor I %NHP**	174	132	157	186	154
CH100 (50-150%NHP)	108	47	99	95	absent
AP100 (50-150%NHP)	92	53	102	95	absent
Serum FHR5 μg/ml % of controls	 5.3 96.2	 3.8 69.9	 4.0 72.5	 ND	 2.1 37.3

*c.485dupA (p.E163Rfs*34) – numbering and nomenclature follows human genome variation society recommendations (methionine = 1, A in ATG = 1, www.hgvs.org).

**no validated reference range exists for FH and FI so results are expressed as % of NHP.

FHR5 levels are expressed as both absolute values in μg/ml and as a % of the median value (5.5μg/ml) of 13 healthy controls.

Abbreviations: FHR5 – factor H-related protein 5, NHP – normal human sera pool, CH100 – total complement haemolytic activity, AP100 – alternative pathway haemolytic activity, ND – not done due to insufficient sample

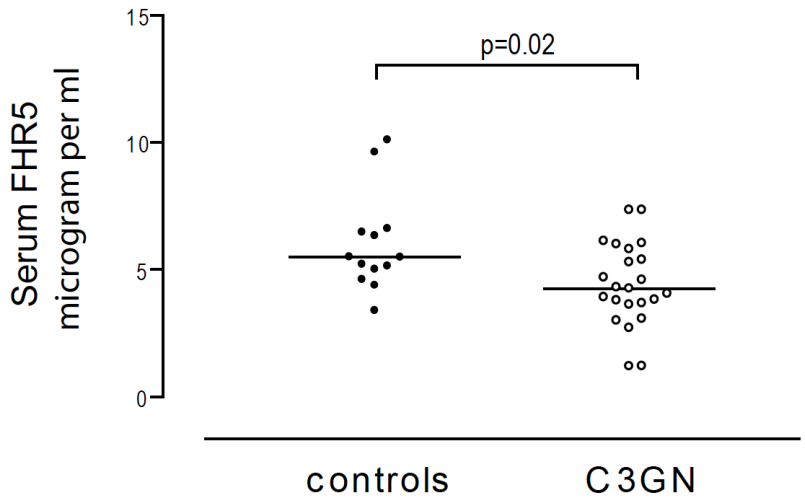


Figure 5.11 **Serum FHR5 levels in individuals with biopsy-proven C3 glomerulonephritis and no *CFHR5* mutation**. The median FHR5 level in the C3GN group (4.3μg/ml, range 1.2-7.4, n=23), was significantly lower than the median in the healthy controls (5.5μg/ml, range 3.4-10.1, n=13, p=0.02, Mann-Whitney test).

5.3.5. Summary

In summary, this was a young girl who developed persistent glomerulonephritis and hypocomplementaemia following a streptococcal throat infection. I demonstrated that she had reduced serum FHR5 levels most likely a consequence of both haploinsufficiency of FHR5 and sequestering of serum FHR5 by complement within the inflamed glomeruli.

5.4. Synthesis of FH and FHR proteins by the kidney

In view of the association between abnormalities in FHR5 and C3 glomerulopathy, I wanted to determine if there was evidence of local production of FHR5 and the other FHR proteins within the kidney. To do this I obtained normal human liver and kidney frozen tissue from the Hospital tissue bank.

I extracted RNA from liver and kidney tissue and then generated cDNA using random primers. This cDNA was then used as a template to determine (i) if I could amplify FH and the FHR proteins and (ii) to compare the relative expression of any of these genes within the kidney to that seen within the liver.

5.4.1. Absolute expression of *CFH* and *CFHR* genes in liver and kidney tissue

Liver and kidney expression was investigated by PCR using liver and kidney cDNA and gene-specific primers. In this analysis I had liver tissue (and therefore cDNA) from 10 samples (L1-10) and kidney tissue from 8 samples (K1-8).

The expected amplicon for FH and all of the FHR proteins was seen in 8 out of 10 of the liver samples (figure 5.12). In the remaining 2 out of 10 liver samples, all had expression of FH, FHR2, FHR4 and FHR5. In one of these samples there was no amplicon for either FHR3 or FHR1 and I speculate that this could be explained by the fact that this sample came from a donor who was homozygous for the *ΔCFHR3-1* deletion. In the other sample there was no amplicon for FHR1.

In the 8 kidney samples only FH and FHR5 were detected in each sample (figure 5.12).

All amplicons were confirmed as gene-specific by sequencing.

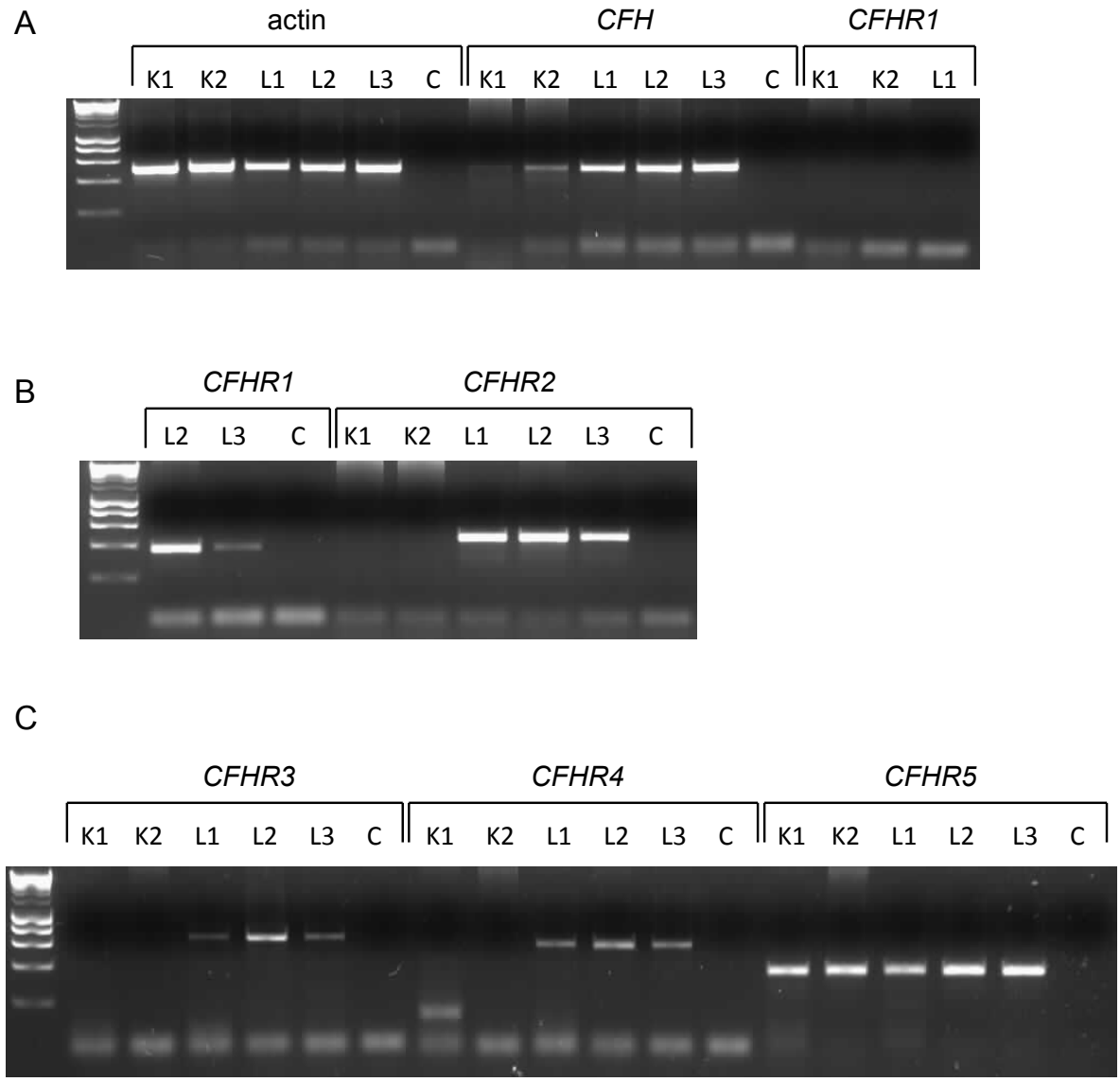


Figure 5.12 **Expression of *CFH* and *CFHR1-5* in human liver and kidney tissue.** Gene-specific primers were used to amplify target sequence from cDNA. L1-3 = liver tissue, K1-2 = kidney tissue, C – water control.

5.4.2. Relative expression of *CFH* and *CFHR5* genes in liver and kidney tissue

I next tried to determine the relative expression of kidney FH and FHR5 both with their respective liver expression by quantitative PCR using cDNA from liver and kidney. In these reactions I amplified two housekeeping genes: beta-actin and GAPDH (glyceraldehyde-3-phosphate dehydrogenase). I then used the comparative Ct (cycle threshold) method to determine relative expression. These calculations showed that (i) liver expression of *CFHR5* was 8-fold less than that of *CFH* and (ii) that kidney expression of *CFHR5* was extremely low with Ct values of greater than 30 (table 5.2).

