Complement activity influences glomerular inflammation and clinical severity in IgA nephropathy and C3 glomerulopathy

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Nicholas Medjeral-Thomas

Centre for Inflammatory Disease

Department of Medicine

Imperial College London

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

Abstract

The mechanisms that link complement activation and glomerular injury are not understood. Recently, novel mechanisms of complement dysregulation mediated by factor H related protein (FHR)1 and FHR5 have been described. IgA nephropathy (IgAN) is important and poorly understood. Exciting recent evidence suggests FHR proteins and lectin complement pathways contribute to IgAN pathogenesis. C3 glomerulopathy (C3G) demonstrates the potential for alternative complement pathway dysregulation to drive glomerular injury. However, in the majority of C3G cases, precise pathogenesis is not understood. I hypothesised IgAN and C3G pathogenesis were dependent on imbalances of FHR1, FHR5 and lectin complement pathway proteins.

I demonstrated circulating FHR1 levels and the ratio of FHR1 to factor H (FH) were increased in patients with IgAN compared to healthy controls and in patients with progressive compared to stable IgAN. I found IgAN patients had higher circulating FHR5 levels than healthy controls and higher circulating FHR5 levels associated with histology markers of IgAN severity. Glomerular FHR5 deposition associated with both clinical and histologic markers of IgAN severity and immunohistolgical markers of complement activation. Glomerular FHR5 deposition also associated with clinical and histology markers of C3G severity and co-localised with glomerular complement (C)3 deposits in C3G. Glomerular FHR5 deposition co-localised with markers of both ongoing (C3b/iC3b/C3c) and previous (C3dg) alternative complement activation in C3G and IgAN. I detected FHR5 in Immunoglobulin (Ig)A containing immune complexes. With regards to the lectin complement pathway, I found IgAN patients had higher circulating levels of M-ficolin, L-ficolin, mannan binding lectin (MBL) associated serine protease (MASP)-1 and MBL associated protein (MAp)19 than healthy controls, and lower circulating levels of MASP-3 than healthy controls. Lower circulating MASP-3 levels also associated with markers of IgAN severity.

My research addresses fundamental gaps in our understanding of glomerular complement activation and IgAN pathogenesis. The results are limited by a number of important confounding factors that need to be addressed. However, they justify researching the mechanisms by which FHR5 contributes to complement activation and disease severity in IgAN and C3G and could contribute to novel and exciting diagnostic tools and therapeutic targets for IgAN, C3G and other complement dependent glomerulopathies.

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Contents

List of Abbreviations

ACEi	angiotensin-converting enzyme inhibitor
ADPKD	autosomal dominant polycystic kidney disease
AF	Alexa Fluor
aHUS	atypical haemolytic uraemic syndrome
ALT	alanine aminotransferase
AMD	age-related macular degeneration
AP-50	50% alternative pathway haemolytic activity of serum assay
APC	antigen presenting cell
APS	ammonium persulfate
ARB	angiotensin receptor blocker
AU	arbitrary units
BSA	bovine serum albumin
C	complement protein (suffixed by numbers and letters)
C1INH	C1 inhibitor
C3G	C3 glomerulopathy
C3GN	C3 glomerulonephritis
C3NeF	C3 nephritic factor
C4bp	C4-binding protein
C5aR1	C5a receptor 1
CH-50	50% haemolytic complement activity of serum assay
CI	confidence interval
CKD	chronic kidney disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CL-K1	collectin kidney 1 (also refferd to as CL-11)
CL-L1	collectin liver 1 (also referred to as CL-10)
CNV	copy number variation
CR1	complement receptor 1
CR2	complement receptor 2
CRP	C-reactive protein
DAB	diaminobenzidine
DAF	decay accelerating factor (CD55)
DAMP	damage-associated molecular pattern
DAPI	4',6-Diamidino-2-Phenylindole
DDD	dense deposit disease
ddH_20	double distilled water
del*CFHR1-4*	deletion of genes encoding FHR1 and FHR4 (*CFHR1* and *CFHR4*)
del*CFHR3-1*	deletion of the genes encoding FHR3 and FHR1 (*CFHR3* and *CFHR1*)
DN	diabetic nephropathy
DNA	deoxyribonucleic acid
E	endocapillary hypercellularity (suffixed with number 1 or 0)
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
eGFR	estimated glomerular filtration rate
ELISA	enzyme linked immune-sorbent assay

ESRD	end-stage renal disease
fB	factor B
fD	factor D
FFPE	formalin-fixed paraffin-embedded
FH	factor H
FHbp	FH-binding protein
FHL1	FH-like protein 1
FHR	factor H related protein
FHR1	factor H related protein 1
FHR2	factor H related protein 2
FHR3	factor H related protein 3
FHR4	factor H related protein 4
FHR5	factor H related protein 5
fI	factor I
FITC	fluorescein isothiocyanate
FPLC	fast protein liquid chromatography
GalNac	N-acetyl-galactosamine
GBM	glomerular basement membrane
Gd-IgA1	galactose deficient IgA1
GPI	glycosyl phosphatidylinositol
GWAS	genome-wide association study
HRP	horseradish peroxidase
HSP	henoch schonlein purpura
ICC	intra-class correlation coefficient
ICHTB	Imperial College Healthcare Tissue Bank
IF	immunofluorescence
Ig	immunoglobulin
IgA	immunoglobulin A
IgA1	IgA subtype 1
IgA2	IgA subtype 2
IgAN	IgA nephropathy
IgG	immunoglobulin G
IgM	immunoglobulin M
IHC	immunohistochemistry
IQR	interquartile range
IS	immunosuppression
LDS	lithium dodecyl sulfate
LN	lupus nephritis
LN IV(A/C)	lupus nephritis class IV (active/chronic)
M	mesangial hypercellularity (suffixed with a 0, 1 or 2)
MAA	malondialdehyde acetaldehyde
mAB	monoclonal antibody
MAC	membrane attack complex (also referred to as TCC or C5b9)
MAp	MBL-associated protein
MASP	MBL-associated protease
MBL	mannan-binding lectin
MCE	mercaptoethanol
MCP	membrane cofactor protein (CD46)
MPGN	membranoproliferative glomerulonephritis

MW	molecular weight
n	number analysed
NFDM	non-fat dried milk
NGS	normal goat serum
NHS	National Health Service
NIHIR	National Institute for Health Research
NR	normal range
OD	optical density
OR	odds ratio
PAMPs	pathogen-associated molecular patters
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PNH	paroxysmal nocturnal haemoglobinuria
PRM	pattern recognition molecule
PTX3	pentraxin 3
PVDF	polyvinylidene fluoride
RAS	renin-angiotensin system
RCA	regulator of complement activation
RES	Research Ethics Service
ROI	region of interest
RPGN	rapidly progressive glomerulonephritis
S	segmental sclerosis (suffixed with number 0 or 1)
SCR	short consensus repeat
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SLE	systemic lupus erythematosus
SNP	single-nucleotide polymorphism
T	tubular atrophy (suffixed with number 0, 1 or 2)
TBM	thin basement membrane disease
TCC	terminal complement complex
TEMED	N,N,N’,N’-tetramethylethylenediamine
TTP	thrombotic thrombocytopenic purpura
UK	United Kingdom
UPCR	urine protein:creatinine ratio
urine PCR	urine protein:creatinine ratio
WCC	white cell count

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Introduction

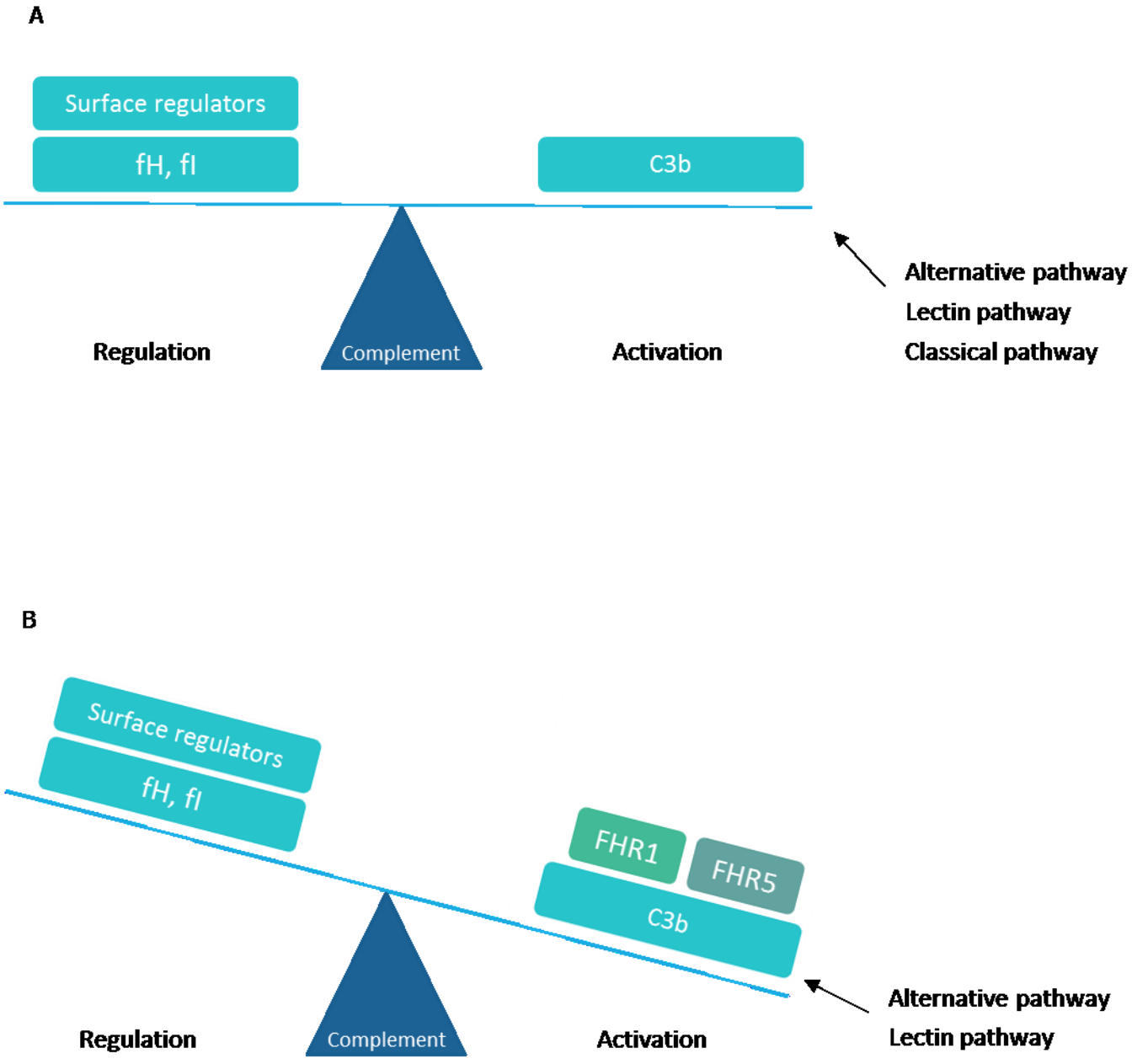
My thesis examines the role of complement in IgA nephropathy (IgAN) and C3 glomerulopathy (C3G). My work investigates how the complement factor H-related (FHR) proteins and the lectin pathway contribute to renal injury in IgAN and the role of complement factor H-related protein 5 (FHR5) in C3G.

My thesis was motivated by two observations. First, a number of large genetic association studies demonstrated common and rare variants in FHR proteins associated with IgAN[2-4]. Second, mutations in the FHR proteins were associated with familial C3G, demonstrating changes in the FHR proteins alone could result in complement-dependent glomerulopathy[5-7]. *In vitro* data suggested FHR proteins interfered with the abiity of factor H (FH), the well characterised negative regulator of complement (C)3 activation, to interact with and inhibit surface C3 deposition. This phenomenon was termed FH deregulation[8, 9]. These observations led me to question whether: (1) glomerular injury in IgAN is dependent on complement activation and dysregulation (Figure 1); (2) complement dysregulation in IgAN is due to increased glomerular binding of FHR proteins, specifically FHR5 and FHR1; (3) the interaction of wild-type FHR proteins and FH could also influence complement activation in C3G.

In my introduction, I discuss the complement system, including its functions, pathways and roles in kidney diseases. I will concentrate on the alternative complement pathway and FH. I will demonstrate the potential for complement activity to cause inflammation and disease. I will next describe the biology of the FHR proteins and how, depending on the context, it is the balance between the actions of FH and the FHR proteins that determine complement activation. I will describe C3G, IgAN, and therapies that inhibit complement activation. In the final section of the introduction, I will discuss my hypotheses and research aims.

Figure 1. The balance of complement activation

(A) In health, a balance of complement activation and regulation provides appropriate and targeted complement activity. Complement activation and amplification results in the cleavage of C3 to C3b, and ongoing complement activity. Complement regulation is dependent on surface-bound regulators and FH and factor I (FI) that can act both on surfaces and in the fluid phase.
(B) I hypothesise that factor H related protein 1 (FHR1), FHR5 and lectin complement pathway activity contribute to IgAN pathogenesis by tipping the balance of complement towards activation.



A. The Complement System

Overview

The complement system is a powerful and important component of innate and adaptive immunity [10]. It forms intricate networks of activating and regulating mechanisms that discriminate healthy and damaged cells, and self and non-self surfaces[11, 12]. Complement activation is tightly regulated. This is essential for preventing inappropriate activation, inflammation and tissue injury. Complement activity is dependent on the alternative pathway because, as will be discussed in more detail, the alternative pathway is responsible for complement amplification regardless of the activating trigger. Complement FH is a major negative regulator of alternative complement pathway activation. Variation in complement activity is associated with susceptibility to both rare and common diseases. These variants have provided insight into complement biology and regulation. Recently, the development of therapeutic agents targeting complement pathway components has expanded. Understanding the contribution of complement to disease pathogenesis and identifying patients likely to benefit from therapeutic complement manipulation is therefore increasingly important.

Complement functions

The complement system was first described as a heat-labile factor that enabled antibodies to lyse bacteria[13]. Traditionally, therefore, the complement system has been viewed as an essential component of immune defence against pyogenic pathogens. However, complement is also essential for many homeostatic processes[14]. Complement mediated functions are dependent on the interaction of complement proteins with surface molecules because this determines appropriate differentiation of self from non-self and localisation of effector functions. The fundamental function of complement is therefore sampling and labelling the surfaces of cells, pathogens, and molecules[12]. This can occur via one of two mechanisms. The alternative pathway constitutively maintains a low level of tick-over activation by spontaneous hydrolysis of a labile thioester bond in C3. Subsequently, a small amount of C3b is generated, even during normal physiological conditions[15]. The C3b is able to bind covalently and tag any surface containing hydroxyl groups in the immediate proximity of its generation. Second, classical and lectin pathway pattern recognition molecules (PRMs) bind damage-associated molecular patterns (DAMPs)[15]. Complement binding can lead to pro-inflammatory and destructive

effects in pathological settings, or immune-regulatory and tolerant effects in physiological settings. Which of these dichotomous fate complement follows is determined by the relative abundance of complement regulators[12].

Pathogen identification and elimination is dependent on the sparsity of complement regulators displayed on pathogenic surfaces[12]. This allows deposited PRMs or C3b to form C3 convertases, with subsequent complement amplification, C3b opsonisation, and cascade to the terminal complement pathway. Pathogens opsonised with C3b are phagocytosed. C3 and C5 convertase activity results in the release of the anaphylatoxins C3a and C5a[16]. Through interactions with their respective receptors, C3a and C5a stimulate inflammation and immune cell recruitment[12]. Terminal pathway activation results in the formation of the membrane attack complex (MAC, C5b9) that disrupts bacterial wall integrity, leading to lysis of gram-negative bacteria[10].

The clearance of immune complexes is dependent on C3b binding and opsonisation. C3b deposited on immune complexes binds complement receptor 1 (CR1) on erythrocytes. The immune complex and complement-coated erythrocytes are cleared by liver and spleen resident macrophages. Body compartments that lack erythrocytes, such as glomeruli and retina, seem predisposed to immune complex deposition and associated pathology. Furthermore, incomplete immune complex clearance associates with autoimmune disases like systemic lupus erythematosus (SLE)[16].

Complement also influences adaptive immunity. If B-cell receptor bound antigens are tagged with C3 fragments, complement receptor 2 (CR2) co-receptors are ligated with the B-cell receptor[14]. This leads to enhanced intracellular B-cell receptor signalling and reduced antigen threshold for antibody response. Complement can also facilitate antigen presentation. For example, follicular dendritic cells bind C3-coated antigens via CR2 receptors and present the antigens to B cells, leading to effector and memory B cell development[14]. T-cells and antigen presenting cells (APCs) express complement receptors and regulators. Complement can polarise T-cell responses and influence T-cell migration[12]. On internalisation by APCs, C3-coated pathogens trigger increased surface major histocompatibility complex (MHC) expression, with subsequent amplified antigen-specific T-cell proliferation. In contrast, cells with relatively deficient surface bound C3 undergo accelerated fusion with lysosomes on internalisation by APCs, leading to impaired antigen-specific T-cell proliferation and immune tolerance. This demonstrates C3 can direct immune responses

towards pathogen destruction or immune tolerance[14]. Complement expressed and secreted by T-cells and APCs has autocrine and paracrine effects leading to cell survival, proliferation, maturation, expression of co-stimulatory molecules and cytokine production.

The balance of complement activation and regulation also directs physiologic immune regulation and tolerance. In healthy individuals, billions of cells undergo apoptosis daily. Complement is essential for maintaining immune tolerance of this potentially immunologically potent process[12]. Cell surface alterations of complement regulator expression leads to low PRM and C3b deposition. When deposited at this relatively sparse density, C3b is cleaved to iC3b. Instead of forming a convertase, the iC3b interact with phagocytes, leading to immune tolerance and preventing immune responses against apoptotic cells[14].

Complement proteins are also involved in a diverse range of non-immune physiologic functions, such as neural crest development, the synaptic pruning essential for normal brain development, and lipid and glucose metabolism[12]. The diversity of complement dependent processes makes predicting the consequences of complement system deregulation or inhibition difficult. To provide more insight into the effects of complement pathway variants and potential complement inhibition, I will next describe complement activation pathways.