Table 5.2 Relative expression of *CFH* and *CFHR5* in liver and kidney tissue

			ΔCT		ΔΔCt *CFHR5-CFH*		fold-change* *CFHR5 vs. CFH*	
Tissue	Gene	Ct	*actin*	*GAPDH*	*actin*	*GAPDH*	*actin*	*GAPDH*
Liver	*CFH*	19.6	1.5	1.2				
Liver	*CFHR5*	22.6	4.5	4.2	3	3	8x less	8x less
Liver	*actin*	18.1						
Liver	*GAPDH*	18.4						
Kidney	*CFH*	26.3	11	11.3				
Kidney	*CFHR5*	33.4	18.1	18.4	7.1	7.1	137x less	137x less
Kidney	*actin*	15.3						
Kidney	*GAPDH*	15						

Ct – cycle threshold, *calculated by $2^{-\Delta\Delta Ct}$

5.5. Discussion

In this chapter I have presented evidence that CFHR5 nephropathy recurs in the transplant kidney. This was the first reported case. Disease recurrence post transplantation is common in patients with DDD, accounting for 70% of allograft failure within five years of transplantation (Lu, Moon et al. 2012). It is not surprising perhaps that transplant recurrence of DDD occurs in the presence of circulating C3 nephritic factors. My report illustrates that renal-derived wild-type FHR5 could not prevent the development of C3 glomerulonephritis. Whilst long-term outcome data are not yet available for CFHR5 nephropathy, it may be that its slow natural history does not preclude transplantation. Even with recurrence, patients may maintain meaningful renal function for a significant number of years without progression to end-stage renal failure. In my published report (Vernon, Gale et al. 2011), I discussed how two other incompletely characterized cases with the *CFHR5* mutation and renal disease, had good allograft function one decade after deceased donor renal transplantation. Firstly, a Cypriot male with a renal biopsy demonstrating C3 glomerulonephritis reached end-stage renal failure at the age of 46. A deceased donor renal transplant was performed at the age of 48 and he died 12 years later from a myocardial infarction. The serum creatinine 3 months before his death was 89μmol/l and the graft was never biopsied. Secondly, a 40 year old Cypriot individual with end-stage renal failure and small kidneys, who presented with macroscopic haematuria at the age of ten but had been lost to follow up, received a deceased donor renal transplant at the age of 43. The transplant functioned well for 10 years until it was lost following atheroembolic complications of diagnostic coronary angiography. He returned to haemodialysis and died 6 years later. These cases, together with the observations that the original disease takes many decades to cause end-stage renal failure, suggest that graft loss due to recurrence of CFHR5 nephropathy is not inevitable.

Acute post-streptococcal glomerulonephritis is a common cause of acute nephritis in childhood. Serum complement C3, C5 and properdin are reduced acutely in contrast to C4 levels which are usually normal, demonstrating selective AP activation. C3 nephritic factor has been identified transiently in some individuals (Fremeaux-Bacchi, Weiss et al. 1994) and streptococcal components may also activate the pathway

(Bellon, Kuhn et al. 1979, Hummell, Swift et al. 1985, Rodriguez-Iturbe and Batsford 2007). Complement levels characteristically return to normal within 12 weeks but a minority of individuals develop chronic disease associated with prolonged hypocomplementaemia (Popovic-Rolovic 1973, Payne, Houtman et al. 2008). In this chapter I have described an association between heterozygous FHR5 deficiency and persistent glomerulonephritis following a streptococcal throat infection. Previously published evidence that FHR5 interacts with glomerular complement (Murphy, Georgiou et al. 2002) suggested to me that this was the reason for the fall in serum FHR5 in the index case. Consistent with this hypothesis was our observation that glomerular FHR5 was detectable in kidney biopsy specimens from the index case, median serum FHR5 levels were low in individuals with C3 glomerulonephritis, and serum FHR5 level in the index patient was lower than levels in other family members with the sequence variant, but no kidney disease. An interesting additional insight based on these data is that serum FHR5 levels may be a useful biomarker of glomerular C3 deposition. My report was the first description of the association of kidney disease with a heterozygous sequence variant causing frameshift and premature truncation of FHR5. Interestingly, the same variant was reported in an individual serving as a control in a study investigating the role of FHR5 in atypical haemolytic uremic syndrome (Monteferrante, Brioschi et al. 2007). No clinical details for this individual were reported. The presence of the *CFHR5* sequence variant in the mother and sister of the index patient (neither of whom had overt evidence of kidney disease) indicated that heterozygosity for this *CFHR5* sequence variant was not sufficient to cause kidney disease. My view was that both the heterozygous *CFHR5* sequence variant and streptococcal infection precipitated chronic kidney disease in the index patient. Interestingly, individuals with other genetic defects within the *CFH* gene family are susceptible to exacerbation of kidney disease during throat infections. Synpharyngitic macroscopic haematuria is characteristic of CFHR5 nephropathy (Gale, de Jorge et al. 2010) and common in FH deficiency states (Levy, Halbwachs-Mecarelli et al. 1986, Pichette, Querin et al. 1994). Furthermore, exacerbation of urinary abnormalities in dense deposit disease has been associated with streptococcal infection (Sibley and Kim 1984). Rarely, kidney biopsies following acute post-streptococcal glomerulonephritis have shown changes consistent with dense deposit disease (Clark, White et al. 1988, Sawanobori, Umino et al. 2009,

Suga, Kondo et al. 2010). I considered the question: is dense deposit disease triggered by streptococcal infection or is it a pre-existing lesion that becomes clinically overt during streptococcal infection. I found one report describing apparent progression from biopsy-proven acute post-streptococcal glomerulonephritis to dense deposit disease (Shahidi-Asl, Ananth et al. 2000). However, in others, it seems that dense deposit disease may have predated the episode of acute post-streptococcal glomerulonephritis (Sibley and Kim 1984, Sawanobori, Umino et al. 2009). In my report the index case had no previous evidence of kidney disease, although it is possible that sub-clinical disease was present before the episode of streptococcal infection. Further studies will be required to determine if haploinsufficiency of FHR5 represents a risk factor for the development of chronic glomerulonephritis following streptococcal infection.

The presence of low serum C3 levels in this case cannot be explained by the heterozygous sequence variant in *CFHR5* alone because serum C3 levels were normal in the index patient's mother and sister, who also carried the sequence variant. Notably, in CFHR5 nephropathy, serum C3 levels typically are normal whilst in DDD a low serum C3 level is typical and commonly associated with the presence of C3 nephritic factor. However, rarer causes such as anti-FB antibodies have recently been reported (Strobel, Zimmering et al. 2010). C3 nephritic factor was consistently undetectable in our case and I could not detect any other factor within the serum of the index case that was causing accelerated C3 consumption. I concluded that the serum C3 depletion was a result of excessive C3 activation within the kidney.

Following recent work demonstrating that FHR proteins mediate deregulation of FH *in vitro* (Goicoechea de Jorge, Caesar et al. 2013, Tortajada, Yebenes et al. 2013), I would have predicted that FHR5 deficiency would not be deleterious in C3 glomerulopathy since this would reduce FHR5-mediated deregulation of FH. It may be that reduction of FHR5 increases the ability of FHR1 to deregulate by altering the composition of FHR homo- and heterodimers. Alternatively, the sequestration and relative abundance of FHR5 in the kidney may act to increase complement deregulation locally, leading to increased complement activation and damage within

the glomerulus. Indeed, the alteration of FHR concentrations locally has been proposed as a mechanism by which complement activation may be modulated through the effect on FH deregulation (Fritsche, Lauer et al. 2010, Goicoechea de Jorge, Caesar et al. 2013). By increasing competition for binding to C3b and thereby the deregulation of FH, higher concentrations of FHR proteins could facilitate complement activation. In circumstances where complement activation is not desirable, lower FHR protein levels would enable FH-mediated inactivation of C3b. A previous study of middle ear effusion fluid from children with otitis media found evidence to suggest AP dysregulation and a relative abundance of the FHR proteins compared to serum (Narkio-Makela, Hellwage et al. 2001). Further investigation of larger patient cohorts with post-streptococcal glomerulonephritis and C3 glomerulopathy is now required in order to clarify the role, if any, of FHR5 deficiency in these settings.

Little is known about the synthesis and expression of the FHR proteins within the kidney. I could only amplify FHR5 cDNA from whole human kidney tissue but when I performed quantitative analysis the relative expression was minimal. This, together with the observation that CFHR5 nephropathy recurs in the transplant kidney suggested to me that the biological contribution of renal-derived FHR5 is minimal.

5.6. Conclusions

5.6.1. Renal-derived FHR5 did not prevent recurrence of CFHR5 nephropathy in the transplant kidney

5.6.2. Haploinsufficiency of FHR5 may influence susceptibility to glomerulonephritis following streptococcal throat infection

5.6.3. Serum FHR5 levels may be a useful biomarker of C3 deposition within the kidney

6. CHAPTER SIX: DISCUSSION

Complement plays a pivotal role in a variety of processes including the defence against invading pathogens and the clearance of apoptotic cells whilst also forming a link between innate and adaptive immunity. Effective regulation of the complement system is critical in preventing unwanted damage to host tissues and complement dysregulation is increasingly associated with disease.

Fundamental to control of the AP pathway of complement is FH, a 150-kDa plasma protein synthesised predominantly in the liver. Encoded by the *CFH* gene within the regulators of complement activation gene cluster on chromosome 1, it forms the FH protein family with the splice variant FHL-1 and the five FHR proteins (FHR1-5). Complete deficiency of FH leads to uncontrolled complement activation with secondary depletion of AP complement components such as complement C3, and is associated with the development of renal disease typified by isolated glomerular C3 deposition. Spontaneous and experimental animal models of total FH deficiency are characterised by the development of MPGN, whilst in humans FH deficiency is typically associated with DDD (now recognised as a form of C3 glomerulopathy) and more rarely, aHUS. aHUS is more frequently associated with mutations affecting the carboxyl terminal of FH responsible for surface binding, and complement regulatory activity mediated by the four amino terminal SCRs typically remains intact.

The ability to regulate complement activation within the circulation has proved critical to the renal pathology observed. A membranoproliferative pattern of disease is seen in the presence of complement dysregulation in complete FH deficiency, whilst mice expressing a FH protein lacking only the five C-terminal SCRs (FHΔ16-20) develop thrombotic microangiopathy and aHUS. Notably, animals with lower circulating levels of this mutant FH protein (*Cfh*$^{-/-}$.FHΔ16-20*low*) were protected from aHUS, indicating that effective plasma C3 regulation is required in addition to dysfunctional surface recognition in order for disease to occur. Furthermore, *Cfh*$^{-/-}$.FHΔ16-20*low* mice with higher circulating C3 levels than *Cfh*$^{-/-}$ animals had reduced mesangial expansion, capillary wall C3 deposition and subendothelial electron dense deposits.
Synthesised by a variety of cell types, the majority of FH originates from the liver. The contribution of extra-hepatic FH to the total circulating pool and plasma C3 regulation has not been defined. To determine the ability of extra-hepatic FH to

prevent the GBM C3 deposition and MPGN seen in *Cfh*$^{-/-}$ mice, I generated a mouse line with hepatocyte-specific FH deficiency (hepatocyte-*Cfh*$^{-/-}$).

What does this model tell us about plasma C3 regulation and its relationship to complement deposition within the kidney?

Median plasma FH levels in hepatocyte-*Cfh*$^{-/-}$ mice were 19% of those observed in wild-type animals and were accompanied by a secondary reduction in circulating C3 levels to 20% of wild-type levels. FH and C3 levels were significantly lower in hepatocyte-*Cfh*$^{-/-}$ mice compared to *Cfh*$^{loxP/loxP}$ animals, whilst C3 levels in hepatocyte-*Cfh*$^{-/-}$ mice were higher than in *Cfh*$^{-/-}$ animals indicating that extra-hepatic FH contributes in part to plasma C3 regulation. At 36 weeks of age, there was no evidence of uraemia, albuminuria or thrombotic microangiopathy in hepatocyte-*Cfh*$^{-/-}$ mice. However, whilst there was mild glomerular hypercellularity comparable to *Cfh*$^{-/-}$ animals, the glomerular C3 staining was completely different. In contrast to the linear capillary wall deposition seen in *Cfh*$^{-/-}$ mice, glomerular C3 in hepatocyte-*Cfh*$^{-/-}$ animals was granular and mesangial in distribution. Furthermore, tubulointerstitial C3 reactivity in hepatocyte-*Cfh*$^{-/-}$ mice indicated the presence of intact circulating C3 not present in *Cfh*$^{-/-}$ animals. Reduced staining relative to the tubulointerstitium in wild-type mice, however, demonstrated that extra-hepatic FH could only partially regulate C3 within plasma.

The administration of murine or human FH to *Cfh*$^{-/-}$ mice also results in a change in the distribution of glomerular C3 from the GBM to the mesangium and is associated with the reappearance of tubulointerstitial C3 staining (Paixao-Cavalcante, Hanson et al. 2009, Fakhouri, de Jorge et al. 2010). This clearance of GBM iC3b/C3d in *Cfh*$^{-/-}$ mice has recently been shown to be properdin-dependent (Ruseva, Vernon et al. 2013). Lack of reactivity of mesangial C3 in hepatocyte-*Cfh*$^{-/-}$ mice with an anti-C3d antibody mirrors that in FH-treated *Cfh*$^{-/-}$ mice, and together with the absence of co-localisation with FH/FHR proteins, appears to support the possibility that mesangial C3 in hepatocyte-*Cfh*$^{-/-}$ mice may also contain C3c. The role of properdin in the C3 deposition seen in hepatocyte-*Cfh*$^{-/-}$ animals remains to be defined.

Lower FH and C3 levels in *Cfh*$^{loxP/loxP}$ mice compared to Alb-Cre animals indicated impaired expression of the targeted *Cfh* locus, likely secondary to the insertion of either the *loxP* sites or the neomycin resistance gene. This precluded a definitive

conclusion regarding the contribution of extra-hepatic FH to the total circulating pool. To circumvent this technical issue, FH and C3 levels need to be measured following removal of the *Neo* cassette using a transgenic mouse expressing the Flp recombinase.

The spontaneous phenotype in hepatocyte-*Cfh*$^{-/-}$ mice can be considered a new model of C3 glomerulopathy characterised by mesangial C3 deposition in the absence of significant immunoglobulin. Furthermore, it illustrates that relatively low levels of FH are sufficient to prevent the renal disease associated with complete FH deficiency.

The presence of C3 deposition along the GBM appears deleterious even in the absence of C5 activation and is associated with exaggerated renal injury in experimentally-triggered nephritis in *Cfh*$^{-/-}$ mice (Pickering, Cook et al. 2002). Additional environmental triggers such as infection are known to exacerbate renal injury in C3 glomerulopathy. To investigate this, I induced NTN in hepatocyte-*Cfh*$^{-/-}$ mice. In the presence of spontaneous mesangial C3, NTN led not only to accelerated and more severe disease, but also to a different phenotype not seen in wild-type animals. Microangiopathic haemolytic anaemia, thrombocytopaenia and renal failure were accompanied by marked glomerular thrombosis on light microscopy in hepatocyte-*Cfh*$^{-/-}$ mice. Immunofluorescence microscopy revealed further C3 deposition in the distribution of thrombosed capillary loops. Although the higher circulating levels of FH resulting from extra-hepatic production in hepatocyte-*Cfh*$^{-/-}$ mice are sufficient to prevent the GBM C3 deposition and MGPN observed in *Cfh*$^{-/-}$ animals, they are not able to adequately control C3 activation in the presence of additional complement activation. With greater circulating C3 and C5 than *Cfh*$^{-/-}$ animals, failure to control surface complement activation on the renal endothelium in the context of NTN culminates in thrombotic microangiopathy.

The induction of NTN in *Cfh*$^{loxP/loxP}$ mice was also associated with more severe disease than wild-type animals consistent with reduced FH levels and underlying complement dysregulation, but this was significantly less than that observed in hepatocyte-*Cfh*$^{-/-}$ mice.

The same genetic mutation in hepatocyte-*Cfh*$^{-/-}$ mice can therefore be associated with two distinct phenotypes depending on the accompanying environment. The spontaneous phenotype in hepatocyte-*Cfh*$^{-/-}$ animals represents a form of C3 glomerulopathy, whilst in the presence of an additional insult such as NTN, is consistent with atypical HUS. The induction of NTN in hepatocyte-*Cfh*$^{-/-}$ mice provides a novel model of atypical HUS which can be used to further delineate the pathophysiological mechanisms involved and enable new therapies to be examined. This model of atypical HUS is not only reproducible and predictable with a rapid onset of disease, it also has the benefit of being able to clearly identify the onset of disease as animals do not have thrombotic microangiopathy prior to the induction of NTN. The other murine model of atypical HUS whereby mice lack the 5 C-terminal SCRs of FH (*Cfh*$^{-/-}$.FHΔ16-20) is associated with the development of spontaneous disease at variable time points. The development of FH-associated or atypical HUS in hepatocyte-*Cfh*$^{-/-}$ mice exposed to experimental nephritis mirrors the pathogenesis of atypical HUS in humans. The requirement for an additional insult in addition to underlying complement dysregulation is now widely acknowledged.

Exacerbation of underlying renal disease in the presence of infection is also well recognised. In addition to IgA nephropathy, synpharyngitic macroscopic haematuria is also a feature of CFHR5 nephropathy and renal disease associated with FH deficiency (Levy, Halbwachs-Mecarelli et al. 1986, Pichette, Querin et al. 1994). The role of infection in the aetiology of DDD remains unclear with some case reports suggesting possible progression from acute post-streptococcal glomerulonephritis to DDD (Shahidi-Asl, Ananth et al. 2000). Here, I have reported a case of persistent hypocomplementaemia and C3 glomerulopathy following streptococcal infection in a patient with heterozygous FHR5 deficiency. Notably, serum FHR5 and C3 levels in the index case were significantly lower than in the mother and sister with the same mutation who did not have renal disease. Marked glomerular C3 deposition accompanied by FHR5 reactivity suggested consumption of C3 within the kidney and sequestration of FHR5 from the circulation. This appears consistent with the finding that serum FHR5 levels in patients with biopsy-proven C3GN are significantly lower than in healthy controls. Serum FHR5 levels may therefore act as a marker of concurrent complement activation within the kidney. Whilst the role of FHR5 in the

development of chronic glomerulonephritis following streptococcal infection remains to be determined, abnormalities in the FHRs are increasingly being associated with the development of other complement-mediated renal diseases including atypical HUS and C3 glomerulopathy.

Similarly encoded within the RCA gene cluster on chromosome 1, the high degree of sequence identity between FHR1-5 and FH has long suggested a role for the FHR proteins in complement regulation. Interestingly, FHR1, 2 and 5 have recently been shown to form homo- and heterodimers through a shared dimerization motif in the two amino terminal SCR domains. By competing with FH for binding to C3b, FHR1, 2 and 5 act to deregulate complement inhibition by FH. Duplication of the dimerization domains in mutant proteins leads to even greater complement deregulation (Goicoechea de Jorge, Caesar et al. 2013, Tortajada, xEd et al. 2013) and a reduction in this competition afforded by the ∆*CFHR3-1* has been proposed as a mechanism by which the deletion is protective in AMD (Fritsche, Lauer et al. 2010). The FHR proteins may therefore play a complement modulatory role locally through the degree of competitive antagonism of FH function.

However, as is the case for atypical HUS associated with FH mutations, renal-derived wild-type FHR5 was unable to prevent the recurrence of CFHR5 nephropathy in an unrelated renal transplant (Vernon, Gale et al. 2011). I have described the first known case of disease recurrence in a graft, identified on a renal transplant biopsy only 46 days after transplantation. Similar to FH, hepatic expression of FHR5 is markedly greater than in the kidney. The relatively higher concentration of circulating mutant FHR5 from the liver may therefore predominate in any deposits within the kidney leading to increased complement deregulation and activation of complement C3 locally. Sequestration thereafter of further abnormal FHR5 from the circulation by glomerular C3 may then exacerbate complement deregulation with uncontrolled C3 activation resulting in C3 glomerulopathy. Glomerular C5b-9 reactivity in the transplant biopsy also provided evidence of TP activation.