Cascade and pathways

The complement system is made up of more than 30 proteins either bound to cell surfaces or soluble in plasma[11]. These proteins are abundant; they constitute 15% of the globulin fraction[10]. As opposed to representing their pathway position, the complement proteins were named in the historical order in which they were discovered, with classical complement pathway components allocated the numbers C1-9, and alternative components named as factors followed by a letter, for example factor B (fB). The fragments produced by complement protein cleavage are referred to with a small-case suffix such as ‘a’ or ‘b’ with the larger fragment normally allocated ‘b’, such as C3b or Bb. Many complement proteins are inactive enzyme precursors, or zymogens, that on activation cleave their own zymogen, producing self-amplifying cascades. Complement forms complex networks with connections to other immunology and inflammatory systems[12].

However, the complement system is traditionally divided into and described as three separate pathways; the classical pathway, the alternative pathway, and the lectin pathway.

The classical pathway is strongly initiated by IgM or IgG clusters, which are typically antibodies bound to antigen. However, the hexameric surface-binding component of the C1 complex that initiates the classical pathway, C1q, is a versatile PRM that can also recognise viruses, gram-negative bacteria, and other endogenous molecules such as the pentraxin C-reactive protein (CRP)[17]. In addition to C1q, the C1 complex consists of two molecules each of the proteases, C1s and C1r. Upon binding of C1q, C1r and C1s are consecutively activated. C1s subsequently cleaves C4 into C4a and C4b. This cleavage exposes a previously hidden thioester domain. The thioester domain covalently binds C4b to surfaces in the immediate vicinity of the activated C1 complex, such as cells, immune complexes, pathogens or molecules. C1s also cleaves C2 into C2a and C2b. C4b then binds the larger protease C2 fragment, C2b, and forms the classical pathway C3 convertase, C4b2b.

The lectin pathway is activated by the interaction of mannan-binding lectin (MBL) with arrays of mannose groups, for example on bacterial cell surfaces. The complex that initiates the lectin pathway is analogous to the C1 complex[18]. The hexameric MBL sits in complex with MBL-associated proteases 1 and 2 (MASP1 and MASP2). MASP1 and MASP2 share structural similarity with C1r and C1s. MBL binding activates MASP2 and, like C1s, cleaves C4 and C2[19] to ultimately form the C3 convertase enzyme C4b2b[11]. MASP1, cleaves C2 but not C4 and amplifies the activated lectin pathway response[20]. In addition to MBL, the lectin pathway can be activated by different PRMs called ficolins and collectins. Three ficolins, H-, M-, and L-ficolin, and two collectins, collectin liver 1 (CL-L1, or CL-10) and collectin kidney 1 (CL-K1 or CL-11) have been described. These lectin pathway PRMs share effector functions with MBL and recognise carbohydrate patterns, such as N-acetylgalactosamine, to which MBL does not bind[21].

The alternative complement pathway is continuously activated at a low rate, a phenomenon termed ‘tick over’. This provides constant probing of surfaces by alternative complement pathway components[22]. Tick over involves the hydrolysis of the otherwise relatively inert C3 to form $C3_{H2O}$, exposing a promiscuous thioester domain binding site[23]. $C3_{H2O}$ recruits and binds the protease zymogen factor B (fB). FB is cleaved by factor D (fD) to form the active serine esterase Bb. The $C3_{H2O}$Bb complex cleaves C3 into its active fragments[22]; C3a, an anaphylatoxin and chemoattractant that is immediately released into the circulation, and C3b. The cleavage of

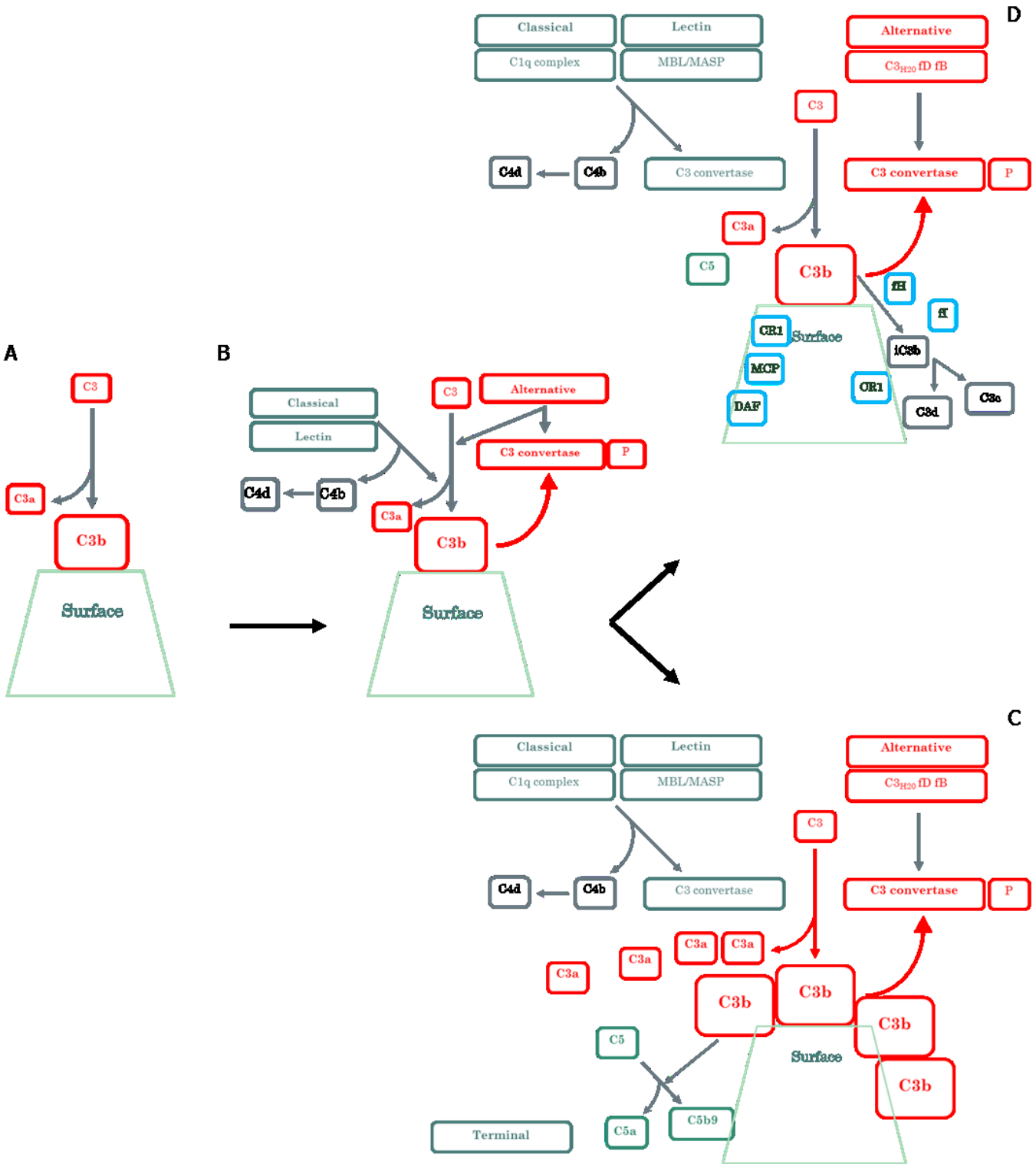
C3 to C3b exposes the thioester domain that covalently attaches to a range of target surface residues. This initial binding of the short-lived C3b thioester moiety is quickly amplified on foreign cells but closely regulated on host surfaces. The thioester domain shows preferential reactivity with specific carbohydrates that may lead to accelerated opsonisation of foreign material, providing an innate pattern recognition mechanism[24]. Surface bound C3b associates with Bb to form the alternative pathway C3 convertase, C3bBb. Properdin stabilises the alternative pathway convertase[25]. Properdin also recognises several pathogen-associated molecular patters (PAMPs) on microbial targets and apoptotic cells, thereby fulfilling similar pathogen recognition functions to MBL and C1q[26]. Once target-bound, properdin attracts fluid-phase C3b and is thereby able to initiate alternative pathway C3 convertase assembly.

The C3 convertase repeatedly cleaves C3 molecules, forming multiple C3b products that are deposited in the activation vicinity (Figure 2). C3b can follow one of three fates. The first is the formation of more C3bBb, leading to complement and C3b amplification. Complement amplification is therefore dependent on alternative complement pathway convertase activity[27]. As the density of deposited C3b increases, C3b bind the C3 convertases C4b2b and C3bBb, forming C4b2b3b or C3bBbBb[12], which are C5 convertases. The second fate of C3b is therefore to form C5 convertases. The C5 convertases cleave C5 to the anaphylatoxin C5a and fragment C5b. C5b binds C6 and C7. The resulting complex inserts into cell membranes and associates with C8, followed by 10 to 16 C9 molecules to form the C5b9 terminal complement complex (TCC) or MAC[28]. The MAC forms a pore-like structure in the membrane's outer lipid bilayer that promotes lysis of non-nucleated cells and gram-negative bacteria, and induces activation of injurious pathways in nucleated cells[29].

The third potential fate for deposited C3b is its proteolysis to C3 fragments unable to participate further in complement activation (Figure 2). Surface-bound C3b is inactivated to iC3b by FI with the release of C3f. This step is dependent on a co-factor for FI, which may be surface bound, such as CR1 or membrane cofactor protein (MCP), or active in the fluid and surface phases, such as FH. iC3b is unable to bind factor B and therefore does not participate in C3 convertase formation. FI performs a second cleavage of iC3b to C3dg, releasing the C3c fragment into circulation. Because C3dg is covalently surface bound but does not form C3 convertases, its presence implies local, but not necessarily acute complement activation.

Figure 2. The complement pathway

(A) The key step in complement activation is the cleavage of inactive circulating C3 to C3b that binds surfaces and molecules, and the anaphylotoxin C3a. (B) C3 cleavage to C3a and C3b occurs following activation via the classical and lectin and alternative pathways and as a result of complement amplification. Complement amplification is dependent on the alternative complement pathway convertase. (C) In the absence of adequate and balanced regulation, C3b production is amplified and cascades to the terminal complement pathway and C5b9 formation. (D) Complement regulation by surface-bound and circulating proteins (blue-bordered shapes) are essential for limiting complement activation and maintaining homeostasis.



Complement regulators

Effective complement system regulation is essential to appropriately target inflammation and prevent host cell injury. Initial complement activation is regulated by inherent physical limitations. The thioester domains exposed on $C3_{H2O}$ and C3b are inherently labile and efficiently inactivated by fluid phase complement regulators FH and fI. The thioester half life is estimated to be about 60µs, suggesting $C3_{H2O}$ and C3b will bind any surface located within about 60nm[30]. Alternatively, $C3_{H2O}$ and C3b may form fluid phase convertases and bind properdin, which attenuates degradation. Classical pathway activation requires binding to an IgM multimer or multiple adjacent IgG molecules. It is not essential for all the IgG FAB regions to be antigen-bound, but the number of engaged IgG molecules, which will itself be influenced by antigen epitope distribution, and the density of non-engaged IgG, determine the strength of pathway activation[15]. Efficient C1q binding depends on the formation of ordered antibody heximers on antigen surfaces[31]. Lectin pathway activation is dependent on PRM binding to clusters of carbohydrate motifs organised in specific ligand patterns. Lectin PRMs bind specific carbohydrate groups and associate with a homodimer of either MASP1 or MASP2. Both MASP1 and MASP2 are required for convertase formation and complement activation. Thereby, to allow activation adjacent PRMs with complexed MASP1 must juxtapose with PRMs complexed with MASP2[32]. In the absence of these events, complement activation will not progress.

Should complement surface binding and convertase formation proceed, a number of surface-bound and soluble complement pathway regulators limit complement amplification and cascade progression. For example, by preventing MAC assembly, CD59 is the major membrane-bound regulator of the terminal pathway. In paroxysmal nocturnal haemoglobinuria (PNH), acquired CD59 absence on erythrocytes renders the affected cells susceptible to complement-mediated lysis. CD55 (decay accelerating factor, DAF) is another important surface-bound regulator of classical, lectin and alternative complement pathways. Classical pathway activation is also controlled by C1 Inhibitor (C1Inh) that binds and inactivates the C1 complex. Lectin pathway regulation is influenced by the presence of MBL associated proteins (MAp) in PRM complexes that lack the ability to cleave C4 and C2[15]. The convertase formed from classical and lectin pathway activation is regulated by C4 binding protein (C4BP) and fI with cofactor activity from MCP and CR1.

Given 80-90% of total complement activation is dependent on the alternative pathway amplification loop, regulation of this component of the complement network holds particular pathophysiological importance[27].

Alternative pathway complement regulation is predominantly performed by FH and fI. In the absence of either of these regulators, alternative pathway activation proceeds unhindered and severe secondary depletion of circulating C3 develops[33]. FI is a protease that cleaves C3b in the presence of different cofactor molecules. This proteolytic conversion of C3b to iC3b by fI is dependent on an additional 'cofactor' protein such as MCP, CR1 and FH.

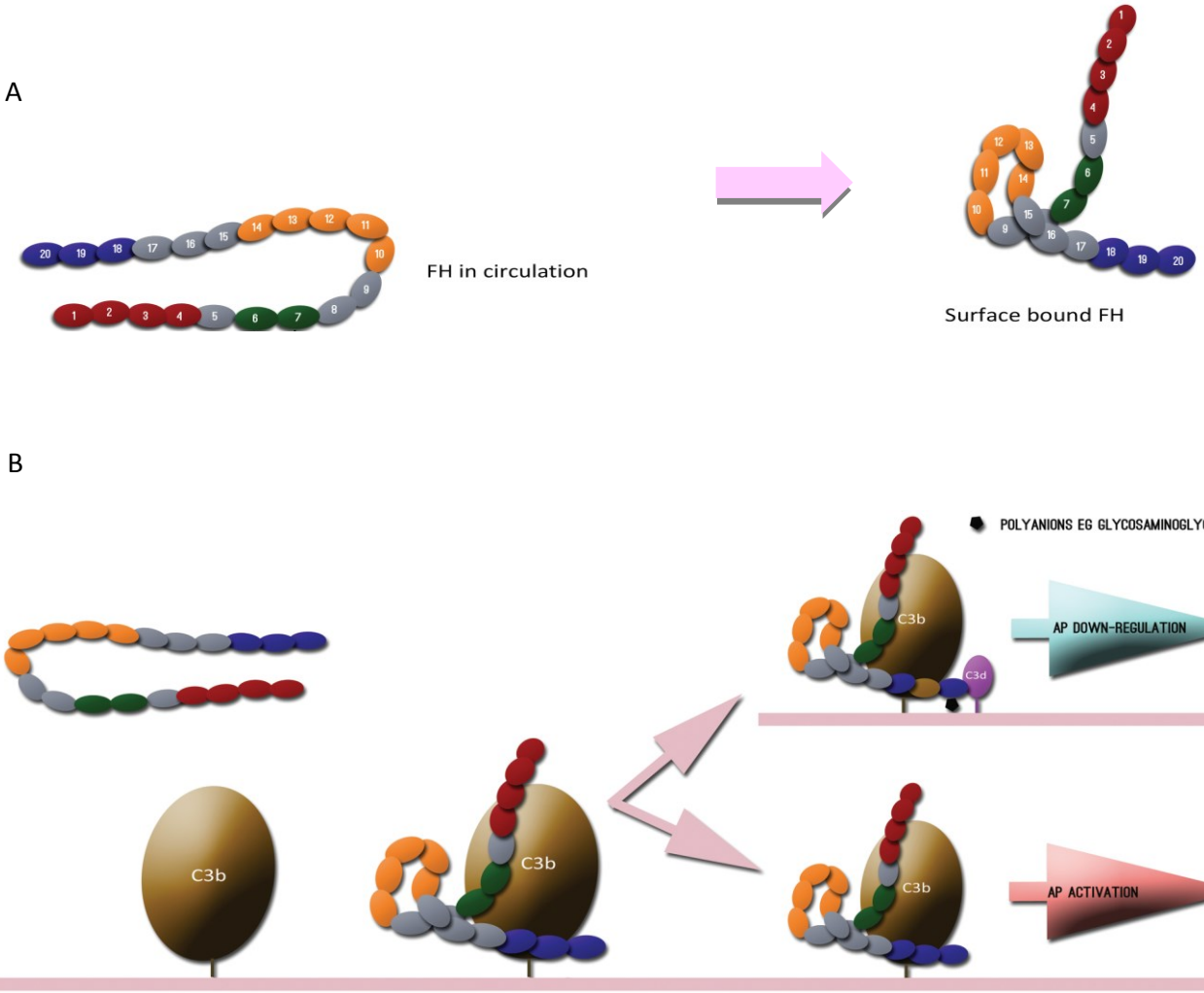
Complement FH is an abundant plasma protein that regulates the alternative pathway in fluid-phase and on cellular surfaces. FH and the FH-like protein 1 (FHL1) are derived by alternate splicing events from the *CFH* gene on chromosome 1q32[34]. Although the precise role of FHL1 is unclear, it is about ten times less abundant than FH in plasma and its potential FH-like actions are often attributed to FH itself. FH has 20 short consensus repeat (SCR) domains and two predominant functional regions; the surface-binding region at the C-terminus, and the complement regulatory region at the N-terminus. FH regulates the C3 convertase step of complement activation; after binding surface deposited C3b and surface expressed glycosaminoglycans by its carboxyl terminus (Figure 3), the FH n-terminus accelerates decay of the alternative pathway C3 convertase[35] and exhibits cofactor activity for fI-mediated proteolytic inactivation of C3b[36]. FH regulation has recently been described in molecular detail. Bound FH accelerates convertase decay by both interfering with C3b contact areas for fB and Bb on C3b and forming binding pockets on C3b that allows fI to attach to and cleave C3b[37]. Complement regulation is particularly reliant on FH on surfaces that do not have surface-bound regulators, such as the glomerular basement membrane.

The complement system is in a constitutive state of activation and regulation that balance to maintain homeostasis. Progression to complement amplification is dependent on a number of factors including the proximity, conformation and density of activating epitopes, and imbalances of complement activation and regulation. The balance of activation and regulation is dependent on the local concentration of complement proteins and regulators, which may be influenced by local activating events, such as pathogen invasion or immune complex deposition, and determinants of protein expression. Whether and how complement activation contributes to specific diseases is the subject of ongoing research and encompasses the subject of this thesis.

Figure 3. Factor H

(A) High-resolution structural studies show FH to adopt a compact hinge-like arrangement in circulation. Following interaction with C3b and molecular surface markers, FH is 'activated', adopting a higher affinity conformation for C3b, as shown.