Graft failure has been reported in up to 60-90% of atypical HUS patients within a year of transplantation and combined liver-kidney transplantation has been used successfully in patients with complement abnormalities affecting circulating components derived from the liver such as FH (Wong, Goodship et al. 2013). Combined liver-kidney transplantation, however, is not without significant risk. Eculizumab has also been used to prevent and treat recurrent aHUS following renal transplantation (Wong, Goodship et al. 2013). A humanized monoclonal antibody directed against complement C5 (Eculizumab) blocks the generation of C5a and C5b-9. Originally approved for the treatment of paroxysmal nocturnal haemoglobinuria, it has recently been evaluated in two clinical trials in patients with atypical HUS with either progressive thrombotic microangiopathy despite plasma therapy, or those with chronic disease requiring ongoing plasma therapy (Legendre, Licht et al. 2013). Treatment with Eculizumab resulted in inhibition of thrombotic microangiopathy and a time-dependent improvement in renal function. The relative contribution of C5a and the MAC in mediating renal injury in atypical HUS is still to be ascertained, and the new model of atypical HUS described here will enable these to be interrogated in more detail. Furthermore, it will also provide a means by which the efficacy of other therapies for atypical HUS can be investigated. It is notable that Eculizumab is currently the only drug licensed for the treatment of atypical HUS, and a recommendation regarding commissioning in the UK is still awaited from the National Institute for Care and Health Excellence.

The success of Eculizumab in atypical HUS has led to consideration of its use in the treatment of other complement-mediated renal diseases. However, whilst the success of C5 blockade in atypical HUS is clear, its efficacy in complement-mediated renal diseases dependent on C3, but not C5 activation, remains to be determined. The GBM C3 deposition and MPGN characteristic of *Cfh*$^{-/-}$ mice and dependent on C3 activation, still occurs in the absence of C5 in *Cfh*$^{-/-}$.*C5*$^{-/-}$ animals. Whilst inhibition of C5 activation in *Cfh*$^{-/-}$ mice did ameliorate spontaneous and experimentally-induced disease (Pickering, Warren et al. 2006), the continuing accumulation of glomerular C3 appears to mediate proteinuria. Inhibition at the level of complement C3 may therefore be preferable in C3 glomerulopathy where glomerular pathology is typified by C3 deposition.

The use of Eculizumab in patients with C3 glomerulopathy has been associated with varying success (Bomback, Smith et al. 2012, Daina, Noris et al. 2012, Herlitz, Bomback et al. 2012, Vivarelli, Pasini et al. 2012, Gurkan, Fyfe et al. 2013). Individuals with either DDD or C3GN (with or without C3 nephritic factor) have been reported to show improvements in serum albumin, renal function and proteinuria following Eculizumab (Bomback, Smith et al. 2012, Daina, Noris et al. 2012, Vivarelli, Pasini et al. 2012). Typically, these have occurred in patients with high levels of soluble C5b-9 (sC5b-9) prior to the initiation of therapy, which subsequently normalised during treatment. Those individuals with normal levels of sC5b-9 prior to Eculizumab showed no improvement or even deteriorated (Bomback, Smith et al. 2012). Of note, whilst a reduction in histological disease activity has been reported with Eculizumab, progression of glomerulosclerosis has also been observed (Bomback, Smith et al. 2012, Herlitz, Bomback et al. 2012, Vivarelli, Pasini et al. 2012). More recently, normalisation of sC5b-9 levels and improving proteinuria in a patient treated with Eculizumab for transplant recurrence of C3 glomerulopathy was followed by a rise in proteinuria and progression of disease on biopsy (Gurkan, Fyfe et al. 2013). C3 glomerulopathy represents a spectrum of pathology and Eculizumab may benefit a subgroup of patients, although these remain to be defined. Elevated sC5b-9 levels may be useful in predicting response. The longer-term implications of Eculizumab binding to C5 within the kidney, mirroring monoclonal immunoglobulin deposition disease, are unknown (Herlitz, Bomback et al. 2012). Inhibition of the TP also increases the risk of infection with encapsulated organisms and meningococcal vaccination is recommended prior to the initiation of Eculizumab.

Recognition of isolated glomerular C3 deposition as a pathological entity has led to interest in the therapeutic inhibition of complement at the level of C3. Successful inhibition of complement C3 has been reported in a number of models by inhibitors including recombinant proteins and Compstatin, a cyclic peptide identified from phage-display libraries. Binding C3b and C4b, complement receptor 1 (CR1/CD35) blocks both the AP and CP, exhibiting decay accelerating and FI cofactor activities. Soluble recombinant human complement receptor 1 (sCR1) reduces inflammation in a variety of models including three models of acute glomerulonephritis in the rat (Couser, Johnson et al. 1995). To avoid immunogenicity, a soluble recombinant form

of Crry fused with non-complement activating mouse IgG1 (Crry-Ig) has been used in rodent models and this agent significantly reduced renal injury in both antibody-mediated glomerulonephritis and in MRL/*lpr* lupus mice (Quigg, Kozono et al. 1998, Bao, Haas et al. 2003).
In addition to systemic inhibition by Crry-Ig, targeted inhibition to sites of complement activation has also been achieved. An iC3b/C3dg-binding fragment of mouse complement receptor 2 (CR2) linked to Crry (CR2-Crry) targeted local and remote sites of complement activation following intestinal ischaemia reperfusion injury (IRI), and required a 10-fold lower dose than Crry-Ig to provide the same protection (Atkinson, Song et al. 2005). Furthermore, susceptibility to infection after CR2-Crry was no different from PBS control in contrast to significantly enhanced susceptibility with Crry-Ig.

More recently, targeted inhibition of the AP has been investigated as a means of avoiding blockade of all pathways either at the level of C3 or C5. With its roles in host defence and immune homeostasis, systemic inhibition of complement is not without potential adverse effects. A fragment of CR2 linked to N-terminal domains of FH (CR2-FH) was protective against intestinal IRI, almost totally abolishing intestinal C3 deposition (Huang, Qiao et al. 2008) and with a therapeutic dose much lower than untargeted Crry-Ig.

Compstatin is a cyclic tridecapeptide and selective C3 inhibitor which through direct binding to native C3, inhibits the convertase-mediated cleavage of C3 to C3a and C3b (Ricklin and Lambris 2008). The single binding site of Compstatin resides within the C3c portion of C3 away from other C3 binding sites and does not appear to interfere with convertase formation or complement regulatory proteins. It was the first complement-specific agent to enter clinical trials for the treatment of AMD (Ricklin and Lambris 2008). The AP is known to be the major contributor to biomaterial-induced complement activation seen during extracorporeal blood circulation such as in haemodialysis (Andersson, Ekdahl et al. 2005) and C3a generation, C3b deposition and MAC formation were all reduced when Compstatin was added to circulating blood in PVC tubes (Nilsson, Larsson et al. 1998). Studies of C3 inhibition in C3 glomerulopathy are awaited.

In summary, how has the data presented here contributed to the literature on complement dysregulation and renal disease? Hepatocyte-*Cfh*$^{-/-}$ mice have demonstrated that relatively low levels of circulating FH are able to prevent the GBM C3 deposition and MPGN typical of complete FH deficiency. The subtotal deficiency of FH in hepatocyte-*Cfh*$^{-/-}$ animals is instead associated with a spontaneous phenotype consistent with C3 glomerulopathy. The induction of NTN in these mice leads to atypical HUS consistent with the paradigm in humans where a further trigger is required in addition to underlying complement dysregulation in order for disease to occur. Hepatocyte-*Cfh*$^{-/-}$ mice therefore illustrate that the same genetic mutation can be associated with a different phenotype depending on the accompanying environment and confirm the ability to regulate complement activation within the circulation is critical to the renal pathology observed.

Hepatocyte-*Cfh*$^{-/-}$ mice notably provide a novel model of both C3 glomerulopathy and atypical HUS. These can be used not only to further define pathophysiological mechanisms, but also facilitate the identification of therapeutic targets and enable such therapies to be tested. Notably, the mesangial C3 deposition characteristic of the spontaneous phenotype in hepatocyte-*Cfh*$^{-/-}$ mice provides an alternative model of C3 glomerulopathy compared to the GBM C3 and MPGN typical of *Cfh*$^{-/-}$ mice, and may therefore enable the efficacy of treatments to be assessed across a wider spectrum of pathology comprising C3 glomerulopathy. Whilst the induction of NTN in hepatocyte-*Cfh*$^{-/-}$ mice is associated with a severe phenotype and may not entirely match disease triggers in human disease, it also adds to the only other animal model of atypical HUS in existence, and has the advantages of predictability and a short interval to disease onset, which is also clearly definable.

During characterisation of the hepatocyte-*Cfh*$^{-/-}$ phenotype, it became evident that insertion of *loxP* sites and/or the neomycin cassette in *Cfh*$^{loxP/loxP}$ animals affected expression at the *Cfh* locus. Removal of the *Neo* cassette using Frt-mediated recombination is currently underway in my supervisor's laboratory. Confirmation of an effect on gene expression will enable a more accurate conclusion to be drawn regarding the contribution of extra-hepatic FH to the total circulating pool through the investigation of hepatocyte-*Cfh*$^{-/-}$ mice in which the *Neo* cassette has been removed.

What has the work presented here added to the literature on the FHRs in renal disease? The recurrence of CFHR5 nephropathy in an unrelated renal transplant illustrates that locally derived normal FHR5 is unable to prevent disease. This may not be surprising in light of the expression studies demonstrating minimal renal expression of FHR5.

The role of FHR5 in chronic GN remains to be determined, but my study of FHR5 haploinsufficiency has shown that this is not sufficient in itself to cause C3 glomerulopathy. Glomerular sequestration of FHR5 may lead to reduced serum levels and represent a marker of concomitant C3 deposition within the kidney. Further work is required to characterise the exact role of the FHRs in renal disease, but this may involve the local modulation of FH deregulation mediated by varying FHR dimer composition and/or concentrations.

7. CHAPTER SEVEN: REFERENCES

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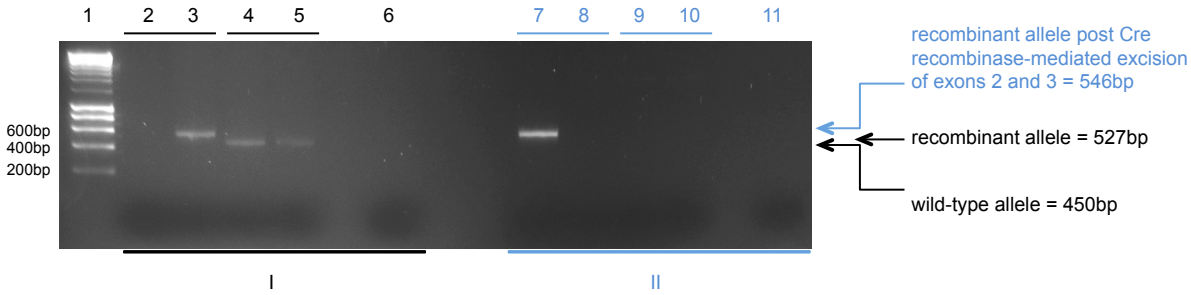
8. CHAPTER EIGHT: APPENDICES

8.1. Appendix 1: Demonstration of hepatocyte-specific Cre-mediated recombination (see Chapter 3, page 91)



Lane 1 – Size marker
Lane 2 and 7 – hepatocyte-*Cfh*$^{-/-}$ hepatic DNA
Lane 3 and 8 – hepatocyte-*Cfh*$^{-/-}$ tail DNA
Lane 4 and 9 – Alb-Cre hepatic DNA
Lane 5 and 10 – Alb-Cre tail DNA
Lane 6 and 11 – H_2O

PCR was performed to confirm hepatocyte-specific Cre-mediated recombination. The 527-bp floxed *Cfh* locus amplified in tail genomic DNA from hepatocyte-*Cfh*$^{-/-}$ mice (lane 3, panel I) was not present in hepatic genomic DNA from the same animals (lane 2, panel I) indicating hepatocyte-specific recombination. This was confirmed by the presence of a 546-bp amplicon only seen following excision of exons 2 and 3, in hepatic genomic DNA from hepatocyte-*Cfh*$^{-/-}$ mice (lane 7, panel II). The 450-bp wild-type amplicon was seen at the *Cfh* locus in both hepatic and tail genomic DNA from Alb-Cre mice (lanes 4 and 5, panel I).

8.2. Appendix 2: Summary of NTN experiments 1-3 (see Chapter 4, page 124)

NTN experiment	Wild-type		Hepatocyte-*Cfh*$^{-/-}$		*Cfh*$^{loxP/loxP}$		Alb-Cre	
	n	Median (range)	n	Median (range)	n	Median (range)	n	Median (range)
Experimental numbers								
NTN1	10		7					
NTN2			6		5		5	
NTN3	7		3					
Haematuria at cull (day 4)								
NTN1	8	0 (0-0)	2	4 (4-4)				
NTN2			4	4 (4-4)[a]	5	0 (0-2)	5	0 (0-2)
NTN3	7	0 (0-3)	3	4 (4-4)				
Proteinuria at cull (day 4)								
NTN1	8	1.5 (0-2)	2	4 (4-4)				
NTN2			4	4 (4-4)	5	3 (2-4)	5	3 (2-4)
NTN3	7	1 (1-3)	3	3 (3-3)				
Urea at cull (day 4) - mmol/l								
NTN1	10	12.0 (7.0-25.2)	7	138.0[b] (77.0-160.8)				
NTN2			5	156.6[c] (12.4-163.3)	5	30.4 (25.5-34.6)	5	20.5 (13.0-50.1)
NTN3	7	17.2 (9.5-50.3)	3	155.6[d] (138.2-163.5)				
Red cell fragments (day 4) - number of red cell fragments per 200 red blood cells counted								
NTN1	10	2 (2-3)	7	37 (16-64)[e]				
NTN2			5	13 (7-18)[f]	5	7 (1-19)	5	2 (0-4)
NTN3	7	2 (0-3)	3	19 (8-25)[g]				
Glomerular thrombosis (day 4) - amount of PAS-positive material per glomerular cross-section								
NTN1	10	0.40 (0.15-0.55)	7	3.50[h] (1.9-3.65)				
NTN2			5	2.10 (1.25-2.60)	5	1.2 (0.50-2.40)	5	1.0 (0.80-2.60)
NTN3	7	1.2 (0.80-2.80)	3	2.80[i] (2.30-3.30)				

n = number of mice examined
There was one death in the NTN2 experiment in the hepatocyte-*Cfh*$^{-/-}$ experimental group
[a] *P*<0.001 versus *Cfh*$^{loxP/loxP}$ and Alb-Cre male mice, Bonferroni multiple comparison test
[b] *P*=0.0001 versus wild-type female mice, Mann-Whitney test
[c] *P*<0.01 versus *Cfh*$^{loxP/loxP}$ and Alb-Cre male mice, Bonferroni multiple comparison test
[d] *P*=0.0167 versus wild-type male mice, Mann-Whitney test
[e] *P*=0.0001 versus wild-type female mice, Mann-Whitney test
[f] *P*<0.01 versus Alb-Cre male mice, Bonferroni multiple comparison test
[g] *P*=0.0167 versus wild-type male mice, Mann-Whitney test
[h] *P*=0.0001 versus wild-type female mice, Mann-Whitney test
[i] *P*=0.0333 versus wild-type male mice, Mann-Whitney test.

Summary of NTN experiments 1-3 (continued)

NTN experiment	Wild-type		Hepatocyte-*Cfh*$^{-/-}$		*Cfh*$^{loxP/loxP}$		Alb-Cre	
	n	Median (range)	n	Median (range)	n	Median (range)	n	Median (range)
Glomerular C3 immunofluorescence (day 4) – arbitrary fluorescence units								
NTN1	9	421.6 (199.5-623.1)	7	1325.0[j] (782.1-1644.0)				
NTN2			5	527.0[k] (370.0-780.9)	5	249.0 (179.0-314.5)	5	232.5 (113.7-419.9)
NTN3	7	292.3 (167.3-1110.0)	3	1122.0[l] (979.8-1326.0)				
Glomerular mouse IgG immunofluorescence (day 4) – arbitrary fluorescence units								
NTN1	9	18.5 (8.2-40.5)	7	39.4[m] (18.0-73.3)				
NTN2			5	320.0 (195.9-339.1)	5	280.6 (154.2-519.6)	5	347.0 (209.2-812.9)
NTN3	7	697.7 (394.3-1293.0)	3	736.1 (715.0-779.3)				
Glomerular sheep IgG immunofluorescence (day 4) – arbitrary fluorescence units								
NTN1	10	1722.0 (1318.0-3019.0)	7	1071.0[n] (920.1-1719.0)				
NTN2			5	1345.0 (945.6-1726.0)	5	1485.0 (1339.0-1734.0)	5	1649.0 (1502.0-1815.0)
NTN3	7	1625.0 (1405.0-2379.0)	3	1337.0 (842.8-1460.0)				

n = number of mice examined
[j] P=0.0002 versus wild-type female mice, Mann-Whitney test
[k] P<0.05 versus *Cfh*$^{loxP/loxP}$ and Alb-Cre male mice, Bonferroni multiple comparison test
[l] P=0.0333 versus wild-type male mice, Mann-Whitney test
[m] P=0.0164 versus wild-type female mice, Mann-Whitney test
[n] P=0.0031 versus wild-type female mice, Mann-Whitney test

9. CHAPTER NINE: PUBLICATIONS

9.1. Publications arising from this thesis

Vernon, K. A., D. P. Gale, E. G. de Jorge, A. G. McLean, J. Galliford, A. Pierides, P. H. Maxwell, D. Taube, M. C. Pickering and H. T. Cook (2011). "Recurrence of complement factor H-related protein 5 nephropathy in a renal transplant." Am J Transplant **11**(1): 152-155.

Vernon, K. A., E. Goicoechea de Jorge, A. E. Hall, V. Fremeaux-Bacchi, T. J. Aitman, H. T. Cook, R. Hangartner, A. Koziell and M. C. Pickering (2012). "Acute presentation and persistent glomerulonephritis following streptococcal infection in a patient with heterozygous complement factor H-related protein 5 deficiency." Am J Kidney Dis **60**(1): 121-125.

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Case Report

Recurrence of Complement Factor H-Related Protein 5 Nephropathy in a Renal Transplant

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Complement factor H-related protein 5 (CFHR5) nephropathy is a familial renal disease endemic in Cyprus. It is characterized by persistent microscopic hematuria, synpharyngitic macroscopic hematuria and progressive renal impairment. Isolated glomerular accumulation of complement component 3 (C3) is typical with variable degrees of glomerular inflammation. Affected individuals have a heterozygous internal duplication in the *CFHR5* gene, although the mechanism through which this mutation results in renal disease is not understood. Notably, the risk of progressive renal failure in this condition is higher in males than females. We report the first documented case of recurrence of CFHR5 nephropathy in a renal transplant in a 53-year-old Cypriot male. Strikingly, histological changes of CFHR5 nephropathy were evident in the donor kidney 46 days post-transplantation. This unique case demonstrates that renal-derived CFHR5 protein cannot prevent the development of CFHR5 nephropathy.

Key words: CFHR5 nephropathy, complement, recurrence, renal transplant

Abbreviations: C3, complement component 3; C3c, complement component 3c; C4d, complement component 4d; C5b, complement component 5b; C9, complement component 9; CD46, cluster of differentiation 46 (membrane cofactor protein); CFH, complement factor H; CFHL-1, CFH-like protein; CFHR5, complement factor H-related protein 5; C3GN, C3 glomerulonephritis; EM, electron microscopy; ESRF, end-stage renal failure; μmol, micromole (s); mg, milligram (s); mmol, millimole (s); PCR, polymerase chain reaction; RCA, regulator of complement activation; SCR, short consensus repeat.

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Introduction

Complement dysregulation is associated with several distinct patterns of glomerular pathology. Common to glomerular abnormalities associated with defective control of the alternative pathway is deposition of C3 in the absence of significant immunoglobulin (1,2). This pathological appearance typifies a number of conditions associated with genetic or acquired complement dysregulation, including dense deposit disease, C3 glomerulonephritis (C3GN) and CFHR5 nephropathy. 'C3 glomerulopathy' has recently been proposed as a new term under which this heterogeneous group of disorders can be classified (1).