(B) Structural studies show that FH domains 19 and 20 contain two distinct binding points for C3b. Furthermore, following binding of 'activated' FH to C3b, simultaneous binding of FH domain 20 to glycosaminoglycans (GAGs) on non-activator surfaces leads to complement alternative pathway (AP) down regulation[1]. This could allow FH-mediated target discrimination. A similar result is achieved if FH simultaneously binds C3b and surface bound C3dg, thus providing a negative feedback control of excessive C3b deposition. However, in the absence of interactions with C3b and either GAGs or C3dg, FH is unable to complete its complement regulatory effects, leading to complement AP activation.



Complement genetics and disease

Genetic variants influence complement activation and regulation. Genetic polymorphisms and mutations determine complement protein function and quantity and therefore affect the contribution of complement proteins to health and disease. Typically, genetic polymorphisms influence the relative quantity of complement components, sometimes without detectable health implications. Contrastingly, genetic mutations and subsequent complement protein deficiencies, with the exception of C9 and C2, are rare. Homozygous deficiencies generally cause clinically significant phenotypes, most commonly increased susceptibility to bacterial infection. This is most problematic in childhood before the antibody repertoire has developed. In general, studying the diseases, or lack thereof, associated with genetically determined complement protein variants has provided insight into the pathophysiological roles of complement pathway components. Complement protein deficiencies also exemplify the potential risks of therapeutic complement inhibition.

a. Genetic polymorphisms influence complement levels

Complement gene polymorphisms are common. They are important because many polymorphisms influence the relative abundance and balance of complement regulators and activators. Common polymorphsisms in alternative pathway components associate with and alter disease risk. For example, a common polymorphism in C3 referred to as C3S/F (for Slow/Fast) was shown to influence alternative pathway activity *in vitro* and is linked with age-related macular degeneration (AMD)[38]. Understanding the influence of genetic variants is complicated by the linkages of many complement polymorphisms to other disorders. To describe this, the mechanisms underlying many genetic associations have been investigated. A complotype has been described that consideres combinations of risk and protective alternative pathway variants in C3, FH and fB. The complotype quantified how polymorphism combinations set plasma levels of alternative pathway activity, and thereby influenced complement-dependent disease risk[38]. This analysis demonstrated how complement polymorphisms that each have modest functional effects in isolation significantly affect marked differences in complement activity in combination. The protective set of FH, C3 and fB polymorphisms (FH_{62I}, $C3_{102R}$, and fB_{32Q}) demonstratred six fold less alternative complement pathway haemolytic activity than the risk set of polymorphisms (FH_{62V}, $C3_{102G}$, and fB_{32R}). In such scenarios, cohorts with the same genetic variant may require

bespoke analysis. AMD and MBL deficiency exemplify the importance of polymorphic variants to complement pathways in patients and healthy individuals.

1. Age-related macular degeneration

Age-related macular degeneration exemplifies the influence of complement genetic polymorphisms on the balance of complement regulation and activation and disease susceptibility. AMD is a heritable and progressive retinal disease that leads to loss of central vision. AMD is a common cause of blindness in older individuals. The early stage of the disease, known as age-related maculopathy, is characterised by the formation of drusen. Drusen are small yellow deposits, external to the outer retina between the retinal pigment epithelium and Bruch's membrane[39]. AMD is associated with polymorphisms in the *CFH* family of genes and FH and FHR protein variants. Large genetic association studies have identified multiple single-nucleotide polymorphisms (SNPs) associated with AMD disease risk, many of which are located within the regulation of complement activation locus on chromosome 1[39]. For example, a polymorphism in the *CFH* gene affecting amino acid 402 in SCR7, known as Y402H, is major risk factor for AMD and has been confirmed in several cohort studies[40, 41]; the aforementioned C3 polymorphism, C3F/S, associated with an odds ratio of 2.6 for AMD when $C3_{R102G}$ is found in homozygosity[42]; and a polymorphism in fB (fB_{R32Q}) had a significant effect on AMD risk, especially when found in combination with the C3 and FH at risk polymorphisms[38]. A genome-wide association study of more than 33,000 patients and controls identified very rare coding variants in the genes for FH and fI, suggesting a causal role for these variants in AMD pathogenesis[43].

2. MBL deficiency

Low MBL levels were first demonstrated in children suffering from unexplained sensitivity to infections and defective opsonising activity[44]. It has since been demonstrated that low MBL levels cause defective antigen opsonisation and phagocytosis and are associated with increased risk of infection in young children and immunosuppressed adults, such as those receiving chemotherapy or solid organ transplant recipients[45]. However, most MBL-deficient adults appear healthy and a wide range of serum MBL levels are seen in healthy individuals[46]. Also, MBL deficiency, as defined as a protein level less than 100ng/ml in plasma, is surprisingly

common; about 5% of people of European decent, and 10% of Sub-Saharan Africans are deficient in MBL. Low MBL levels are influenced by homozygosity of one of three common SNPs in the coding region of the MBL gene (*MBL2*), termed alleles B, C and D. The variants are found with markedly different frequencies in different ethnic groups. These alleles have a dominant effect over the wild type allele A. For example, almost all individuals homozygous for allele A, and without alleles B, C or D have a serum MBL level above 1µg/ml; heterozygous individuals with allele A on one gene and B, C or D on the other mostly have MBL levels between 0.5 and 1 µg/ml; individuals with a polymorphic variant on both genes have MBL levels below 50ng/ml[47]. The polymorphism with the largest effect on low MBL concentrations in Caucasians is allele B and is located at codon 54 of *MBL2* exon 1 [48]. Allele B has a gene frequency of about 13% in Caucasians. Additionally, polymorphisms within the promoter region of the *MBL2* gene influence MBL levels[49]. These promoter polymorphisms are in linkage disequilibrium, resulting in three promoter region haplotypes, each associated with different MBL levels[49]. The number of haplotypes that determine MBL levels are increased further by polymorphisms in the 5'-untranslated region of the *MBL2* gene[50, 51]. In summary, although MBL deficiency is associated with disease in some cohorts, MBL concentrations are influenced by a number of genetic variants and are distributed across a wide range in healthy individuals.

b. Genetic mutations to classical complement pathway components

Homozygous deficiency of complement classical pathway proteins is, with the exception of C2, extremely rare. In addition to recurrent encapsulated bacterial infections, classical pathway protein deficiency is associated with increased susceptibility to a SLE-like illness[52] characterised by rash, glomerulonephritis and a low incidence of anti-DNA antibodies[53]. Although rare, SLE cases associated with complement deficiency provide insight into SLE pathogenesis. They demonstrate classical complement pathway activity protects against SLE development. This seems counterintuitive to the paradigm that SLE activity correlates with the classical complement activation that follows immune complex formation. However a number of potential mechanisms could link complement deficiency and SLE development; complement deficiency may disable apoptotic cell and inflammatory material clearance[54], and contribute to ineffective clearance of immune complexes[55] and failure of negative autoreactive B cell selection[56].

Deficiencies of complement regulators are also associated with disease. Deficiency of the key classical pathway inhibitors, C1 inhibitor (C1INH) and C4-binding protein (C4bp) are associated with recurrent episodic angioedema[57] and a disease with similar phenotype to Behcet's syndrome[58], respectively. The angioedema caused by C1INH deficiency may be hereditary or acquired and, interestingly, is a reflection of the additional roles of C1INH in the contact system where it inhibits factor XIIa and kallikrein. Lack of inhibition of the contact system in the setting of C1INH deficiency, leads to inappropriate bradykinin generation, vascular permeability, and angioedema[57].

c. Genetic mutations to lectin complement pathway components

As described above, MBL deficiency is common and the result of polymorphic variants. Conventional genetic deficiencies of lectin components are rare. A patient with immunodeficiency, autoimmune diseases and non-functional MASP-2 has been identified[59]. Homozygous missense mutations in MASP-3, and the genes that encode CL-L1 and CL-K1 (*COLEC10* and *COLEC11* respectively), lead to the 3MC syndrome[60], manifesting with a spectrum of developmental features and congenital abnormalities. H-ficolin deficiency has been described in association with nectrotizing enterocolitis in neonates[61] and one adult patient with mixed symptoms of immunodeficiency[62].

d. Genetic mutations to alternative complement pathway components

Factor B, fD and properdin deficiency are rare and associated with meningococcal infection, often complicated with septicaemia[63-66]. These cases emphasise the importance of the alternative pathway in defence against meningococci. Homozygous C3 deficiency is also rare and associated with recurrent and life-threatening infections by encapsulated bacteria[53]. Childhood recurrent otitis media, meningitis and pneumonia are characteristic. This again provides insight into complement pathophysiology; it demonstrates the importance of C3 as an opsonin for bacteria, particularly in childhood before protective antibodies and memory immune responses have developed[52]. There is also a low but significant incidence of SLE[53] and membranoproliferative glomerulonephritis (MPGN)[67] in C3-deficient patients, and a single case of discoid lupus erythematosus in a properdin-deficient individual has been reported[66].

Deficiency of either of the alternative complement pathway regulators, FH and fI, lead to uncontrolled complement activation and consumption of circulating intact C3[33]. Deficiency of fI can be differentiated from FH by the presence of circulating C3b and reduced but not absent FH. These findings are explained by fI being essential for the cleavage of C3b to iC3b, and some of the excess C3b binding to and reducing circulating FH levels[68]. Increased alternative pathway activation secondary to deficient or impaired alternative pathway regulation is associated with C3G and atypical haemolytic uraemic syndrome (aHUS). C3G will be discussed in a separate section.

1. Atypical haemolytic uraemic syndrome

Atypical haemolytic uraemic syndrome refers to the triad of microangiopathic haemolytic anaemia, thrombocytopenia, and renal thrombotic microangiopathy in the absence of Shigatoxin-producing Escherichia coli, Streptococcus pneumoniae infection or ADAMTS13 deficiency thrombotic thrombocytopenic purpura (TTP). aHUS is associated with a number of complement protein variants, both inherited and acquired. Characterisation of these variants has provided insight into aHUS pathogenesis; all the variants impair regulation of the alternative complement pathway on surfaces, with subsequent renal endothelium C3 and C5 activation. The complement variants include loss-of-function mutations in the regulators FH[69-73], fI[74], and CD46[75, 76], and gain-of-function mutations of fB[77] and C3[78]. FH mutations associated with aHUS preferentially affect FH surface recognition domains. This results in impaired targeting and binding to the renal endothelium by FH, but does not affect its ability to regulate complement in circulation. Consequently, plasma C3 levels are frequently normal in patients with CFH mutations associated with aHUS.

Acquired variants that cause complement dysregulation and aHUS include autoantibodies against FH[79] and, rarely, anti-fI autoantibodies[80]. Anti-FH autoantibodies are seen in about 9% of aHUS cases and most commonly develop among individuals with the homozygous deletion polymorphism of *CFHR1*[81]. Using recombinant FH fragments, the binding sites of FH autoantibodies from five unrelated aHUS patients has been shown to recognise the FH surface-recognition domains at FH SCR19 and SCR20[82]. Functionally, these autoantibodies achieve the same outcome as loss-of-function mutations of the FH surface recognition domains. The development of these autoantibodies is explained by the surface recognition domains of FHR1 sharing high similarity with the FH surface recognition domains. The anti-FH antibodies are likely to develop as

anti-FHR1 antibodies that then cross-react with FH[83], although why these arise *in vivo* remains to be determined.

The relationship between complement mutations and aHUS phenotype is complex. Multiple genetic factors, which include additional mutations and polymorphic variants, may be required for the syndrome to develop[84]. Environmental factors such as intercurrent infection, drugs, pregnancy, and kidney donation are also important precipitants[85-87].

e. Genetic mutations to terminal complement pathway components

Deficiencies in terminal complement pathway components have been identified in patients with recurrent and invasive *Neisseria* infections[88]. Interestingly, however, terminal pathway deficiencies may remain undetected and C9 deficiency is relatively common in Japan, where it is found at a prevalence of approximately 1 in 1000 and does not associate with disease[89].

The absence on erythrocyte surfaces of CD59 leads to complement-mediated red cell lysis in PNH. PNH is caused by a clonal disorder of red cells that do not express glycosyl phosphatidylinositol (GPI) molecules and therefore lack all GPI-linked membrane proteins, including CD59 and CD55. CD59 is the key regulator of the terminal complement pathway. The absence of surface-bound complement regulators renders eyrthrocytes susceptible to complement-mediated autolysis, leading to haemolytic anaemia. Interestingly, the single reported case of systemic CD59 deficiency also developed PNH, while haemolytic anaemia has not been documented in individuals deficient in CD55 a GPI-linked surface-bound complement regulator of the classical, lectin and alternative, but not terminal pathways[90].

Summary

The complement system is essential to a wide range of homeostatic processes including immune defence, the processing of apoptotic cells and immune complexes, development and metabolism. It is both potent and delicately balanced between activation and regulation. Effective and appropriate complement activity depends on protein structure and and abundance. Complement protein deficiencies may be tolerated in health and become apparent only with additional triggers, such as pathogen invasion. Furthermore, the same protein may

have a range of roles dependent on, for example C- or N-terminus interaction, the surface to which it is bound or whether the protein is in complex with activating proteins and zymogens. Consequently, depending on the specific effect on protein structure and concentration, variants to the same genes and proteins can lead to a range of diseases and severities.

B. Factor H related proteins

The FHR proteins are part of the FH protein family and can influence the balance of complement activation and regulation. The primary role of the FHR proteins and their influence on pathogen defence and autoimmune disease is the subject of ongoing research and yet to be conclusively determined. My research aimed to contribute to our understanding of the role of the FHR proteins in kidney diseases. Unlike FH, a potent negative regulator of complement C3 activation, the FHRs appear to promote C3 activation. Physiologically they are likely to direct and target complement activation to specific surfaces and thereby facilitate accurate and rapid complement response to potential injury, while selectively avoiding host damage. However, they can also drive complement deregulation leading to cell damage and disease. I will outline the discovery, structures and functions of the FHR proteins. I will then describe example diseases caused by imbalanced FHR activity and complement deregulation.

Discovery

The initial characterisation of the FHR proteins derived from molecular cloning experiments in the late 1980s[91]. cDNA clones for FH extracted from human liver were noted to include a clone for a fragment of FH. This fragment encoded most of the FH N-terminal and C-terminal but not the region in between. When this FH fragment cDNA clone was used as an mRNA probe, multiple FH-specific mRNAs were identified[92]; the corresponding proteins have since been described as the FHR proteins. There are five FHR proteins: FHR1, FHR2, FHR3, FHR4 and FHR5. They are produced primarily in the liver and secreted in plasma, mostly in glycosylated forms[93]. The FHR genes are located on human chromosome 1q32 within the regulator of complement activation (RCA) gene cluster [94]. The genes are located downstream of the *CFH* gene in the order *CFHR3, CFHR1, CFHR4, CFHR2, and CFHR5* [70, 95]. The region contains several large regions of sequence similarity and is susceptible to genomic duplication and deletion events [34]. For example, deletion of a region containing CFHR3 and CFHR1 (del*CFHR3-1*) results in alleles containing only the *CFHR4, CFHR2, and CFHR5* genes and is a common polymorphism [96].

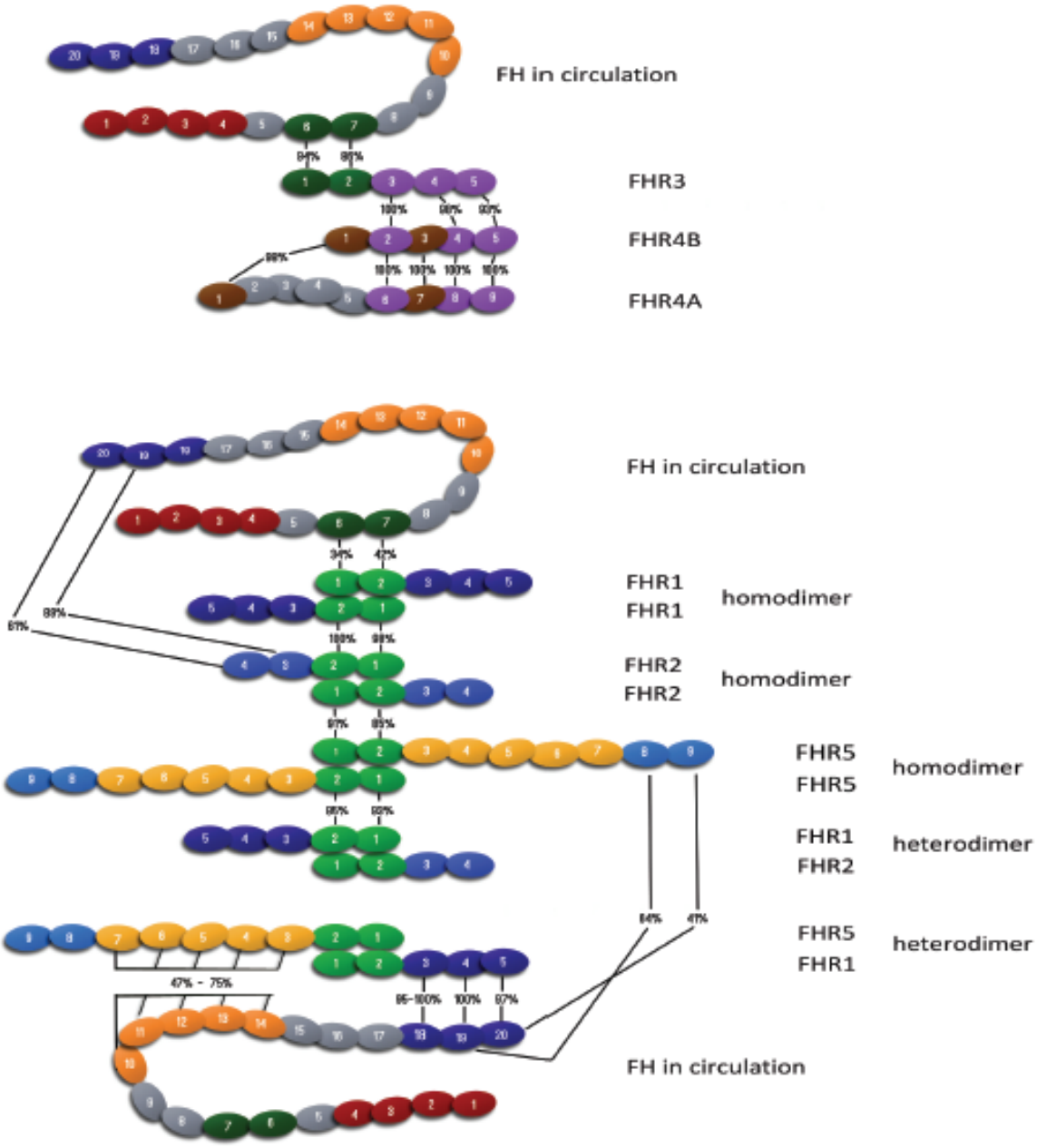
Structural homology to factor H

The FHR proteins, like FH, are composed of SCR domains. The C-terminal regions of FH and the FHR proteins show high levels of sequence identity (Figure 4). For example, the FHR1 C-terminal SCR domains, SCR3-5, differ from the equivalent FH C-terminal domains, SCR18-20, by only two amino acids in FHR1 SCR5 and FH SCR 20; L290 is substituted for S1191 and A296 for V1197[97]. The C-terminal of FH is key for surface-binding[98] and facilitates cell-surface glycosaminoglycan, heparin, complement C3b[99] and *in vitro* C3dg binding[100]. The FH and FHR C-terminal sequence similarities suggest FHR proteins bind similar surface ligands to FH. Consistent with this, albeit with a lower affinity than FH, FHR1 binds to C3b, an interaction that depends on SCR3-5[101]. Recombinant FHR1 also binds plasma incubated HUVEC cells, epithelial cells and C3b-treated rabbit erythrocytes[101]. The sequence uniformity of the C-termini has an additional implication on investigating the FH protein family; antibodies that react with the FH C-terminal are also likely to detect at least some of the FHR proteins.