C3GN is a feature of CFHR5 nephropathy, a familial renal disease characterized by persistent microscopic hematuria, synpharyngitic macroscopic hematuria and progressive renal failure (3). C3GN may be associated with membranoproliferative or mesangial proliferative features. Endemic in Cyprus, affected individuals have a heterozygous internal duplication in the *CFHR5* gene. Previously, mutations in complement factor H, CD46 (membrane cofactor protein) and factor I were identified among patients with biopsy-proven C3GN (2), but CFHR5 nephropathy is the first description of C3GN associated with a mutation in the *CFHR5* gene. CFHR5 is a member of the complement factor H (CFH) family, a group of highly related proteins encoded by genes located within the regulator of complement activation (RCA) gene cluster on chromosome 1. Comprising CFH, CFH-like protein (CFHL-1) and complement factor H-related proteins 1–5, the proteins are composed of individual domains termed short consensus repeats (SCRs), which display varying degrees of amino acid sequence similarity to each other. CFHR5 is a 65 kDa protein composed of nine SCRs, and the internal duplication in exons 2 and 3 characteristic of CFHR5 nephropathy results in an expressed protein with duplicated SCRs 1 and 2 respectively. Although the role of CFHR5 is not yet fully understood, its complement regulatory activity *in vitro* (4) and co-localization with renal complement deposits *in vivo* (5) suggest that it may play a role in complement regulation within the kidney. Furthermore, the mutant protein has been shown to have reduced affinity for glomerular-bound complement, raising the possibility of impaired targeting to

complement within the kidney (3). We report the case of a 53-year-old gentleman with end-stage renal failure (ESRF) secondary to CFHR5 nephropathy, who underwent renal transplantation from a deceased donor and was found to have evidence of disease recurrence in a transplant biopsy 46 days later.

Case Presentation

A previously healthy British male with Cypriot ancestry was referred at the age of 36 with persistent microscopic hematuria, episodes of macroscopic hematuria coinciding with upper respiratory tract symptoms, and renal impairment (serum creatinine 178 μmol/L). He was not aware of any family history of renal disease at that time although relatives with CFHR5 nephropathy have subsequently been identified. Physical examination and blood pressure were normal. However urinalysis demonstrated 1+ blood and 1+ protein, and a renal biopsy was performed. Light microscopy revealed 30% glomerular obsolescence with a fibrous crescent in one glomerulus, and a few tubular red cell casts. Immunoperoxidase staining showed capillary wall C3 but notably was negative for IgA. Subendothelial electron dense deposits and rare subepithelial 'humps' were seen on electron microscopy (EM), as were mesangial deposits associated with an increase in mesangial cells and matrix. A further decline in renal function 6 years later led to a second biopsy (Figure 1). This demonstrated large segmental scars, capillary wall thickening with double contours and mesangial cell interposition. Granular capillary wall C3 was again evident in the absence of immunoglobulin staining. On EM there were subendothelial and mesangial deposits, and rare subepithelial deposits. These histological features are consistent with C3 glomerulonephritis.

Over the following 4 years he suffered progressive renal impairment and required renal replacement therapy at the age of 47. Six years after commencing hemodialysis, during which he had further episodes of macroscopic hematuria, he received a deceased donor renal transplant. The donor was a 65-year-old female with no significant past medical history, who had a creatinine of 82 μmol/L. HLA matching demonstrated a 2,1,1 mismatch, but there were no recipient class I or II HLA antibodies at the time of transplantation, and this proceeded with no complications. Immunosuppression included alemtuzumab and corticosteroids peri-operatively, with tacrolimus monotherapy continued at discharge. Although his renal function initially improved, his creatinine stabilized at 186 μmol/L, and in the presence of persistent microscopic hematuria and following a single episode of macroscopic hematuria (with a urine protein: creatinine ratio of 56 mg/mmol), he underwent a renal transplant biopsy, 46 days after transplantation (Figure 2).

Light microscopy showed occasional neutrophils in the capillary loops of one glomerulus and a small area of tubulointerstitial fibrosis. Immunoperoxidase staining showed capillary wall granular C3 and complement component 9 (C9), whilst electron microscopy showed increased mesangial matrix associated with scattered mesangial and subendothelial deposits (with new basement membrane beneath in two areas; Figure 2C). Complement component 4d (C4d) staining was negative. The findings in the renal transplant were therefore consistent with a recurrence of his original disease. Serum C3 was 0.73 g/L (normal range 0.7–1.7 g/L) at the time of biopsy, whilst CFH and factor I levels were 320 mg/L (207% of normal control) and 32 mg/L (177% of normal control), respectively.

A second transplant biopsy performed 3 months later on account of a rise in the serum creatinine to 250 μmol/L, showed granular (mainly mesangial) C3 staining associated with subendothelial, mesangial and subepithelial deposits. Indirect immunofluorescence also showed the presence of glomerular complement components 5b (C5b)-9 (Figure 2D). Polymerase chain reaction (PCR) using genomic DNA isolated from peripheral blood monocytes

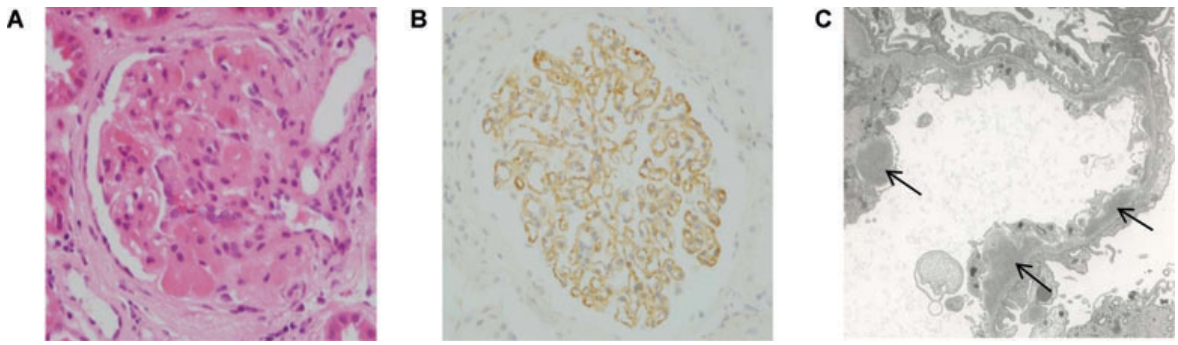

Figure 1: Native renal biopsy. (A) Light microscopic appearance showing segmental glomerulosclerosis and hyalinosis and increased mesangial matrix (hematoxylin and eosin). (B) Immunoperoxidase staining showing granular capillary wall C3 deposition. C3 was detected using a polyclonal rabbit anti-human C3c antibody (Dako Ltd., Ely, United Kingdom). (C) Electron micrograph showing multiple subendothelial electron dense deposits (denoted by arrows).

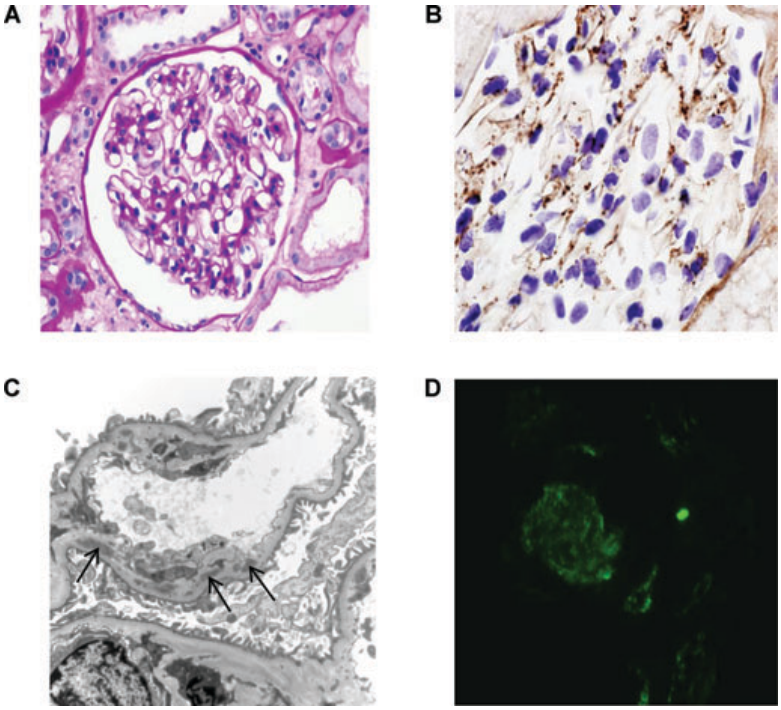

Figure 2: Renal transplant biopsy. (A) Light microscopy showing a normal glomerulus (periodic acid-Schiff). (B) Immunoperoxidase staining showing granular capillary wall and mesangial C3 deposition. C3 was detected using a polyclonal rabbit anti-human C3c antibody (Dako Ltd., Ely, United Kingdom). (C) Electron micrograph showing subendothelial deposits (denoted by arrows) with new basement membrane beneath. (D) Glomerular C5b-9 by indirect immunofluorescence. C5b-9 was detected using a monoclonal mouse anti-human C5b-9 antibody (DakoCytomation Ltd., Ely, United Kingdom) and a fluorescein isothiocyanate-labeled goat anti-mouse IgG (Sigma-Aldrich Company Ltd., Dorset, United Kingdom).

and serum CFHR5 western blot analysis (Figure 3) revealed the presence of the heterozygous internal duplication in the *CFHR5* gene, confirming the diagnosis of CFHR5 nephropathy.

Discussion

To our knowledge this is the first description of recurrence of CFHR5 nephropathy in a transplant. The recurrence of CFHR5 nephropathy in an unrelated kidney demonstrates that local synthesis of normal CFHR5 by the kidney is not sufficient to prevent disease. However, we are aware of two other incompletely characterized cases with the CFHR5 mutation and renal disease, in which good allograft function was evident one decade after deceased donor renal transplantation. Firstly, a Cypriot male with a renal biopsy demonstrating C3GN reached ESRF at the age of 46. A deceased donor renal transplant was performed at the age of 48 and he died 12 years later from a myocardial

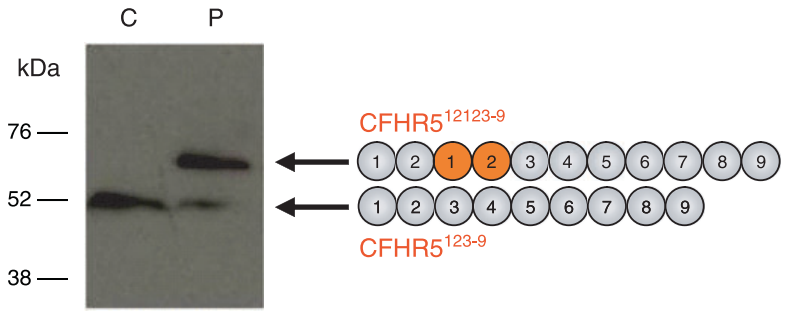

Figure 3: Internal duplication of CFHR5. Western blot of serum for CFHR5 and schematic depicting normal and mutant CFHR5 proteins (denoted CFHR5$^{123-9}$ and CFHR5$^{12123-9}$ respectively). A polyclonal anti-CFHR5 antibody was used to detect the wild-type and mutant CFHR5 proteins in sera from the patient (P) and control (C). The wild-type CFHR5 protein consists of nine protein subunits termed short consensus repeat (SCR) domains. The internal duplication of exons 2 and 3 in the mutant *CFHR5* gene results in a mutant protein that contains two additional SCR domains (duplicated protein domains depicted in orange in the schematic). The mutant CFHR5 protein therefore has a higher molecular weight than the wild-type protein. The mutant protein was readily detectable in the patient but not in the control sample. The presence of the wild-type protein in the patient sample confirmed that the mutation is present in heterozygosity in this individual.

infarction. The serum creatinine 3 months before his death was 89 μmol/L and the graft was never biopsied. He was a member of a family described previously and genotyping of his daughter and the offspring of his cousin demonstrated that he was an obligate carrier of the CFHR5 mutation (individual III-1 from family 1 in reference (3)). Secondly, a 40-year-old Cypriot individual with ESRF and small kidneys, who presented with macroscopic hematuria at the age of ten but had been lost to follow up, received a deceased donor renal transplant at the age of 43. The transplant functioned well for 10 years until it was lost following atheroembolic complications of diagnostic coronary angiography. He returned to hemodialysis and died 6 years later. Subsequent molecular testing on stored genomic DNA demonstrated the presence of the CFHR5 mutation. There was no record of either native or allograft renal biopsy. These cases, together with the observations that the original disease takes many decades to cause ESRF, suggest that graft loss due to recurrence of CFHR5 nephropathy is not inevitable. Clearly larger studies will be required to establish the clinical course of CFHR5 nephropathy in renal transplantation. In summary, we describe the first reported case of recurrence of CFHR5 nephropathy in an unrelated renal transplant. Notably histological recurrence was demonstrable only 46 days after transplantation.

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Disclosure

Commercial Organizations

This manuscript was neither prepared nor funded in any part by a commercial organization.

Conflict of Interest

The authors of this manuscript have conflicts of interest to disclose as described by the *American Journal of Transplantation*. These are as follows:

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Consultant: Bio Nano Consulting (Paid Director); Ownership: ReOx Ltd.—Director and stockholder;

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DP Gale

Honoraria: Alexion

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Case Report

Acute Presentation and Persistent Glomerulonephritis Following Streptococcal Infection in a Patient With Heterozygous Complement Factor H–Related Protein 5 Deficiency

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Acute poststreptococcal glomerulonephritis is a common cause of acute nephritis in children. Transient hypocomplementemia and complete recovery are typical, with only a minority developing chronic disease. We describe a young girl who developed persistent kidney disease and hypocomplementemia after a streptococcal throat infection. Kidney biopsy 1 year after presentation showed isolated glomerular complement C3 deposition, membranoproliferative changes, and subendothelial, intramembranous and occasional subepithelial electron-dense deposits consistent with C3 glomerulopathy. Complement gene screening revealed a heterozygous single nucleotide insertion in exon 4 of the complement factor H–related protein 5 gene *(CFHR5),* resulting in a premature stop codon. This variant was not detected in 198 controls. Serum CFHR5 levels were reduced. The mother and sister of the index patient were heterozygous for the sequence variant, with no overt evidence of kidney disease. We speculate that this heterozygous CFHR5 sequence variant is a risk factor for the development of chronic kidney disease after streptococcal infection.
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INDEX WORDS: Complement; kidney; streptococcus; C3 glomerulopathy.

Acute poststreptococcal glomerulonephritis (APSGN) is a common cause of childhood acute nephritis. Serum complement C3, C5, and properdin levels typically are reduced acutely, whereas C4 levels are normal.[1,2] This pattern of hypocomplementemia reflects selective activation of the complement alternative pathway. Streptococcal components have been shown to activate this pathway,[3-5] and transient C3 nephritic factor (C3NeF) has been shown in some cases.[6] C3NeF potentiates activation of the alternative pathway and typically is seen in dense deposit disease, a condition strongly associated with alternative pathway dysregulation. In APSGN, most children recover completely and complement levels return to normal within 12 weeks. For reasons that are unknown, a minority develop chronic kidney disease associated with continuing hypocomplementemia.[7,8] We report a case of persistent kidney disease after streptococcal infection in association with a heterozygous mutation in the gene encoding complement factor H–related protein 5 (*CFHR5*).

CASE REPORT

A previously healthy 7-year-old Caucasian female presented with a 10-day history of lethargy, fever, sore throat, and cough associated with abdominal pain, vomiting, and dark colored urine. Mild periorbital edema was accompanied by abnormal urinalysis results (protein [3+], blood [3+]). Results of investigations included the following values: serum urea nitrogen, 50.1 mg/dL (17.9 mmol/L); creatinine, 0.98 mg/dL (87 μmol/L); hemoglobin, 10.9 g/dL (109 g/L); C-reactive protein, 77 mg/L; C3, 0.36 mg/mL (0.36 g/L; reference range, 0.75-1.65); C4, 0.29 mg/mL (0.29 g/L; reference range, 0.11-0.43); and urine protein-creatinine ratio, 5,221 mg/g. Both serum anti-streptolysin O (>200 U/mL) and anti-DNase antibody titers (360 U/mL) were elevated. Kidney ultrasound showed 8.3-cm kidneys with loss of corticomedullary differentiation.

The patient improved after treatment with phenoxymethylpenicillin, although proteinuria and microscopic hematuria persisted. Four months later, she developed macroscopic hematuria after an upper respiratory tract infection.

Nine months after presentation, kidney biopsy showed mesangial hypercellularity, segmental endocapillary hypercellularity, and segmental capillary wall double contours on light microscopy. Immunoperoxidase staining showed marked granular capillary

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wall and mesangial C3, C9, and CFHR5 deposition (Fig 1A). Glomerular C1q and immunoglobulins were absent. On electron microscopy, glomerular basement membranes appeared thickened, with segmental duplication of the basement membrane and mesangial cell interposition. There were intramembranous electron-dense deposits with occasional subendothelial deposits, as well as scattered hump-like subepithelial deposits. Mesangial deposits were absent. Angiotensin receptor blockade reduced urinary protein loss and kidney function remained normal.

Twenty months after presentation, a second kidney biopsy (Fig 1A) showed persistent membranoproliferative glomerulonephritis with tubulointerstitial scarring involving ~40% of the cortex. Electron microscopy showed intramembranous electron-dense deposits and some mesangial deposits. The findings in both biopsies are consistent with C3 glomerulopathy with a membranoproliferative pattern of glomerulonephritis.[9] Proteinuria improved with glucocorticoid therapy. Since the onset of disease, circulating C3 levels have remained low (Fig 1B). She has not developed ocular drusen or lipodystrophy. C3NeF has been consistently undetectable and anti-factor H autoantibodies have not been detected.

To determine whether there was any other serum factor enhancing C3 activation, we added purified C3 (0.5% solution; Merck, www.merck.com/index.html) to serum from the index case and compared its hemolytic activity at 2 and 4 hours with that of