The regions between FH SCR6 to SCR8 and FHR3 SCR1 to SCR3, and FH SCR6, SCR8 and SCR9 with FHR4 SCR1 to SCR3[97] are other areas of high amino acid similarity. Specifically, the amino acid identities of FHR3 SCR1 are 91% similar to FH SCR6, FHR3 SCR2 shows 85% identity with FH SCR7, and FHR3 SCR3 shows 62% amino acid similarity with FH SCR8. FHR4 SCR1 shares 71% amino acid identify with FH SCR6, FHR4 SCR2 shares 62% identity with FH SCR8 and FHR4 SCR3 shows 68% amino acid identity with FH SCR9 [100]. This region of FH SCR6 to SCR9 binds heparin and modified/monomeric C-reactive protein. The lack of complement regulatory domains similar to FH (FH SCR1-4) in any of the five FHRs indicates the FHRs do not possess direct complement regulatory activity. As will be discussed, this observation has been supported by experimental data.

Figure 4. Factor H and the factor H related (FHR) proteins

Short consensus repeats (SCRs) of FH and FHR1 to FHR5 are represented by ovals and are numbered from the N-terminal end. Colour similarity illustrates SCRs of FH and different FHRs that exhibit relative amino acid sequence homology. Black numbers with lines linking two SCRs from different proteins show the percentages of amino acid similarity. This figure demonstrates similarities between the following SCRs; SCRs 1 and 2 of FHR3 and SCRs 6 and 7 of FH; SCRs 3,4 and 5 of FHR3 and SCRs in FHR4A and FHR4b; SCR 1 and 2 of FHR1, FHR2, and FHR5 with SCRs 6 and 7 of FH; SCRs 3-7 of FHR5 and FH SCRs 10-14; FHR5 SCR 8 and 9 and FH SCRs 19 and 20; and SCRs 3,4 and 5 of FHR1 with FH SCRS 18, 19 and 20. FHR1, FHR2 and FHR5 are found exclusively as dimers in the circulation. In addition to FHR1, FHR2 and FHR5 homodimers, FHR1:FHR2 heterodimers have been identified. As depicted in the figure, structural data suggests SCR1 and SCR2 from these proteins form dimers in a head-to-tail orientation.



a. Factor H related protein 1

Factor H related protein 1 was identified in 1991 when human plasma was analysed by immunoblotting with a goat antiserum against human FH[102]. In addition to the FH signal at 150kDa, two antigens corresponding to molecules of 42 and 37kDa (termed Factor H-related Ag h42 and h37) were identified [102]. Enzymatic deglycosylation transformed h42 and h37 to molecules of the same 33kDa mass, suggesting h42 and h37 are glycosylation isoforms of the same protein, FHR1. Unlike FH, the newly described protein neither accelerated the dissociation of C3bBb nor demonstrated factor I cofactor activity [103].

FHR1 consists of five SCR domains with variable similarity to FH (Figure 4). As mentioned above, FHR1 SCR3-5 have high amino acid sequence similarity to the FH C-terminal domains SCR18-20, with subsequent functional consequences as FHR1 shares some surface-binding characteristics with FH[97]. FHR1 exists as two glycosylated forms in human plasma [104]; FHR1α displays one carbohydrate side chain; FHR1β has two [101]. Two allelic variants of FHR1 have been identified, FHR1A and FHR1B. FHR1A (the acidic isoform) expresses the amino acids HLE in SCR3. FHR1B (the basic isoform) expresses YVQ in SCR3 [104, 105]. These differences distinguish exon 4 of *CFHR1* from exon 21 of *CFH*, suggesting the FHR1B allele originated from a gene conversion event between *CFHR1* and *CFH* [104, 105]. Interestingly, the FHR1B allele (*CFHR1*B*) frequency is significantly increased in cohorts of patients with aHUS suggesting this allotype has increased ability to interfere with the function of FH to protect cellular surfaces from complement damage. This is an example of how minor amino acid changes in complement proteins can lead to important increases in disease risk.

Deletion of the *CFHR1* and *CFHR3* genes is a common polymorphism. The del*CFHR3-1* allele frequency varies with ethnicity. The highest frequency of the deleted allele is seen within African populations. In sub-Saharan Africa the frequency is approximately 54.7% whilst in European Caucasoid populations the frequency is approximately 15-25% [96]. The frequency is lowest in populations from East Asia and South America and almost absent in Japanese populations. This equates to del*CFHR3-1* homozygosity frequencies of around 30% in Nigeria, 3-10% in Europe, and almost 0% in East Asia and South America[96]. The FHR1 plasma concentration has been measured at between 70-100μg/mL[101], equating to 1.7-2.5μM[106], although there is significant population variation. Besides the presence of del*CFHR3-1* alleles, factors that may influence its circulating concentration include its ability to form heterodimers with FHR2 and to interact with high density lipoprotein particles[107, 108].

Comparing the molarity of FH and FHR1 is complicated by the wide range of FHR1 and FH in plasma concentrations. The concentration of FH ranges from 110 to 615µg/mL, equating to 0.7-3.6µM[34]. Therefore, the serum concentrations of FH and FHR1 may be equimolar in many individuals[8]. Other groups, however, have estimated the molar ratio of the FHR1 dimer to FH at approximately 0.3:1[108].

The two FHR1 N-terminal SCR domains (SCR1-2) show remarkable sequence identity with the corresponding two N-terminal SCR domains of both FHR2 and FHR5. These domains have been shown to mediate obligate 'head to tail' dimerisation[8]. As discussed below, dimerisation has implications for ligand avidity and implies that heterodimers could exist. To date, FHR1, FHR2 and FHR5 homodimers have been demonstrated from plasma[8, 9]. Due to the inability of FHR2 to bind heparin in the absence of FHR1, the FHR1-FHR2 heterodimer has been readily identified in human plasma using heparin chromatography. This has been replicated with enzyme linked immune-sorbent assay (ELISA) techniques using recombinant and serum-derived proteins[109]. Although FHR5 would be expected to from heterodimers with FHR1 and FHR2, these have not been consistently identified and it seems FHR5 circulates exclusively as homodimers[8, 9, 109].

b. Factor H related protein 5

Factor H related protein 5 is composed of nine SCR domains [108]. FHR5 is glycosylated and has a molecular mass of 62kDa. Through a dimerization domain in the two N-terminal domains, it exists as an obligate homodimer[8, 109, 110]. FHR5 plasma concentrations are approximately 5ug/mL, which equates to 0.05-0.09µM[111]. The molar concentration of FH far exceeds that of FHR5 and it seems unlikely FHR5 directly interferes with FH function in circulation. However, FHR5 dimers show a stronger binding affinity for heparin than FH at physiological salt concentrations[109]. FHR5 may interfere with FH binding to polyanionic residues on cellular surfaces, which are essential to localise FH-dependent regulation to areas of complement activity[109]. Furthermore, the relative concentrations of FH and FHR5 in localised compartments may be different to circulation. Therefore, despite marked differences in circulating concentrations, FHR5 may interfere with FH functions in localised compartments. It has been detected in complement-containing kidney deposits[112]. In fact, FHR5 was first detected using an antibody raised by immunizing mice with glomerular basement membrane preparation from glomerulonephritic human kidneys. In a prospective study of 100 consecutive patient kidney biopsies stained

with anti-FHR5 monoclonal antibody, FHR5 glomerular staining distribution appeared similar to C3 and C5b9 staining[111].

Factor H related proteins: Functions

Defining the biological functions of the FHRs has been elusive. The presence of the del*CFHR3-1* allele among healthy individuals indicated that both FHR1 and FHR3 are biologically non-essential. Likewise, the presence of a del*CFHR4-1* allele in healthy individuals, although much less common than the del*CFHR3-1* allele, indicated CFHR4 also lacks an essential biological role[105]. This assumption has been questioned.

Furthermore, the structural and sequence similarity of FHR and FH domains led to debate about whether the FHRs could replicate or interfere with FH functions[113]. Because none of the FHR proteins contain domains analogous to the complement regulation domains of FH (SCR1-4), the FHR proteins were not expected to replicate FH regulatory functions. Consistent with this, FH regulatory activity was not demonstrated for FHR1 and FHR2[102]. FHR1 and FHR2 binding to heparin or components of lipoprotein complexes was repeatedly demonstrated, although the relevance of this was unclear [107]. FHR1, FHR2 and FHR5 circulate exclusively as dimers, and their dimerization increases avidity for C3b and heparin[109]. The obligate nature of the dimerization indicates this is important for biological function and recent evidence suggest the FHR proteins augment FH dependent regulation by interfering with FH binding to C3b and surfaces. However, in order to provide a balanced literature review, I will first summarise data showing complement regulatory roles for the FHRs, the majority of which is derived from *in vitro* assays. I will next review data demonstrating a role for FHRs in promoting complement activation.

a. Evidence of FHR complement regulatory activity

Using a sheep erythrocyte lysis assay, FHR1 reduced C5 deposition and the generation of C5a, but not C3a, suggesting FHR1 has an inhibitory effect on the C5 convertase[101]. FHR1 was shown to bind C5 and the activation product C5b6, and inhibited haemolysis in a C5b9-dependent erythrocyte lysis model[101]. However, further studies have not replicated these findings[8].

FHR2 has been reported to inhibit the C3 convertase and terminal complement complex assembly *in vitro*[114]. Using human sera, FHR2 inhibited the production of C3a from C3 in a dose-dependent manner, reduced terminal complement complex surface deposition on sheep erythrocytes[114], and inhibited in vitro C3 convertase assembly but did not demonstrate factor I cofactor activity[114].

Using surface plasmon resonance techniques, recombinant FHR3 and FHR4 were shown to bind C3b and, in the presence of factor H, promote cleavage of the C3b alpha chain [115]. This suggested FHR3 and FHR4 have factor I cofactor activity. However, in the absence of factor H, FHR3 and FHR4 factor I cofactor was very low and only detectable at non-physiological FHR concentrations [115]. When added to FH-depleted, complement active plasma, FHR3 reduced complement-mediated haemolysis of sheep erythrocytes by approximately 15%. This compared with approximate 80% inhibition by FH [41]. At non-physiologically high concentrations, FHR3 also reduced production of C5a and C5b from C5 in an *in vitro* C5 convertase model.

Recombinant FHR5 binds C3b in a dose-dependent and saturable manner [116]. The addition of FHR5 to C3b and fI led to C3b cleavage, suggesting that FHR5 has fI cofactor activity [111]. Furthermore, using a C3 convertase assay, FHR5 appeared to inhibit C3 activation [111]. However, 10µg of FHR5 was needed to achieve a similar level of fI cofactor activity as 50ng of FH, and 5µg of FHR5 had a similar C3 convertase inhibition effect as 1µg of FH. The dependence on high FHR5 concentrations makes the biological relevance of these *in vitro* observations questionable. This, in addition to subsequent data described below, suggests FHR5 and the FHR proteins in general have no complement regulatory effects and actually accelerate complement activity.

b. Evidence of FHR proteins accelerating complement activation

There has been substantial evidence that the FHR proteins increase complement activation at physiological concentrations. The result is a reduction in the ability of FH to negatively regulate surface C3b activation. This process has been termed FH deregulation[8, 9]. I will first discuss experiments that demonstrate C3 convertase formation and complement activation by the FHR proteins. I will then describe data showing FHR proteins interact with FH binding and subsequently associate with complement deregulation and activation.

1. FHR protein and C3 convertase formation

As opposed to regulating C3b activation, investigations of FHR4 binding to C3b suggested that the FHR4-C3b interaction actually promoted complement activation. FHR4B immobilized on microtitre plates bound C3b and C3dg[115], while FHR4A was also able to bind inactivated C3b (iC3b) but not C3c. FHR4A binding to fluid-phase and plate-immobilized C3b was stronger than FHR4B and comparable to FH[117]. Analysis of recombinant FHR4A mutants showed the C3b and C3dg binding site was localized within the SCR8 and SCR9 domains. Importantly, no significant factor I cofactor or C3 convertase decay accelerating activity could be demonstrated. Addition of Mg-EGTA buffered human serum, which restricts complement activation to the alternative pathway, to FHR4A and FHR4B led to C3 activation and the deposition of C3b fragments. C3 activation was dependent on the FHR4A SCR8-9 domains and associated with factor B and properdin deposition [118]. This demonstrated that FHR4-C3b complex could promote C3 convertase formation and consequently alternative pathway activation.

Recently, FHR5 has been shown to directly promote C3 convertase formation. When incubated with C3b, purified factor B, factor D and properdin, C3 convertases can form on surface-immobilised FHR5 and function to produce C3a and C3b[119]. Direct alternative complement pathway activation can be replicated in the fluid phase; the addition of normal human serum to FHR5 immobilised on microtitre plates leads to C3, factor B and properdin deposition[119].

These data question what are the ligands for FHR4 and FHR5 in vivo that promote local complement activation? FHR4 interacts with C-reactive protein. Using recombinantly expressed FHR4 fragments, binding was localized to the SCR1 domain of FHR4. Sequence comparisons with FH and the other FHR proteins showed this site to be unique to FHR4[117]. FHR4 binds preferentially to the native pentameric form of CRP, while FH predominantly binds to the monomeric form. FHR4-bound CRP leads to enhanced opsonisation of necrotic cells by CRP[120]. Furthermore, as demonstrated by C3 fragment deposition, the bound FHR4 was able to activate complement via C2 and C4-dependent pathways[117], independent of FH. The ability for FHR4 to allow C3 convertase formation on bound C3b may contribute to enhanced damaged cell and pathogen opsonisation.

Interestingly, although FHR5 appears to interact with pentraxin 3 (PTX3), the subsequent complement activation seems to involve FH deregulation. Pentraxins are a family of pattern recognition molecules of the innate immune system which include CRP. Under inflammatory conditions, PTX3 is produced locally by neutrophils, macrophages, dendritic cells, fibroblasts and endothelial cells and can recruit, initiate and control

complement regulators and activation. FHR5 binds PTX3-coated wells in a dose-dependent manner, and is able to do this at lower concentrations than FH[119]. Recombinant FHR5 and serum-derived FHR5 are able to interfere with the binding of FH to PTX3 in an extracellular matrix model[119]. The interaction of FH and PTX3 is potentially important in down-regulating PTX3-induced complement activation. These studies describe a potential functional interaction at physiological concentrations of FHR5 with pro-inflammatory molecules and the extra cellular matrix (ECM). They also provide a mechanism for FHR5-driven local complement deregulation by competition with FH for C3b and other physiological ligands. FHR5 also binds laminin-521 and laminin-211, major constituents of glomerular basement membrane and mesangial compartments, and malondialdehyde acetaldehyde (MAA) epitopes on necrotic human cells[121]. The binding of FHR5 to surface expressed non-complement ligands, including laminin-521, MAA epitopes and heparin occurs via SCR5-SCR7, which are distinct from the C-terminal domains involved in C3b binding. FHR5 is able to complete with FH for binding to the MAA epitopes, thus forming a platform for complement activation. This shows how FHR5 can compete with FH to trigger complement activation on specific surface bound epitopes[121].

2. FHR proteins and FH deregulation

Factor H-dependent C3 convertase decay is reduced in the presence of FHR3 and FHR1 *in vitro*[41]. This observation led to the theory that FHR proteins interfere with FH-mediated regulation of C3b activation. Furthermore, the addition of exogenous FHR3 and FHR1 to complement-dependent haemolytic assays significantly increased complement-dependent haemolysis[41]. This FH deregulation was confirmed in subsequent experiments using FHR1 and FHR5. FHR5, like the other FH protein family members, recognises and binds to C3b on surfaces[111]. FHR5[8] and FHR1[9] have been shown to compete with FH for binding to surface C3b. In erythrocyte lysis assays using guinea-pig erythrocytes, both FHR5[8] and FHR1[9] promote the lysis of cells in a FH-dependent manner.

The ability of FHR1, FHR2 and FHR5 to form dimers[8] and higher order complexes[9] results in increased ligand avidity[8]. This phenomenon is predicted to potentiate FH deregulation and is supported by *in vivo* observations that abnormal FHR proteins, in which there is duplication of the dimerization domains, are associated with C3G, a condition characterised by abnormal C3 deposition within the kidney[5-7, 9, 122]. *In vitro* evidence that dimerization enhances FH deregulation includes the superior ability of FHR1 dimers, compared to monomers,

to inhibit the binding of FH to surface C3b [8] and to interact with glomerular bound C3 in a FH-deficient mouse model [8]. These data suggest mechanisms by which dimeric FHR1 and FHR5 could compete with FH binding for C3b, interfere with alternative pathway regulation, and amplify C3 activation. The ability to interfere with C3b regulation may be particularly important to autoimmunity because C3b is continuously produced from continuous alternative pathway tick-over and complement amplification regardless of the triggering pathway.

FHR protein associated diseases

a. Interactions with pathogens

FHR protein binding to several microbes and microbial proteins has been demonstrated[123], including Neisseria meningitidis[124, 125], Streptococcus pyogenes[126], Borrelia burgdorfei[127], Leptospira interrogans[128], Pseudomonas aeruginosa[129] and Candida albicans[130]. However, few studies have described the functional mechanism and effect of these interactions. Where there is both a FHR-pathogen and FH-pathogen interaction, the FHR interaction may, by promoting complement activation, facilitate complement-mediated pathogen damage [110]. The FHR-pathogen interaction may also influence immune cell recruitment, as FHR1, has been shown to bind complement receptor type 3 on neutrophils [123].

Neisseria meningitidis is an important human pathogen, causing septicaemia and meningitis. As evidenced by the susceptibility of individuals with alternative or terminal complement pathway deficiencies to Neisseria meningitidis, complement activation is critical for preventing infections[124]. In a recent genome-wide association study (GWAS), all SNPs associated with meningococcal disease were located within the *CFH-CFHR* locus. Specifically, three SNPs in *CFH* and three in *CFHR3* showed repeated independent association with disease susceptibility in an original UK based study population and two European replication populations[124]. Neisseria meningitidis binds FH with high affinity via FH-binding protein (FHbp), a surface lipoprotein with mimicry to host cell carbohydrates[124]. FHbp binds FH SCR6 and SCR7 domains. These are very similar to FHR3 SCR1 and SCR2 (Figure 2). Using full length FHR3, mutant FHR3 proteins lacking specific SCR domains and serum from FHR3-deficient individuals, FHR3 interactions with FHbp were demonstrated and were dependent on SCR domains 1-2[125]. Furthermore, FHR3 was shown to compete with FH for both FHbp and C3b on the bacterial surface and significantly influenced Neisseria meningitidis survival in serum assays[125]. This important

study demonstrated susceptibility to an infectious disease was governed by the relative abundance of FH and FHR3 through their shared affinity for pathogen FHbp. Interestingly, Neisseria meningitidis FHbp shows variation in FH and FHR3 binding affinity which may explain the prevalence of certain strains[125]. The findings also demonstrated how genetic variants and the consequent competition between two opposing complement factors influences infectious disease susceptibility, and suggests the distribution of complement proteins, particularly the FHR proteins, should be considered in epidemiology disease mapping and immunisation strategies.

b. Age-related macular degeneration

The FHR proteins are also implicated in AMD pathogenesis. The *delCFHR3-1* is associated with a lower risk for AMD; the deletion haplotype is present in 20% of healthy controls and 8% of individuals with AMD[40]. Regression analysis confirmed the del*CFHR3-1* remains significantly associated with the risk for AMD even without the effect of the Y402H FH polymorphism[41]. The pathogenic role of FHR-dependent complement deregulation in AMD is further demonstrated by the composition of the drusen that characterise the condition. The drusen contain complement activation products and regulators that are remarkably similar to the FHR-containing deposits found in C3G.

c. Atypical haemolytic uraemic syndrome

As discussed above, aHUS is associated with mutations or variants of the complement system, commonly affecting FH[85]. Four *CFHR* gene rearrangements are also associated with aHUS. Three of these rearrangements result in the generation of hybrid genes between *CFH* and *CFHR1* or *CFH* and *CFHR3*, and affect the FH C-terminal SCR20[131-134]. The *CFH:CFHR1* hybrid gene involves the substitution of the C-terminal SCR 20 of FH with the C-terminal SCR of FHR1. The *CFH:CFHR3* hybrid gene substitutes FH SCR20 with the whole FHR3. The *CFHR1:CFH* hybrid gene leads to the substitution of the two C-terminal SCRs of FHR1 with those of FH[131-134]. Despite the aforementioned close sequence homology between FH SCR20 and the C-terminal FHR1 SCR5, when substituted into the FH C-terminal the FHR1 SCR domain renders FH ineffective in binding to endothelium and complement regulation. This demonstrates FHR1 lacks appropriate activity to substitute FH

C-terminal dependent protection of host surfaces from complement-driven injury. It is also a striking example that small changes in amino acid sequences result in remarkable differences in molecular binding and surface interaction[134].

The fourth *CFHR* gene rearrangement associated with aHUS is with the common del*CFHR3-1* polymorphism or the less common deletion of genes *CFHR1-CFHR4* (del*CFHR1-4*). As discussed above, although these deletion polymorphisms are associated with protection from other conditions, they are also associated with and may contribute to the presence of anti-FH autoantibodies[105]. The autoantibodies mimic C-terminal FH mutations by binding the C-terminus of FH, impairing FH cell surface recognition, binding and complement regulation, leading to the development of aHUS[135]. The FHR3-FHR1 deletion and associated generation of anti-FH autoantibodies is highly prevalent in haematopoietic stem cell transplantation related thrombotic microangiopathy[135]. Interestingly and potentially because of a predisposition to produce anti-FH antibodies, the polymorphism is also a risk factor for SLE[136].

Summary

Despite sharing significant sequence homology with FH, the FHR proteins lack the FH N-terminal complement regulatory domains. A common deletion polymorphism of the *CFHR3* and *CFHR1* genes, and less frequently, a combined deletion of the *CFHR1* and *CFHR4* genes, result in significantly reduced or absent levels of the respective proteins in healthy individuals. Genetic studies in both rare and common diseases indicated that these proteins have important roles in complement-associated diseases. The appreciation of two other structural characteristics has been key to understanding FHR protein functions. First, the FHR proteins are characterised by C-terminal domains that share sequence similarity with the C-terminal domains of FH. They therefore display similar, but not identical, surface binding to FH and can act as competitive antagonists. Second, FHR1, -2 and -5 share a conserved dimerization domain within their first two N-terminal domains which, through polymerisation, enhances ligand avidity. Studies of Neisseria meningitidis illustrates that both protein availability, influenced for example by the presence of del*CFHR3-1* alleles, and the surface upon which complement activation is occurring, influenced for example by the type of FHbp on the Neisserial surface, is biologically relevant to disease susceptibility. In the next section, I will describe C3G, a group of diseases caused by alternative complement pathway imbalance and deregulation. The characterisation of rare cases of

C3G caused by FHR gene mutations provides further evidence that FHR1 and FHR5 may be of particular importance to renal diseases. This is supported by the identification of glomerular FHR5 in a number of glomerulopathies characterised by complement deposition such as IgAN. Data derived from these conditions suggests that FHR1 and FHR5 proteins antagonise the ability of FH to negatively regulate surface complement C3b within the kidney.

C. C3 glomerulopathy

C3 glomerulopathy is a term used to designate a disease process due to abnormal control of complement activation, deposition, or degradation and characterized by predominant glomerular C3 fragment deposition with electron-dense deposits on electron microscopy[137]. It is strongly associated with abnormal regulation of the alternative pathway[138].

Definitions and subtypes

The first described cases of glomerulopathy associated with dominant complement activation were patients with MPGN light microscopy patterns and dense intramembranous deposits by electron microscopy[139]. MPGN describes the presence of glomerular lesions with thickened glomerular capillary walls and increased mesangial matrix size and cellularity. The cases were associated with low circulating C3 levels and dominant glomerular C3 deposition, both evidence of alternative pathway activation. However, the majority of dense deposit disease (DDD) cases do not have MPGN, so DDD is the preferred term[140]. Furthermore, cases were increasingly recognised with dominant glomerular C3 deposition and absent dense intramembranous deposits. This led to the term C3 glomerulopathy being used to describe glomerulopathies in which alternative pathway dysregulation was considered to be the major mediator of glomerular damage, as evidenced by glomerular C3 deposition without immunoglobulins and C1q[137]. Based on EM appearances, C3G can be subclassified as DDD, if dense osmiophilic intramembranous deposits are seen or C3 glomerulonephritis (C3GN) if the EM deposits are light in density, amorphous or located in other glomerular or membrane compartments[141]. There are no light microscopy inclusion or exclusion criteria. This reflects the range of light microscopic appearances that are seen in C3G which include mesangial proliferative, membranoproliferative and endocapillary proliferative patterns, with or without crescents. Rarely, glomeruli may be normal by light microscopy[141].

The original definition of C3 without any immunoglobulins was shown to be too stringent since many cases of DDD may have some immunoglobulin deposition rather than none. So the current working definition is to suspect C3G when the is a C3 dominant glomerulonephritis defined as C3c intensity 2 orders of magnitude more than any other immune reactant on a scale of 0 to 3 (including 0, trace, 1+ , 2+, 3+)[142].

We have gained insights into C3G pathophysiology through an number of cases and animal models. Overall, these findings demonstrate the range of variants, including the FHR proteins, that can imbalance complement regulation and drive disease.

Complement pathophysiology

a. Genetic causes of C3 glomerulopathy

In the majority of C3G cases, the precise pathogenesis of complement dysregulation and glomerular injury is not defined. However, in some cases, genetic and acquired complement variants have been identified. DDD is associated with homozygous CFH deficiency[143, 144], loss-of-function mutations in the regulatory domains of CFH and a gain-of-function C3 mutation[145]. The characterization of the C3 gain-of-function mutation in a family with DDD was especially informative because this mutant protein resulted in specific enhanced activation of C3 in plasma, providing convincing evidence that DDD is a disorder of fluid-phase C3 activation[145]. Similarly, abnormal C3 molecules that form C3 convertases resistant to FH inactivation, have also been described in familial non-DDD C3G[146, 147].

Whilst mechanistically revealing, pathogenic mutations are rare in patients with C3G. Overall, the presence of identifiable complement-associated genetic variants ranges from 18% to 25% of C3G patients screened[144, 148]. Also, in the majority of cases, genotype-phenotype correlations have not been established and it is difficult to determine whether a variant is likely benign or pathogenic. There seems to be little difference in the prevalence of complement associated genetic variants in C3GN and DDD cohorts. Complement-associated genetic variants were demonstrated in 11 from 56 (19.6%) individuals with C3GN from France[144] and 9 out of 42 (21.4%) patients with C3GN from North America[148]. Comparably, five of 29 patients (17.2%) with DDD from the French cohort[144] and three of nine (33.3%) DDD patients from North America[148] had complement-associated genetic variants. It is notable that DDD associated with fI deficiency has not been described. This replicates the finding that fI-deficient mice neither accumulate C3 along the GBM[149] nor develop MPGN, as will be discussed below. The French patient series also analysed 48 individuals with MPGN type 1, a different lesion from C3 glomerulopathy as it is an immune complex mediated disorder. In the MPGN type 1 cohort, eight complement-associated genetic variants were identified, five in *CFH* and three in *CFI*. In the French series,

homozygous FH deficiency was associated with both DDD, C3 glomerulonephritis, and MPGN type 1[144]. This demonstrates that the relationship between mutation and renal phenotype is influenced by other factors, including environmental triggers like infections, and polymorphic genetic variation that influence, for example, disease penetrance. In most series, associations between complement-associated genetic variants and renal outcomes have not been demonstrated[148]. However, as discussed below, genotype-phenotype correlation is seen with a mutation of the FHR5 gene; males of Cypriot enthnicity with an internal duplication of *CFHR5* exons 2 and 3 in heterozygosity develop more severe renal disease than females.

b. FHR protein associated C3G

A major development in C3 glomerulopathy research was the recognition of familial C3 glomerulonephritis associated with mutations within the *CFHR* loci. The seminal observation was the characterization of familial C3G associated with a heterozygous mutation of the *CFHR5* gene in a large Greek Cypriot cohort[5]. Patients with CFHR5 nephropathy have an internal duplication of *CFHR5* exons 2 and 3 that encode FHR5 SCR1 and SCR2 respectively. CFHR5 nephropathy is characterized by persistent microscopic haematuria, episodes of synpharyngitic macroscopic haematuria, recurrence in renal transplantation and, curiously, a more severe clinical course in males[5, 106, 150]. One clear distinction with DDD is the absence of fluid-phase dysregulation. Low plasma C3 levels, common among DDD patients, were not seen in patients with CFHR5 nephropathy, suggesting that the mutant FHR5 causes abnormal C3 regulation within the kidney. The pathogenicity of this associated mutant FHR5 protein was supported by the identification of the same mutant FHR5 protein, with duplicated SCR1 and SCR2, caused by a novel intronic genetic mutation in a British family without Cypriot ancestry[7].

A number of other cases of familial C3G have identified additional mutant FHR proteins associated with C3G[5-7, 9, 122]. These include a heterozygous mutation in a large Irish kindred whereby deletion of *CFHR3* exons 4-6 and *CHFR1* exon 1 produced a hybrid *CFHR3-1* gene and protein; SCR1 and SCR2 of FHR3 were linked to full length FHR1[6]. A Spanish family was identified with a heterozygous mutation causing duplication of FHR1 SCR1 to SCR4[9]. And a German sibship was reported with heterozygous deletion of *CFHR2* exons 4 and 5, producing a hybrid protein of FHR2 SCR1 and SCR2 linked to full-length FHR5. This hybrid is structurally similar to the abnormal FHR5 identified in the aforementioned Cypriot and British families[122].

The pathogenic FHR proteins are mostly characterised by duplication of their dimerization domains. The abnormal dimerization domains likely increase C3 avidity. This results in enhanced FH deregulation within glomeruli, driving abnormal C3 accumulation. Data supporting this derives from the detailed study of a mutant FHR1 associated with familial C3 glomerulopathy in which the mutant FHR formed higher-order oligomers and multimeric complexes[9]. The mutant FHR1 contained a duplication of the N-terminal SCR1 and SCR2; the location of the dimerization motif. The mutant protein bound to C3b, iC3b and C3dg immobilised on a Biacore chip with greater affinity than native FHR1. Compared to assays of non-mutant FHR1 mentioned earlier, the mutant FHR1 showed enhanced competition with FH in complement-dependent haemolysis assays of guinea pig erythrocytes[9]. A comparable assay assessed the complement-activating ability of FHR1, FHR2 and FHR5 from healthy individuals and from patients with familial C3G and either mutant CFHR5[5], or a FHR3-1 hybrid protein[6,8]. In this analysis, both mutant FHR proteins were characterised by duplication of their respective dimerization motifs. When added to a FH-dependent complement haemolysis assay, the serum-derived preparations from patients with mutant FHR proteins showed significantly greater haemolysis and complement deregulation than healthy controls[8]. If the mutant protein had the same effect *in vivo*, it may explain the clinical findings, although the potential influence of the mutation on non-quantified factors that may augment this mechanism, such as expression levels and molecule clearance, must also be considered.

An important observation that demonstrates our incomplete understanding of FHR-driven complement deregulation is the effect of a hybrid protein with SCR1 and SCR2 from FHR2 added to a full length CFHR5 protein. Although structurally similar to a mutant FHR5 with duplicated SCR1 and SCR2, unlike other FHR mutants it causes severe plasma C3 depletion. This effect is similar to C3 nephritic factor, suggesting this mutant FHR may have C3 convertase stabilising properties[151]. How this occurs is unclear.

c. Acquired causes of C3G

Antibodies to complement components can be detected in about 10% to 50% of C3G patients. The commonest acquired abnormality is C3 nephritic factor (C3NeF). C3Nef prevalence varies widely in different C3G cohorts[144, 148]. This may reflect, in part, the technique used for C3Nef detection. C3NeF activity was first described in patients with glomerulonephritis whose serum contained a factor that potentiated C3 activation[152]. The factor was subsequently demonstrated to be an immunoglobulin[153]. C3NeF interacts with the C3 convertase and

prevents its spontaneous decay and inactivation by regulators[154]. C3NeF (more appropriately termed C3 nephritic antibody) may result in predominant activation of C3 alone (properdin-independent C3NeF) or activation of both C3 and C5 (properdin-dependent C3NeF)[155-157]. Consequently, C3NeFs are typically but not invariably associated with reduced plasma C3 levels[144] and, for properdin-dependent C3NeF, a reduction in C5 and other terminal pathway components as well. They may be detected among individuals with partial lipodystrophy with or without renal disease[158] and even among healthy individuals[159]. As a consequence, whilst their association with enhanced plasma C3 activation seems clear, their relationship to renal disease might be an epiphenomenon[160]. C3 glomerulopathy is also associated with autoantibodies distinct from C3NeF that trigger uncontrolled C3 activation. These include inhibitory autoantibodies to the regulatory domains of CFH[161, 162] and stabilizing autoantibodies against factor B[163] and C3[164].

d. C3 glomerulopathy animal models

Animal models of complement deregulation have provided important experimental models of C3G and, specifically DDD. Norwegian Yorkshire piglets developed spontaneous and lethal mutations to the gene coding FH, resulting in no circulating FH[165]. The affected piglets were characterised by severe renal disease that resembled human DDD and was called hereditary porcine MPGN II[166]. Gene-targeted FH deficient mice, denoted *CFH-/-* mice, show alternative complement pathway dysregulation with low plasma C3 and spontaneously develop renal disease pathologically similar to DDD[167]. This model has provided a number of important insights into C3G pathogenesis. When the *CFH-/-* mice were crossed with mice deficient in fB, a key component of the alternative pathway C3 convertase, C3 consumption and renal disease were eliminated[167]; demonstrating the phenotype is dependent on alternative complement pathway activation. Furthermore, injecting the *CFH-/-* mice with purified FH rescued the phenotype; both circulating plasma C3 levels and glomerular C3 deposition improved[168, 169]. Regular infusions of porcine MPGN II piglets with plasma containing FH also reduced GN severity and improved survival[170].

Complement knockout mouse models also demonstrate the complexity of the complement system and potential pitfalls in therapeutic complement component inhibition. For example, mice with homozygous deficiency of fI (*CFI-/-)* have low circulating C3 levels but only mild renal pathology characterised by mesangial C3 accumulation and expansion, and no MPGN or glomerular basement membrane (GBM) C3 deposition[68]. It

was predicted that mice lacking both FH and fI, would have more severe disease than mice lacking just fI. However *CFH-/- .CFI-/-* mice were very similar to *CFI-/-* mice and injecting fI into *CFH-/- .CFI-/-* mice switched the renal phenotype to GBM C3 deposition that resembled *CFH-/-* mice[149]. Complement fI is essential for the cleaveage of C3b to iC3b, and it seems this fI-dependent iC3b production is critical for C3 GBM deposition.

A similarly unexpected finding was revealed when generating *CFH-/-* mice deficient in properdin, a positive regulator of the C3 convertase. It was hypothesised that properdin knockout would ameliorate the renal pathology seen in *CFH-/-* mice. However, *CFH-/-* mice also deficient in properdin had more severe MPGN and C3 GBM deposition than *CFH-/-*mice[171]. A suggested explanation for this is the absence of properdin leads to less rapid complement dysregulation and C3 cleavage. The subsequent higher preserved levels of C3 and C5 in the properdin deficient mice may cause more renal damage through the prolonged production of C3a, C5a and C5b9.