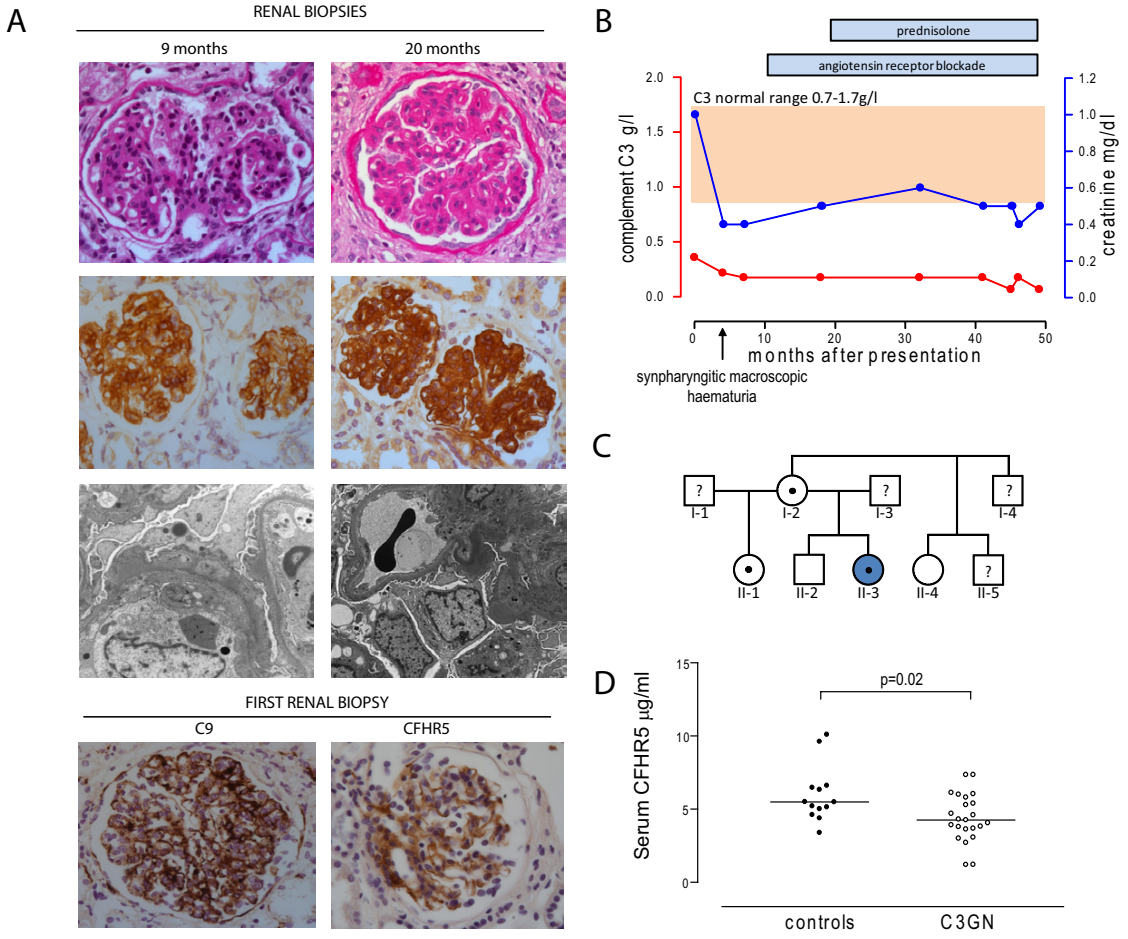

Figure 1. (A) Kidney biopsies performed at 9 and 20 months postpresentation. Light microscopy (first row) shows mesangial hypercellularity, segmental endocapillary hypercellularity, and capillary wall double contours with marked capillary wall C3, C9, and CFHR5 seen on immunoperoxidase staining (second [C3] and fourth [C9, CFHR5] rows). On electron microscopy (third row), there were intramembranous and occasional subendothelial electron-dense deposits. Rare hump-like subepithelial deposits were seen in the first biopsy specimen. Rabbit anti-human C3 (Dako, www.dako.com), mouse anti-human C9 (Leica, www.leica.com), and mouse anti-human monoclonal CFHR5 antibodies (a gift from Dr J. McRae) were used for immunoperoxidase staining. (B) Serum complement C3 and creatinine levels versus time. Serum C3 levels remained profoundly depressed throughout the illness. Conversion factor for serum creatinine in mg/dL to μmol/L, $\times$88.4. (C) Family pedigree. II-3 (filled circle) denotes the index patient. A central dot within the symbol (circle and square denoting female and male individuals, respectively) indicates the sequence variant is present, empty symbols denote absence of the sequence variant, and symbols with a question mark indicate genetic status unknown. (D) Serum CFHR5 levels in individuals with biopsy-proven C3 glomerulonephritis and absence of the CFHR5 sequence variant. Median CFHR5 level in the C3 glomerulonephritis group (4.3; range 1.2-7.4 μg/mL; n = 23) was significantly lower (P = 0.02, Mann-Whitney test) than the median in healthy controls (5.5; range 3.4-10.1 μg/mL; n = 13). CFHR5 was measured by enzyme-linked immunosorbent assay using rabbit anti-human CFHR5 and mouse anti-human CFHR5 antibodies (both from Abcam, www.abcam.com) as capture and primary antibodies, respectively. The standard curve was generated using recombinant CFHR5 (R&D Systems, www.rndsystems.com).

C3-deficient human serum reconstituted with purified C3 in an identical fashion. Hemolytic activity at 2 (60% vs 57%) and 4 hours (43% vs 47%) did not differ between the test and control sera, indicating that there was no evidence of accelerated serum C3 conversion in serum of the index case.

We performed screening for the known genetic causes of alternative pathway dysregulation. No coding mutations were detected in the complement genes CD46, complement factor H (CFH), factor B, factor I, and C3. No copy number variation within the *CFH-CFHR* gene locus was seen using a multiplex ligation-dependent probe amplification assay. CFHR5 gene sequencing revealed a single heterozygous nucleotide duplication in exon 4 (c.485dupA) which generates a reading frameshift at amino acid 163 and a premature stop codon at amino acid position 197 (p.Glu163Argfs*34). This variant was not detected by sequencing of 198 ethnically matched DNA samples (obtained from the UK Blood Services Collection of Common Controls) and was not present in dbSNP (www.ncbi.nlm.nih.gov/snp, accessed October 2011). The healthy mother (I-2) and sister (II-1), but not the 2 other siblings examined (II-2 and II-4), were heterozygous for this sequence variant (Fig 1C). The complement profile of the kindred is shown in Table 1. Serum CFHR5 levels in unaffected members with the gene variant were within the range seen in healthy controls (3.4-10.1 μg/mL). However, serum CFHR5 level was decreased in the index case (2.1 μg/mL). Notably, serum CFHR5 levels also were found to be decreased in individuals with biopsy-proven C3 glomerulonephritis (Fig 1D).

DISCUSSION

CFHR5 is a member of the CFH family of proteins encoded within the regulators of complement activation gene cluster on chromosome 1.[10] CFHR5 has been shown to have complement-regulatory function in vitro[11] and to colocalize with complement deposits within the kidney in a variety of glomerular pathologic states.[12] The biological role of CFHR5 is unclear, but it is postulated to have a physiologic role in the processing of complement within the kidney.[13] This hypothesis has derived from reports of genetic variation in CFHR5 and susceptibility to kidney disease. A heterozygous CFHR5 mutation has been shown to be associated with the development of familial C3 glomerulonephritis in individuals of Cypriot ancestry,[13] and allelic variants of CFHR5 have been associated with dense deposit disease.[14] Previously published evidence that CFHR5 interacts with glomerular complement[12,13] suggests that this phenomenon contributed to the decrease in serum CFHR5 levels in the index case. Consistent with this hypothesis was our observation that glomerular CFHR5 was easily detectable in kidney biopsy specimens from the index case, median serum CFHR5 levels were low in individuals with C3 glomerulonephritis, and serum CFHR5 level in the index patient was lower than levels in other family members with the sequence variant, but no kidney disease. An interesting additional insight based on these data is that serum CFHR5 levels may be a useful biomarker of glomerular C3 deposition.

Our report is to our knowledge the first description of the association of kidney disease with a heterozygous sequence variant causing frameshift and premature truncation of CFHR5. Interestingly, the same variant that we describe was reported in an individual serving as a control in a study investigating the role of CFHR5 in atypical hemolytic uremic syndrome.[15] No clinical details for this individual were reported. The presence of the CFHR5 sequence variant in the mother and sister of the index patient (neither of whom had

Table 1. Complement Profile

	Pedigree No.					
	I-2	II-1	II-2	II-4	II-3[a]	Reference Range
CFHR5 sequence variant	Yes[b]	Yes[b]	No	No	Yes[b]	
C3 (g/L)	1.43	1.03	1.08	1.14	0.07	0.7-1.7
C4 (g/L)	0.24	0.17	0.21	0.16	0.22	0.16-0.54
CFH (%)[c]	181	132	132	147	148	
CFI (%)[c]	174	132	157	186	154	
Total complement hemolytic activity (%)[d]	108	47	99	95	ND	50-150
Alternative pathway hemolytic activity (%)[d]	92	53	102	95	ND	50-150
Serum CFHR5						
Absolute amount (μg/mL)	5.3	3.8	4.0	NA	2.1	3.4-10.1
Relative amount (%)[e]	96.2	69.9	72.5	NA	37.3	

Abbreviations: CFH, complement factor H; CFHR5, complement factor H-related protein 5; CFI, complement factor I; NA, not available (insufficient sample); ND, not detectable.

[a]Index patient.

[b]c.485dupA (p.Glu163Argfs*34); numbering and nomenclature follows Human Genome Variation Society recommendations (for cDNA, A in ATG start codon is position 1; for protein, the methionine encoded by the start codon is position 1).

[c]No validated reference range exists for CFH and CFI, so results are expressed as percentage of the level in pooled serum from apparently healthy controls.

[d]Percentage of the activity in pooled serum from apparently healthy controls.

[e]Compared with the median value in 13 apparently healthy controls, which was 5.5 μg/mL (Fig 1D).

overt evidence of kidney disease) indicates that heterozygosity for this CFHR5 sequence variant is not sufficient to cause kidney disease. We speculate that it was the presence of both the heterozygous CFHR5 sequence variant and streptococcal infection that precipitated chronic kidney disease in our index patient.

Interestingly, individuals with other genetic defects within the CFH gene family are susceptible to exacerbation of kidney disease during throat infections. Synpharyngitic macroscopic hematuria is characteristic of CFHR5 nephropathy[13] and common in factor H deficiency states.[16,17] Furthermore, exacerbation of urinary abnormalities in dense deposit disease has been associated with streptococcal infection.[18] Rarely, kidney biopsies following APSGN have shown changes consistent with dense deposit disease.[19-21] This raises the difficult question of whether dense deposit disease is triggered by streptococcal infection or is a pre-existing lesion that becomes clinically overt during streptococcal infection. We found one report describing apparent progression from biopsy-proven APSGN to dense deposit disease.[22] However, in others, it seems that dense deposit disease may have predated the episode of APSGN.[18,20] Our patient had no previous evidence of kidney disease, although it is possible that subclinical disease was present before the episode of streptococcal infection.

The presence of low serum C3 levels in this case cannot be explained by the heterozygous sequence variant in CFHR5 alone because serum C3 levels were normal in the index patient's mother and sister, who also carried the sequence variant. Notably, in CFHR5 nephropathy, serum C3 levels typically are normal.[13] In contrast, in dense deposit disease, low serum C3 level is typical and commonly associated with the presence of C3NeF. However, rarer causes such as anti–factor B antibodies recently have been reported.[23] C3NeF was repeatedly undetectable in our case and we could not detect any other factor within the serum of the index case that was causing accelerated C3 consumption. The serum C3 depletion in our case most likely results from excessive C3 activation within the kidney.

In summary, we have identified a heterozygous sequence variant causing frameshift and premature truncation of CFHR5 in an individual with chronic kidney disease after streptococcal infection. We speculate that CFHR5 mutations may be a risk factor for the development of chronic glomerulonephritis after streptococcal infection.

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