The *CFH-/-* mice have also provided insight into the potential role of C5 activation in C3G. Mice produced from crossing *CFH-/-* mice with those lacking C5 had different disease phenotype to the *CFH-/-* mice; spontaneous MPGN was observed but glomerular crescents, renal function and mortality improved[172]. These mice were also less susceptible to renal injury from injected sheep nephrotoxic serum. Interestingly, this protection was not seen in *CFH-/-* mice lacking C6 instead of C5[172]. This suggested production of the anaphylatoxin C5a, as opposed to C5b9 formation was pathogenic in the nephrotoxic nephritis model. The finding was replicated by injecting the *CFH-/-* mice with an anti-C5 monoclonal antibody before nephrotoxic serum. These data also suggested that C3G patients may benefit from C5 inhibition at times of disease flares, but that it would not be expected to improve the underlying C3 dependent glomerulopathy.

Clinical features and outcomes

C3G is rare, with an incidence of about 1 per million per year, and currently incurable. The mean age of diagnosis is between the 2rd and 5th decades, although it can occur at any age[173]. At ten years from diagnosis, 40%-50% of patients will have reached advanced CKD stage 5 (estimated glomerular filtration rate less than 10ml/min/1.73m^2) or renal failure[144, 148, 173]. Although this group of diseases is defined by pathogenic dependence on uncontrolled complement activation leading to glomerular C3 deposition, C3G patient cohorts

include a wide spectrum of clinical severity and outcomes. The most common clinical course is progressive deteriorating chronic renal disease with persistent alternative pathway complement activation[141]. Clinical presentation is frequently preceded by infectious episodes[138]. However, presentations range from asymptomatic proteinuria with preserved renal function to nephrotic syndrome and rapidly progressive glomerulonephritis[144, 148, 173]. This variation is not understood.

About two thirds of patients present with serum C3 levels below the normal range. This may be a more common finding in paediatric cohorts[148]. Low C3 levels at diagnosis have been documented more commonly in DDD in one cohort[173] but not others[148]. Reduced serum intact C3 at presentation does not associate with disease severity or clinical outcomes[148]. Whether markers of specific alternative or terminal complement pathway activity may associate with disease severity is not known.

Circulating paraproteins associate with C3G. The association is more pronounced in older patients. In a recent cohort of 111 patients from North America, monoclonal paraproteins were identified in 14 of 38 patients tested, and the median age of these patients was 53 years. In the same cohort, 4 (28.6% of those with a detectable paraprotein, or 10.5% of those tested) of the patients with paraproteins had detectable autoantibodies directed at the alternative complement pathway[148]. A separate cohort of C3G pateints with a mean age at diagnosis of 60 years demonstrated monoclonal immunoglobulins in 36 patients[174]. Amoung patients of at least 50 years age, 65.1% had a detectable monoclonal immunoglobulin. In comparison, monoclonal gammopathy of undetermined significance is present in approximately 3% of the general population aged 50 years and older[175]. 89% C3G patients with detectable monoclonal gammopathy had haematuria at presentation[174]. Similar associations between C3G and paraproteinaemia were identified in two other cohorts[148, 176]. These papers showed therapy targeted towards the haematological dyscrasia and achieving haematological response associated with better renal outcomes than immunosuppression or conservative treatment targeted to the glomerular injury[174]. This suggests circulating monoclonal immunoglobulins play a role in C3G pathogenesis, at least in some cases. However, in all series the influence of complement autoantibodies on disease course seemed variable[141, 148].

Non-specific markers of glomerular inflammation, such as crescentic GN[173] or scarring, such as tubular atrophy and/or interstitial fibrosis[148], predict C3G progression to renal failure. The histologic C3G subtype does not seem to influence outcome; DDD has been associated with a younger age of onset and more rapid progression

to end-stage renal disease (ESRD) in some cohorts[173] but not others[148]. More severe renal impairment and older age at presentation associate with worse prognosis[148, 173]. Pathogenesis-specific features, such as the presence of autoantibodies, low C3, or complement-associated genetic variants, do not currently universally associate with outcome. C3G transplant recurrence rates also seems similar between C3GN and DDD cohorts. Anecdotally, in the cohort followed-up at Imperial College Healthcare NHS Trust, we have found recurrence to be universal in patients with a C3Nef and evidence of pre-transplant C3 consumption (low serum C3 secondary to complement activation). Overall C3G recurrence rates are reported between two-thirds to 100% of patients. About one half of the patients with transplant recurrence will progress to ESRD[177].

Areas of controversy and research needs

C3G cases with specific inherited or acquired drivers of alternative complement pathway activation have been informative in demonstrating C3G pathogenesis. However, in the majority of C3G patients, disease pathogenesis is poorly understood. This limits our ability to stratify patient groups into cohorts likely to benefit from targeted complement inhibition. Currently, the only clinicopathologic features that consistently correlate with disease outcome in different cohorts are markers of non-specific renal impairment and chronicity. There is also a need to identify markers that correlate with both ongoing complement dysregulation and disease severity as patients with this combination of features are most likely to benefit from targeted alternative pathway inhibition in the future. These shortcomings underpin my motivation for assessing specific immunohistologic markers of complement activation in C3G and their correlation with disease activity.

D. IgA nephropathy

Clinical features

IgA Nephropathy is the most common primary glomerular disease and a common cause of renal failure, especially in young people [178]. The natural history of IgAN is poorly understood. IgAN encompasses a range of clinical presentations, severities and outcomes, the relative prevalence of which vary with ethnicity and geography. In North America, the majority of children and young adults present with macroscopic haematuria during upper respiratory or gastrointestinal infection[179]. Older adults usually present with proteinuria, hypertension, microscopic haematuria and established renal impairment[180]. In India, however, the majority of patients present with nephrotic syndrome and raised serum creatinine[181]. In Europe and North America, IgAN affects males roughly twice as frequently as females[180]. In patients of Pacific Asian origin, IgAN affects males and females equally[182]. The median age of presentation is between 22 and 40 years of age[183].

The most common reported clinical course is indolent and slow progression to chronic renal impairment. However, about 5% of patients will experience a benign course with preserved renal function[183]. Conversely, some patients will present with rapidly progressive glomerulonephritis or vasculitis associated with glomerular IgA deposition (previously called henoch schonlein purpura, HSP). The crescentic form of IgAN (defined as more than 50% glomeruli exhibiting crescents) is relatively rare but leads to end stage renal disease (ESRD) by one year in 42.5% of patients despite immunosuppression therapy[184]. Pooled cohorts of patients from across the world suggest an average of 27% of patients will either reach ESRD or lose half their estimated glomerular filtration rate (eGFR) within 10 years of diagnosis[185], and up to 40% of patients will progress to ESRD over 30 years[183]. IgAN therefore has significant clinical impact on both a personal and societal level.

Clinical outcomes are also influenced by ethnicity and geography. In Canada, all patients with microscopic haematuria, normotension and minimal proteinuria at diagnosis had stable renal function at ten years follow-up[186]. In contrast, 44% of IgAN patients from Hong Kong with similar clinical characteristics at presentation had developed proteinuria, hypertension or renal impairment at seven years follow-up[187]. Of IgAN patients resident in Canada, individuals of Pacific Asian origin have a faster rate of eGFR decline and higher risk of progression to ESRD[188]. Therefore, the variation in outcomes is unlikely to simply be the result of lead-time bias from regional differences in biopsy practices.

Clinical features that predict IgAN outcomes are not specific to proposed IgAN pathogenesis and largely reflect established chronic kidney disease. For example, impaired GFR, hypertension and substantial proteinuria predict poor clinical course[189]. Of patients with a combination of more than 1g/day proteinuria, blood pressure of more than 140/90mmHg and severe histology lesions at biopsy, 64% will need dialysis after 20 years[190]. In cohorts of either predominantly Caucasian or Chinese ethnicity, less than 10% of patients with normal GFR at biopsy will reach ESRD at 10 years follow-up[189, 191]. Patients with more than 1g/day proteinuria have a risk of ESRD 46 times the risk of patient with less than 0.5g/day proteinuria[192]. The risk of ESRD is greater for patients with proteinuria of 0.5-1g/day than those with less than 0.5g/day[192].

The histology features of IgAN that predict clinical outcome at diagnostic biopsy have been defined. The Oxford Classification of IgA nephropathy revealed robustly identifiable histology features that predicted progression to ESRD or 50% loss of eGFR in IgAN[193]. Mesangial hypercellularity, segmental glomerulosclerosis and tubular atrophy and interstitial fibrosis independently predicted outcome of renal function, even after accounting for clinical features at biopsy and during follow-up[194]. Endocapillary hypercellularity predicted outcome and showed an interaction with corticosteroid or immunosuppression therapy suggesting a benefit of such treatment in cases with endocapillary proliferation[193]. The Oxford classification has since been validated in replication studies of paediatric[195] and adult[196] patients. Endocapillary hypercellularity has also been confirmed as an independent predictor of rate of renal function loss in a cohort of IgAN patients who received no immunosuppression[197]. The addition of the Oxford classification significantly improved outcome prediction compared with proteinuria, serum creatinine and blood pressure alone, and seemed as accurate as using two years follow-up data[198]. Therefore, the Oxford classification can be added to clinical information at the time of biopsy to accurately predict which patients are at risk of adverse outcome.

The Oxford classification was derived from IgAN populations with eGFR of at least 30ml/min/1.73m^2, proteinuria of at least 0.5g/day and no progression to ESRD within 12 months of the biopsy[194]. Its prognostic application is limited to patients that meet these criteria. Because these criteria excluded most patients with crescentic or rapidly progressive disease, the assessment was underpowered to determine the impact of glomerular crescents on outcome. However, since then, the presence of cellular or fibrocellular crescents in at least 25% of glomeruli on diagnostic biopsy has been shown to predict ESRD or a 50% loss of eGFR, regardless of immunosuppression use. Consequently, the Oxford Classification has recently been updated to include a

crescents C score of C0 for no glomeruli, C1 for 1-25% glomeruli and C2 for more than 25% glomeruli with cellular or fibrocellular crescents[199].

Incomplete understanding of IgAN pathogenesis: Implications

Despite significant progress identifying features associated with future renal failure in IgAN, we are unable to reliably and accurately identify patients who will benefit from immunosuppression therapy. This limitation is reflected by a number of negative clinical trials of immunosuppression in IgAN. Early trials of corticosteroid use in IgAN suggested benefit for patients with persistent proteinuria of more than 1g/day[200, 201]. However, these studies have been criticised for lack of uniform renin-angiotensin system (RAS) blocker use and small sample sizes[202]. More recently, two large randomised trials demonstrated the limitations of current patient selection methods for immunosuppression. The STOP-IgAN study compared immunosuppression with conservative therapy in patients with persistent proteinuria after six months blood pressure control with RAS blockers[203]. There was no significant difference in the rate of loss of kidney function between the two groups. More infections, weight gain and impaired glucose tolerance were found in the immunosuppression group. Despite demonstrating improved proteinuria and a lower rate of progression to 40% eGFR loss, ESRD or death with corticosteroids, a recent randomised trial of corticosteroid use versus RAS blockade alone was stopped early due to an excess of serious infections in the treatment cohort[204]. These disappointing results may be partly explained by study design flaws, particularly the failure to incorporate Oxford Classification histology features in inclusion criteria for immunosuppression. However, they also reflect the current inadequacies of our understanding of IgAN natural history and pathogenesis. These limit our ability to identify appropriate markers of disease activity, identify effective disease-specific therapeutic targets, and select patients for specific and targeted therapies.

Current pathogenic theory: Four-hit hypothesis

Two observations suggest circulating factors are important in IgAN pathogenicity. First, IgAN may recur in patients who receive an unaffected transplant[205]. Second, IgA deposits in kidneys inadvertently transplanted into non-IgAN renal patients clear after transplantation[206]. The most popular theory of IgAN pathogenesis

describes a multi-hit process. This is based on the observation that IgAN patients have, on average, a greater proportion of poorly O-galactosylated IgA subtype 1 (IgA1) in circulation compared to healthy individuals[207]. This has been reproduced in populations of different ethnicities and geographic locations[208, 209]. Also, IgA1 is the only IgA subclass identified in mesangial IgA deposits and poorly galactosylated IgA1 has been eluted from these deposits[210]. The IgA1 glycoforms are not completely deficient in galactose. They are characterised by relative galactose deficiency following failed O-galactosylation of the extended hinge region of IgA1, which differentiates it from the other human subclass, IgA2[211]. The absence of galactose leaves N-acetylgalactosamine residues exposed. Measurement of gd-IgA1 by ELISA with N-acetyl-galactosamine (GalNac)-specific HAA lectin in a cohort of 50 Japanese IgAN patients demonstrated mean serum levels of 117 (SD 46) units/mg IgA[212]. Poorly O-galactosylated IgA1 also circulates in healthy individuals, albeit at a lower proportion of the total circulating IgA pool[213]. Comparison of pooled serum from three patients with IgAN and three healthy controls showed a ratio of gd-IgA1 to total IgA of 1.32 in patients and 0.83 in healthy controls[214]. The IgA1 glycoforms are normally produced at mucosal surfaces as part of innate immune systems[215]. Incomplete post-translational O-galactosylation of IgA1 may be an evolutionary response to bacterial proteases, against which galactose-deficient IgA1 could have relative resistance[211]. How the poorly O-galactosylated IgA1 glycoforms become more prevalent in circulation is unclear but there is evidence that both imbalances of relevant enzymes in[216], and altered homing of IgA1-secreting cells between mucosal and systemic compartments[217] contribute. Interestingly, poorly galactosylated IgA levels are elevated in 25% of healthy IgAN patient relatives and is a heritable trait[218, 219]. Delineating the mechanisms that underlie IgAN pathogenesis is hindered by a lack of comparable small-animal models[220]. Although a number of mouse strains that model human IgAN have been described[221], only humans and hominoid primates have the pathogenic IgA1 subclass. The applicability of findings from IgA animal models to human IgAN is therefore limited.

Almost all circulating poorly O-galactosylated IgA is found within immune complexes, bound to either an IgG or IgA antibody. The next hit in IgAN pathogenesis is therefore the production of glycan specific antibodies. These immunoglobulins probably prevent the IgA1 binding the asialoglycoprotein receptor on hepatocytes, that would be its normal route of catabolism[222]. The glycan specific IgG and IgA are often characterised by a specific replacement of serine with alanine in the complementarity-determining region 3 of the variable region of the heavy chain[223]. This change increases the affinity of binding to the O-glycans on poorly O-galactosylated

IgA, and particularly to exposed N-acetylgalactosamine. This alteration arises from a somatic mutation during an active immune response, perhaps following exposure to viruses or bacteria that express N-acetylgalactosamine[222].

The formation of immune complexes containing anti-glycan auto-antibodies and poorly O-galactosylated IgA1 is a critical hit in IgAN pathogenesis. Uncomplexed poorly O-galactosylated IgA1 does not stimulate human mesangial cells in culture. However, patient derived immune-complexes of poorly O-galactosylated IgA1 glycoforms increase mesangial cell activity and proliferation, suggesting the complexes are nephritogenic[224]. The pro-inflammatory nature of the immune complexes is likely to be amplified by other factors such as bound C3 fragments[222].

The next step in IgAN pathogenesis sees the immune complexes deposit in glomeruli, most commonly in the mesangium. The complexes may attach to extracellular matrix components[225] and interact directly with mesangial cell receptors. Although the receptor via which IgA-containing immune complexes interact with mesangial cells is debated, it seems likely to be the transferrin receptor CD71[226], possibly enhanced by immune-complex bound soluble CD89[227]. However, canonical immune complex binding to mesangial cell receptors may not drive mesangial cell activation. Immune complexes trapped in mesangial extracellular matrix could activate complement locally, with subsequent activation of mesangial cells via complement receptors[228]. The pathogenic mechanisms subsequently leading to glomerular injury are imprecisely defined, but probably involve mesangial release of mediators of renal injury such as angiotensin II[229], proinflammatory cytokines[224], growth factors[230] and fibrotic cytokines[229] and complement activation[228, 231, 232]. Over time, this leads to the histopathologic features of IgAN, renal injury, and chronic renal disease.

Gaps in current understanding

The current theory of IgAN pathogenesis fails to explain a key disease characteristic; why does glomerular IgA deposition lead to a range of glomerular injury and disease severity in different patients? The single criterion for a diagnosis of IgAN is the presence of dominant or co-dominant IgA deposits in the glomerular mesangium. However, there is significant heterogeneity in the histopathological response to IgA deposition and the subsequent clinical course[211]. Furthermore, indolent mesangial IgA deposition is a common finding without

clinical or histology evidence of disease. Its prevalence varies with geography and ethnicity; it is found in autopsy series in about 1.3% Finnish[233] and 15.6% of Japanese[234] healthy individuals. It is not clear whether the quiescent and apparently tolerated mesangial IgA shares similar galactose-deficient characteristics as the supposedly pathogenic IgA in IgAN patients. However, as healthy individuals have circulating galactose-deficient IgA, albeit at a lower proportion of the total circulating IgA pool than patients, I would expect at least a proportion of the deposited IgA to be galactose deficient IgA1 (gd-IgA1). It may be that the formation and deposition of immune complexes as opposed to IgA specifically is key to IgAN pathogenicity. Direct binding of immune complexes to glomerular cells has been difficult to demonstrate, partly due to the absence of an obvious receptor. It is tempting to speculate that the pathogenic link between immune complex mesangial deposition and glomerular cell activation is yet to be described. This suggests inherent non-cellular factors within the kidney determine whether deposited IgA is tolerated, leads to mild mesangial reaction, or triggers significant inflammation and injury. A growing body of evidence implicates the complement system in determining the glomerular inflammatory and injurious response to deposited IgA and IgAN disease severity.

Evidence of complement activity in IgA nephropathy

a. Alternative complement pathway

Mesangial co-deposition of C3 and IgA is a well-established characteristic of IgA nephropathy, being present in at least 90% of biopsies[235]. In fact, the earliest descriptions of IgA nephropathy by Jean Berger in 1968 and 1969 noted that C3 deposits often accompanied the mesangial IgA[236]. These observations were followed by the findings that glomerular IgA and C3 deposition is often accompanied by properdin, C3dg, terminal complement pathway proteins and the MAC[235, 237]. Also, plasma levels of C3 fragments, as evidence of alternative complement pathway activation, were higher in IgAN patients than healthy controls[238, 239]. More recently, this risk of developing ESRD in IgAN was shown to correlate with the quantity of deposited glomerular C3 and the extent of alternative complement pathway activation and C3 consumption[240]. In IgAN. C3 is almost never accompanied by C1q, indicating the classical pathway is unlikely to contribute to pathogenesis[237]. Together these data demonstrate robust associations between alternative complement pathway activation and IgA nephropathy pathogenesis. However, the mechanisms through which IgA activates C3 and deposited complement proteins cause glomerular inflammation and injury has not been demonstrated.

Although IgA has been shown to activate C3 *in vitro*, this is only seen with polymeric or surface bound IgA1 and under conditions specific for alternative complement pathway activation[241-243]. A rat model for IgA-mediated glomerular inflammation demonstrated that polymeric but not monomeric IgA triggered mesangial C3, but not C4 or C1q, deposition.[244] This leaves important questions of pathogenicity unanswered, for example, why does glomerular IgA deposition cause significant alternative complement pathway activity, as opposed to the classical complement pathway activation normally associated with IgA-IgG immune complexes? Why does the alternative pathway activation seem to be dependent on polymeric IgA? Why is marked C3 deposition demonstrable in some patients but not others? And how does this relate to glomerular injury and inflammation?

b. Factor H related proteins 1 and 5

A recent series of international genome-wide association studies provided a watershed moment in our understanding of how alternative complement deregulation might influence IgAN severity. These large genetic studies implicated a role for the FHR proteins in IgAN pathogenesis. An initial GWAS of 1194 cases and 902 controls of Chinese Han ancestry identified a strong association between reduced IgAN risk and SNP rs6677604 that tags the delCFHR3-1 deletion[2]. The latest meta-analysis of more than 20,000 individuals of different ethnicities confirmed this variant to have a protective effect in IgAN, with inheritance of a single allele calculated to reduce disease risk by 36%, while two alleles reduce the disease risk by 45% [3]. Interestingly, the allelic frequency of the deletion exhibits marked differences across worldwide populations, with the highest frequency of the protective alleles in ethnicities of African ancestry (55%), in whom the prevalence of IgAN is least common, while people from South America and East Asia, where IgAN is most common, have the lowest prevalence of protective alleles (0-5%). Subsequently, an association was demonstrated between the same SNP rs6677604, serological and histopathological evidence of complement deregulation, and histologic markers of disease severity[245].

The genetic studies triggered interest in a role for other FHRs in IgAN. Glomerular FHR5 deposition was present in all IgAN cases from a series of glomerular disease biopsies, and was found in mesangial locations similar to IgA and C3[112]. Interestingly, we noted that C3G associated with mutant FHR proteins shared many clinical, phenotype and histology features with IgAN. The similarity was particularly striking for cases of CFHR5

nephropathy. Also, rare variants in the FHR5 gene (*CFHR5*) are significantly more common in IgAN patients than healthy controls[4].

Our appreciation of associations between FHR1, FHR3 and FHR5 and IgAN coincided with the aforementioned insights in FHR biology, particularly the ability of FHR1 and FHR5 homodimers to interfere with the physiological actions of FH and deregulate complement activation. This led to a possible pathogenic mechanism that could link FHRs and IgAN incidence and severity; in response to a trigger, such as mesangial IgA1 deposition, imbalances or gain-of-function variants in the FHRs compared to FH would increase alternative pathway activation and C3 cleavage leading to amplified complement-dependent inflammation and renal injury.

c. Lectin complement pathway

IgAN is characterised by disease flares following respiratory or gastrointestinal tract inflammation[238]; both IgA and the lectin complement pathway are important mediators of innate immunity at these sites. IgAN patients have higher levels of gd-IgA1 than healthy controls[214, 246]. Gd-IgA1 is characterised by exposed GalNac motifs[247, 248]. GalNac may trigger lectin pathway activation due to interaction of ficolins with patterns of acetyl-groups[21]. Furthermore, MBL binds polymeric IgA and triggers complement activation *in vitro*[249]. Both high and very low circulating MBL levels were associated with poor renal outcomes in a Chinese IgAN population[250]. Endo *et* al detected MBL and MASP-1 in renal biopsies from 24% of a cohort of 45 patients with IgAN[251]. Roos et al demonstrated glomerular MBL, L-ficolin, MASP1/3 and C4d deposition in 25% of a cohort of 60 IgAN patients[252]. These associated with features of IgAN disease severity, specifically proteinuria, higher serum creatinine and the presence of renal failure [252]. This finding is supported by the association of glomerular C4d deposition with poor prognosis in IgAN[253, 254].

E. Complement therapeutics

Interest in therapeutic inhibition or modulation of complement system components is increasing[255]. This has been driven by three factors. First, the number of clinical conditions that involve pathological complement system activity to trigger disease or amplify injury is growing. Second, the mechanisms by which complement

deregulation and activation drives disease pathogenesis has been precisely defined in some conditions, such as PNH and aHUS. Third, inhibition of terminal complement activation by the anti-C5 monoclonal antibody, eculizumab, has been demonstrated as effective treatment for PNH and aHUS. In this section, I will summarise the evidence for eculizumab use and highlight examples of other agents in development that target complement components. The growing interest and availability of agents that inhibit specific complement components adds clinical urgency to understanding the mechanisms driving complement-dependent diseases. Without understanding the natural history and mechanisms driving complement-associated disease, we will be unable to appropriately identify patients for specific complement protein inhibition. This will limit effective treatment and increase the risk of inappropriate and excessive complement inhibition, which is predicted to have similar clinical consequences to acquired deficiency of the targeted proteins.

Eculizumab

Eculizumab is a fully humanised monoclonal antibody (mAb) that binds to C5 and prevents its cleavage by the C5 convertases to C5a and C5b. This blocks both the formation of C5b9 and terminal complement pathway progression, and release of the anaphylotoxin C5a. Understanding the mechanism of PNH, and its pathologic dependence on absent CD59 on erythrocytes and unregulated terminal pathway activation allowed eculizumab to be used with marked clinical success[256]. Eculizumab impairs C5b9 formation on erythrocytes and thereby prevents haemolytic anaemia in the majority of patients with PNH. Predictably, it is also associated with an increased risk of meningococcal infection, although this can be limited by antibiotics and appropriate vaccination[257]. Eculizumab is also an effective treatment for aHUS[258, 259]. This suggests the severity of TMA and renal injury associated with aHUS is determined by C5 activation.

The successful treatment of patients with PNH and aHUS led to the use of eculizumab to treat other conditions associated with complement activation. Eculizumab has been used to treat cases of C3G. The results have been mixed and difficult to interpret. A small prospective trial from 2012 reported an improvement in renal function or proteinuria in three of six patients[260], while clinical improvement has been reported more frequently in case reports. Although this difference might be explained by publication bias[138], it is also important to consider that most of the patients had significant renal impairment despite receiving other immunosuppression. As discussed, C3G encompasses a range of disease severity and the mixed results may

reflect this. For example, eculizumab may provide most benefit to C3G patients with acute renal inflammation as this is likely to be mediated significantly by C5a release. The results also likely reflect the important contribution of C3 activation via the alternative complement pathway to C3G pathogenesis[261]. These data emphasise the importance of understanding the natural history and mechanisms linking complement dysregulation to renal injury and impairment in C3G, including developing techniques to select patients most likely to benefit from inhibition of a particular complement protein.

Three case reports have documented the use of eculizumab in IgA nephropathy. Two of these report its use in progressive IgAN with clinical deterioration despite anti-proteinuria treatment and other immunosuppression use[262, 263]. Five weekly doses of eculizumab seemed to stabilise the renal function of a 16-year-old with crescentic IgAN for about six months, but did not affect proteinuria or biopsy markers of disease chronicity[262]. Three months' eculizumab treatment coincided with improved proteinuria and renal function stabilisation in another 16-year-old patient with progressive crescentic IgAN, but the patient deteriorated after completing the treatment course[263]. The third case was a patient with recurrent crescentic IgAN causing graft dysfunction two months after renal transplantation. The patient required haemodialysis for transplant dysfunction before eculizumab was started and four doses did not rescue renal function[264]. As for C3G, the published experience of eculizumab in IgAN is difficult to interpret and may reflect the heterogeneity of IgAN pathogenesis and severity. The findings may also be consistent with a dominant role for C3 activation via the alternative complement pathway, as opposed to terminal pathway activation in IgAN pathogenesis. Overall, the data suggest effective treatment of IgAN with complement inhibition will depend on being able to identify appropriate patients, the most effective time point in disease natural history, and the pathogenic complement protein to target. These are dependent on understanding and identifying mechanisms of complement activity contributing to disease pathogenesis and being able to identify markers of ongoing and previous complement activation.

Other therapeutic agents

Understanding the mechanisms of complement activation in renal conditions is in part motivated by the approaching availability of therapeutic agents that target other proteins. For example, inhibiting antibodies for MASP2 and MASP3 are in development and the anti-MASP2 antibody OMS721 received orphan drug and

breakthrough therapy designation for IgAN in 2017 and is in phase III trials[255]. A number of agents that inhibit components of the alternative complement pathway are in development. These include, but are not limited to, small-molecule inhibitors of fD[265], anti-fB antibodies[255], and compstatin analogues that bind to C3 substrate and prevent it binding to C3 convertases[266], thereby inhibiting C3 activation. Another approach targeting alternative pathway activation is the development of engineered regulators, such as mini-FH proteins. These are comprised of the key functional FH domains but avoid the size and complexity of full-length FH that limits its production for therapeutic use[267, 268]. They are currently at the pre-clinical stage of development. Finally, downstream effector functions of complement can be targeted. For example, C5a signalling is targeted by C5a receptor 1 (C5aR1) antagonists, such as avacopan. This is effective in replacing high-dose steroids in ANCA-associated vasculitis[269] and has received orphan drug designation for C3G and aHUS.

In summary, therapies that manipulate a range of complement components are increasingly available. Deciding which therapeutic agent to use depends on identifying pathogenic mechanisms and complement components. This makes understanding complement dependent renal injury in C3G and IgAN essential.

Hypotheses

The central step to complement activation is the cleavage of C3 to C3b. Although this can be triggered by different pathways, the alternative pathway is responsible for complement system amplification. The key regulator of the alternative complement pathway is FH. Complement associated genetic mutations have provided insight into the function and importance of complement components, and potential consequences of inappropriate therapeutic complement inhibition. There is growing evidence that FHR1 and FHR5 homodimers compete with FH for C3b binding and deregulate complement activation. Some C3G cases are associated with FHR variants, which suggest the potential for FHR proteins to modify complement-associated glomerular disease. However, the pathogenesis underlying most cases of C3G is poorly understood. Although associations between alternative complement pathway activation and IgAN are well established, the mechanisms linking complement activation and IgAN pathogenesis are also not understood. Recent evidence suggests FHR proteins and the lectin complement pathways are important in IgAN pathogenesis.

These observations led to my two research hypotheses:

1. Complement activity influences IgA nephropathy disease severity. Specifically, complement activation determines whether IgAN patients develop glomerular inflammation and renal impairment in response to glomerular IgA deposition.
2. The factor H related proteins contribute to the pathogenesis of complement-dependent glomerular disease by deregulating complement activation and amplification and thereby accentuating glomerular inflammation and renal injury.

My aims were:

1. To explore whether or not FH deregulation and lectin pathway activity correlate with IgAN severity.

Using a large cohort of IgAN patients and healthy controls, I planned to describe circulating and immunohistochemical evidence of (a) FH deregulation by FHR1 and FHR5 and subsequent alternative pathway

complement activation, and (b) lectin pathway activation. I planned to correlate the findings with clinical and pathology markers of IgAN severity. I predicted these would provide insight into progressive IgAN pathogenesis and identify biomarkers of disease severity and future therapeutic targets.

2. To identify mechanisms by which complement deregulation contributes to renal damage.

Alternative complement pathway activation is pathogenic in C3G. I therefore aimed to describe immunohistochemistry evidence of complement activation and FH deregulation by FHR1 and FHR5 in C3G. This would demonstrate the ability for this process to contribute to alternative pathway activation and renal inflammation. I planned to complete detailed complement protein immunohistochemical and co-deposition studies of C3G and IgAN renal tissue to describe the pathogenic interaction of FHR1, FHR5 and other complement proteins in vivo. I also planned to investigate whether circulating IgA interacts directly with FHR1 and FHR5 to drive FH deregulation and complement activation.

Methods

A. Patient and healthy control cohorts

I collected demographic, clinical and histologic information and performed assays on clinical samples from a number of groups. I analysed clinical and histological data and used clinical samples from patients with IgAN and C3G. As experimental controls, I also used surplus stored clinical samples from patients receiving care at Imperial College Healthcare National Health Service (NHS) Trust with other conditions including autosomal dominant polycystic kidney disease (ADPKD), thin basement membrane disease (TBM), diabetic nephropathy (DN), lupus nephritis (LN), membranous nephropathy, ANCA-associated vasculitis, C3 deficiency and selective IgA deficiency. All patients treated and investigated at Imperial College Healthcare NHS Trust consented to their clinical records and stored surplus clinical samples being used for research. Human samples used in this research project were obtained from the Imperial College Healthcare Tissue Bank (ICHTB). ICHTB is supported by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Imperial College Healthcare NHS Trust and Imperial College London. ICHTB is approved by NRES to release human material for research (12/WA/0196). IgAN and C3G patients underwent additional consent as part of their enrolment into retrospective cohort studies (details are below). I also enrolled healthy control cohorts. Healthy individuals consented to provide serum, plasma, or DNA samples and demographic information. All person-identifiable data were stored electronically on a password-secured computer on the Imperial College Healthcare NHS Trust Information Technology (IT) network. Access to person identifiable data was limited to individuals with appropriate clinical training and authorisation from Imperial College Healthcare NHS Trust. Person identifiable data was removed after initial data collection. Only anonymised data was analysed and presented.

Patient samples were collected at the time of study enrolment. 14ml of blood was collected in two ethylenediaminetetraacetic acid (EDTA) treated vacutainers, one for DNA extraction and one for plasma. Also, 6ml of blood was collected for serum in an untreated vacutainer. Blood samples for serum and plasma were processed and stored within 24 hours of collection. Blood for DNA extraction was immediately stored at -80°C until processing. Blood for serum collection was left to clot fully for one hour, centrifuged at 3000rpm for 15 minuts, and serum supernatant was extracted with Pasteur pipettes in1ml aliquots and stored in microtubules

at -80°C. Blood obtained for plasma was centrifuged at 3000rpm for 15 minutes and the supernatant was aliquoted into two microtubules with a Pasteur pipette and stored at -80°C.

The time of sample collection was different to the time of diagnostic biopsy in all cases. Patients were included regardless of the duration from diagnostic biopsy to enrolment, which varied markedly in all study cohorts, or progression to ESRD. Patients who had received renal transplant for ESRD were also included. Thereby, the study included patients receiving regular maintenance dialysis and post-transplant immunosuppression regimens. These features added innumerate confounding factors to interpreting the results that will be detailed in the relevant Results chapters. The time difference between blood sampling and renal biopsy is a major limitation to interpreting correlations between immunohistology features and circulating complement levels. This will also be described in more detail in the relevant results chapter. However, the strengths of the study design were that large cohorts of patients with rare diseases were collected, the cohorts were representative of contemporaneous clinical populations managed in clinical settings, and the patient samples avoid the inherent uncertainty that animal and *in vitro* disease models may not be representative of the human disease.

B. Complement factor H related protein 3 and 1 gene copy number assessment

Deoxyribonucleic acid (DNA) was extracted from whole blood samples using QIAamp DNA Blood Mini Kits (Qiagen). Quantitative real–time polymerase chain reaction (PCR) was performed on the ViiA Real–Time PCR System (Applied Biosystems). Copy number variation (CNV) within the *CFHR3* and *CFHR1* genes was assessed using the Taqman Copy Number Real–Time Detection System (Applied Biosystems). CNV calls were determined using the Copy Caller Software (Applied Biosystems). Assay readings were normalized to control samples and the values represent mean copy number plus and minus standard deviation (SD). All probes were validated using genomic DNA from controls with either heterozygous or homozygous polymorphic deletion of the *CFHR1* and *CFHR3* genes. The *CFHR1* gene copy number in the renal transplant cohort was inferred from the rs6677604 genotype, which is in linkage disequilitbrium with the *CFHR1* and *CFHR3* gene copy number[270]. Genotyping was performed using the Taqman genotyping assays (Applied Biosystems). I assisted with the copy

number assessment and genotyping. However, the majority of the assays were performed by Dr Hannah Lomax-Browne.

C. Circulating factor H, factor H related protein -1 and -5 quantification

Factor H and Factor H related protein 1

I used a sandwich ELISA designed by Tortajada *et al*[254] to measure plasma levels of FH and FHR1. Although FHR1 and FHR2 can form homodimers, heterodimers and heterooligomeric molecules *in vivo*, the levels detected by this ELISA are referred to as FHR1 levels as FHR1 is the major component in these complexes. 96-well Costar 3590 microtiter plates (Corning) were coated overnight with 100µl rabbit polyclonal antibody that recognises both FH and FHR1 (provided by Professor Santiago Rodriguez de Cordoba, Madrid, Spain) diluted 1:1000 in 0.1M sodium bicarbonate (pH9.5). After washing with Tris-Tween (Tris 50mM (pH7.4), sodium chloride 150mM, Tween 20 0.2%) and blocking with Tris-Tween bovine serum albumin (BSA) (Tris 50mM (pH7.4), sodium chloride 150mM, Tween 20 0.2%, BSA 1%) we added 100µl plasma samples diluted in Tris-Tween BSA at ratios of 1:3000 for FH and 1:12000 for FHR1. Samples had ben thawed on ice for one hour and at room temperature for 10 minutes, and were mixed thoroughly before use. Samples were tested in duplicate at two dilutions. A standard curve was generated using serial dilutions of a plasma sample of known FH and FHR1 concentration. FH was detected with a mouse monoclonal anti-FH antibody that recognises SCR10 and 11 of FH, diluted 1:100 in Tris-Tween BSA. FHR1 was detected with a mouse monoclonal antibody that recognises an epitope within SCR1 and 2 of FHR1 (both provided by Professor Santiago Rodriguez de Cordoba, Madrid) diluted 1:5000 in Tris-Tween BSA. Bound antibody was detected using HRP-conjugated goat anti-mouse IgG antibody (Dako) and o-phenylenediamine dihydrochloride substrate (Thermo Scientific) and optical absorbance was measured at 492nm. Inter and intra-assay coefficient of variance were 8.7 for the FH ELISA and 9.9 for the FHR1 ELISA.

Factor H related protein 5

Factor H related protein 5 levels were measured by ELISA. Nunc-Immuno MaxiSorp high protein-binding capacity 96-well ELISA plates (Thermo Scientific) were coated overnight with 50µl of rabbit monoclonal anti-

FHR5 antibody (Abcam) 1:500 in in 0.1M sodium bicarbonate (pH9.5). After washing with PBS Tween (1x phosphate buffered saline (PBS), 0.1% Tween) and blocking with PBS Tween 1% BSA (1x PBS, 0.1% Tween, 1%BSA) we added 50µl plasma samples diluted 1:800 in PBS Tween 1% BSA. Samples had ben thawed on ice for one hour and at room temperature for 10 minutes, and were mixed thoroughly before use. Samples were tested in duplicate at two dilutions. A standard curve was generated using serial dilutions of recombinant FHR5 protein (R&D Systems). FHR5 was detected with a mouse monoclonal anti-FHR5 antibody (Abcam). Bound antibody was detected using a HRP-conjugated rabbit anti-mouse IgG antibody and tetramethylbenzidine substrate (BD Biosciences). The absolute optical densities were calculated by subtracting the absorbance at 540nm from that at 450nm. Inter and intra-assay coefficient of variance value was 12.1.

D. Lectin pathway protein plasma concentrations

Plasma assessment strategy and control cohort assessment

I analysed circulating plasma levels of the 11 known lectin components in the IgAN cohort and healthy controls in collaboration with Professor Steffen Thiel, Aarhus University, Denmark. I anonymised, aliquoted and shipped patient and healthy control samples to Professor Thiel. Professor Thiel and members of his team including Dr Anne Troldborg measured plasma lectin levels by immunofluorescence assays (more details below). The anonymised data was sent to me. I analysed the data and compared levels in IgAN and healthy control cohorts.

We assessed lectin complement pathway levels in two stages. We first screened a subgroup of 125 IgAN patients for quantitative differences in circulating lectin pathway components compared to healthy controls. We quantified plasma levels from samples taken at study recruitment for the following proteins; MBL, M-ficolin, H-ficolin, L-ficolin, CL-L1, CL-K1, MASP-1, MASP-2, MASP-3, Map19, and Map44. We compared levels with a cohort of 211 healthy Danish volunteers of Caucasian ethnicity. The lectin components that showed differences in the screening assessment were measured in the remaining 198 IgAN patients and a second control cohort of 51 healthy volunteers living in London, UK, four of whom (7.8%) were of non-Caucasian ethnicity. The Danish and London control cohorts showed significant differences in plasma lectin complement

protein levels, including plasma MBL. The differnces are detailed in the Results chapter. Based on more similar ethnicity with our study group, we used the London-based control cohort for the remaining analyses.

Plasma levels of lectin pathway components

Plasma levels of MBL[258], M-ficolin[259], H-ficolin[260], CL-L1[261], CL-K1[262], MASP-1[263], MASP-2[264], MASP-3[265], MAp19[266] and MAp44[265] were measured using time-resolved immunofluorometric assays in duplicates. All assays and specific antibodies for the assays were produced in-house by Professor Steffen Thiel. Detailed description for each assay can be found in the references for each protein. L-ficolin plasma concentrations were estimated using ELISA according to the manufacturer's instructions (Hycult Biotech). We defined MBL deficiency as a plasma level of less than 100ng/ml.

E. Antibody specificity analyses

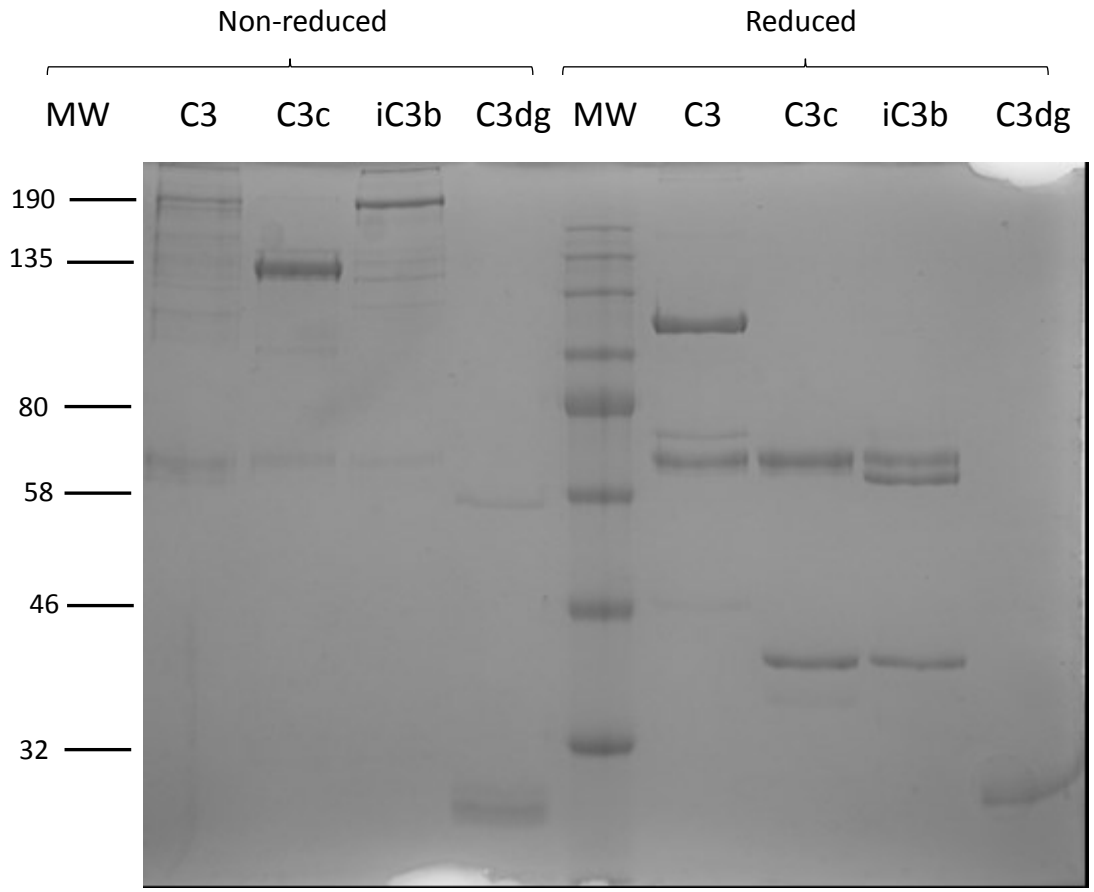
I estimated antibody specificity for target antigens using ELISA for anti-C3c, anti-C3dg and FHR5 antibodies (antibody details below). (1) Nunc-Immuno MaxiSorp high protein-binding capacity 96-well ELISA plates (Thermo Scientific) were coated with purified human C3c (2µg/ml, Comptech #A116), iC3b (2.5µg/ml, Comptech #A115), C3dg (5µg/ml, Comptech #A117) or 10% BSA overnight at 4°C. Professor Matthew Pickering and Dr Hannah Lomax-Browne had previously established the purity of the commercially produced proteins by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). In brief, purified human intact C3, C3c, iC3b and C3dg (1µg of each) were separated by SDS-PAGE on a 10% gel under both non-reducing and reducing conditions. Following electrophoresis bands of interest were visualised by staining with Coomassie and confirmed to be of appropriate molecular weight (Figure 5). The predicted molecular weights of the complement fragments are shown in the figure 5[271, 272]. (2) After washing, wells were incubated with either 5µg/ml purified human FHR5 (R&D Systems #3845-F5) or blocking buffer (1% BSA in 0.1% Tween phosphate buffered saline (PBS) (1x)) for 45 minutes. (3) After washing, wells were blocked for a further 45 minutes, before (4) the addition of polyclonal rabbit anti-C3c (Dako #A0062), rabbit anti-C3dg (Abcam #136916) or rabbit anti-FHR5 (Abnova #81494-D01P). (5) Bound antibody was detected using horseradish peroxidase

(HRP)-conjugated swine anti-rabbit IgG (Dako #P0260) and 3,3',5,5'-tetramthelybenzidine substrate (Dako #S1599). Absorbance was calculated by subtracting the optical density (OD) at 540nm from 450nm.

In order to estimate the specificity of the fluorescein isothiocyanate (FITC)-conjugated anti-C3c antibody used in immunofluorescence (IF) assays, we used an identical method but at stage (4) sheep anti-human C3c-FITC (ThermoFisher PA1-36179) was added. Subsequently for step (5), bound antibody was detected by measuring relative fluorescence with a BMG, FLUOstar OMEGA plate reader.

Figure 5. Purity of commercially produced complement protein preparations.

Professor Matthew Pickering and Dr Hannah J. Lomax-Browne had previously established the purity of the commercially produced proteins by SDS-PAGE. Bands representing the complement pathway component are shown after Coomasie stain at appropriate molecular weights (MW, in kilodaltons) under non-reduced and reduced conditions. The predicted molecular weights of complement fragments and alpha (α) and beta (β) chains are shown in the table below. Under reduced conditions, α and β chains dissociate, and α chains separate into α1 and α2 fragments, producing multiple bands.
Image courtesy of Dr Hannah J. Lomax-Browne.



Molecular Weight (KDa)	Non-reduced	Reduced conditions	
C3	190	α	115
		β	75
C3c	138	α1	23
		α2	40
		β	75
iC3b	178	α1	63
		α2	40
		β	75
C3dg	40		40

F. Immunohistochemistry

Protocol

I optimised immunohistochemistry (IHC) protocols with stored surplus unstained renal biopsy tissue from cases of thin basement membrane disease, membranous nephropathy, and lupus nephritis, and an unstained healthy liver section (provided by Dr Candice Roufousse, Imperial College Healthcare NHS Trust). Two-micron sections from formalin-fixed paraffin-embedded (FFPE) renal tissue were mounted on organo-silane coated slides, deparaffinised (xylene for 10 minutes followed by an ethanol gradient), washed in distilled water and subjected to antigen retrieval. I tested heat and enzyme-dependent antigen retrieval techniques to determine the optimal technique for each antibody. Examples of this process for an anti-FHR5 antibody (rabbit anti-human FHR5 antibody (Abnova #81494-D01P)) are shown in Figure 6. This provides examples of images obtained from different methods of antigen retrieval; proteinase XXIV enzyme dependent antigen retrieval, and pressure cooker antigen retrieval. It exemplifies how different antigen retrieval techniques can produce markedly different antigen distribution patterns, using the antibody. For FHR5, proteinase XXIV antigen retrieval resulted in glomerular staining with minimal background, interstitial or tubular staining (the first column of images on the left). Pressure cooker antigen retrieval produced different images. As I was interested in studying glomerulopathies, I aimed to optimise staining protocols to provide glomerular antigen deposition in cases known to be driven by complement activity (C3G and lupus nephritis (LN)). When this had been achieved, I used a biopsy section from a case with non-complement dependent pathology, such as thin basement membrane disease (TBM), to indicate the specificity of the staining. I verified this by pre-incubating the antibody with a purified form of its canonical antigen; FHR5 in this case. This eliminated the FHR5 staining but introduced tubular staining, both in C3G, IgAN, LN and TBM. Pre-incubation of a TBM section with heparin that would limit non-canonical glycosaminoglycan interactions had no effect on tubular staining. For pre-incubation experiments, we used recombinant full-length human FHR5 (R&D Systems #3845-F5) and heparin sodium (Sigma-Aldrich #H3393) at 30x the antibody concentration. The tubular staining was reproduced in a case of IgAN using an anti-FHR5 antibody raised in mouse, not rabbit (Abnova #81494-B01P) and a different method of detection (immunofluorescence using an AF-488 conjugated goat anti-mouse IgG antibody (Thermofisher #A-11029). The cause of the tubular staining has not been determined, but did not correlate

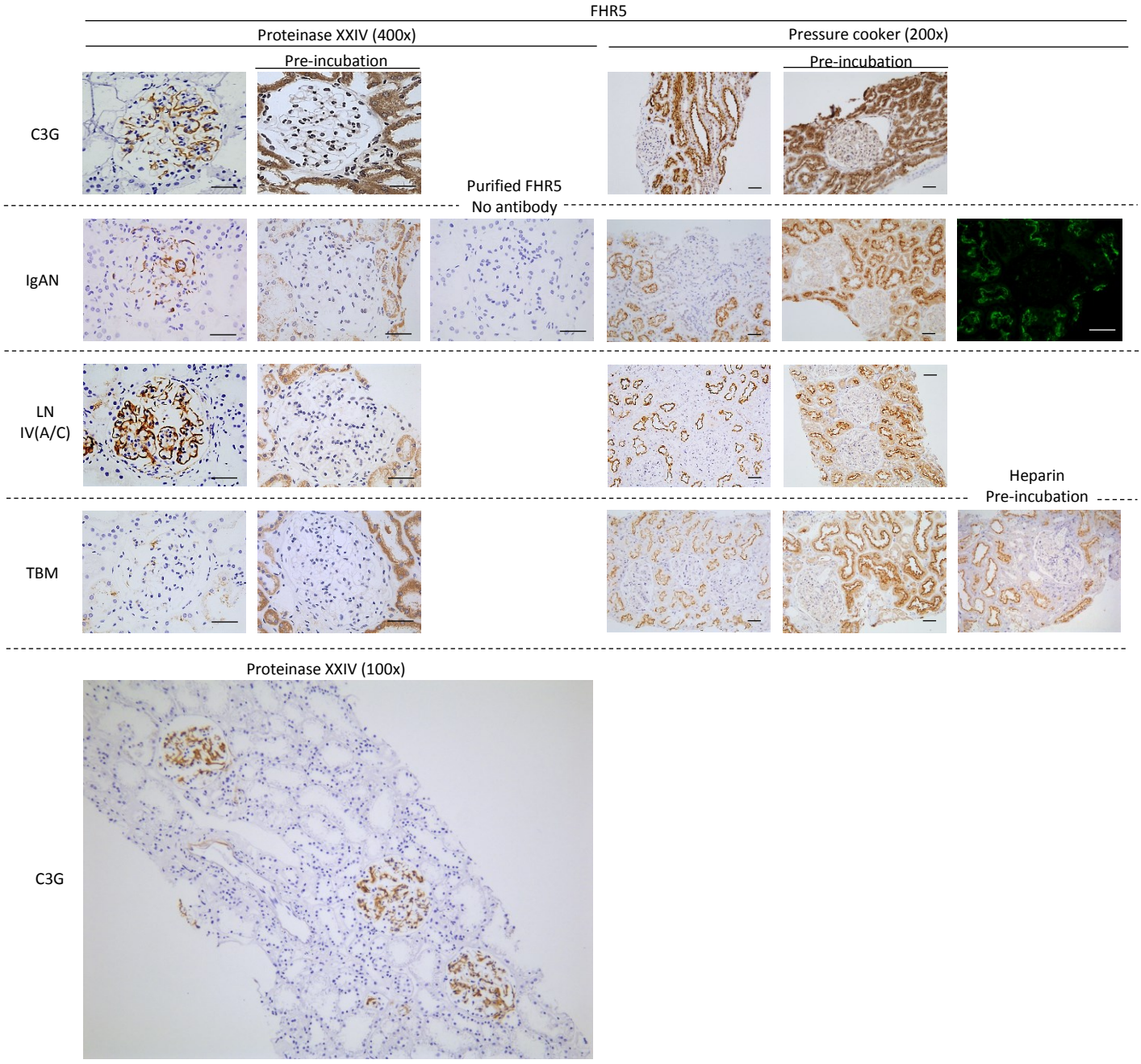
with markers of disease severity (as will be discussed in the Results section) and could be explained by Fc interactions.

I subsequently designed the following protocols for immunoshistochemistry analysis. For C3b/iC3b/C3c, C5b9 and FHR5: bacterial proteinase XXIV (Sigma-Alrich #P8038) at 37°C for 30 minutes. For C3dg, C4d, FH, and C1q: sodium citrate tribasic buffer at 95°C for 30 minutes. For properdin, FHR1 and CD68: Tris- ethylenediamine tetraacetic acid (EDTA) at pH 9 and heating in a pressure cooker for 30 minutes. Sections were blocked for 60 minutes at room temperature with peroxidase (EnVision, Dako #K4007 or #K4011)) followed by 30% normal goat serum (Sigma-Aldrich #G9023). Primary antibody was applied overnight at 4°C followed by application of secondary antibodies for 60 minutes at room temperature: HRP-conjugated goat anti-mouse (EnVision+System-HRP, Dako #K4007) or rabbit (EnVision+System-HRP, Dako #K4011) IgG. 3'-Diaminobenzidine (DAB) substrate (EnVision+System-HRP, Dako #K4007 or #K4011) was applied for up to 15 minutes and sections counterstained with filtered haematoxylin, washed and dehydrated and fixed in xylene for 10 minutes prior to mounting using xylene-based media (Pertex, CellPath #SEA-0104-00A). DAB reaction times were optimised to allow complete reaction with minimal background, non-specific staining and differed slightly for each antibody. DAB was applied for the same duration to all sections stained with the same antibody. I tested each secondary antibody in the absence of a primary antibody to ensure my results were not influenced by non-specific secondary antibody binding. Images were taken at 100x, 200x and 400x magnification. Images are shown at 400x magnification if the aim is to demonstrate glomerular staining, 200x magnification to demonstrate tubular staining and 100x magnification to demonstrate staining uniformity throughout the biopsy core.

Figure 6. Complement factor H related protein 5 immunohistochemistry protocol optimisation

I optimised our IHC protocols to stain stored surplus paraffin embedded renal biopsy tissue. When bacterial proteinase XXIV was used for antigen retrieval, glomerular FHR5 was detectable in C3 glomerulopathy C3G and other renal pathologies associated with glomerular complement deposition. TBM cases, that are not associated with complement activation and deposition, showed neither glomerular nor tubular staining. This glomerular FHR5 staining was eliminated when the primary antibody was pre-incubated with purified recombinant full length human FHR5 (column of images labelled 'Pre-incubation'). An example image of FHR5 staining using the adopted protocol at 100x magnification exemplifies inter-glomerular staining consistency and minimal background or interstitial staining with this protocol.

LN IV(A/C); lupus nephritis class IV (active/chronic). Images at 400x, 200x or 100x magnification. Bars represent 100 µm.



Antigens tested

Depending on the availability and quality of stored surplus tissue, renal biopsy sections were examined for the following proteins: FHR5, FHR1, FH, C3b/iC3b/C3c, C3dg, C5b9, properdin, C4d, C1q and CD68. Due to availability of stored surplus clinical biopsy material, it was rare to be able to analyse serial sections of the same glomerulus. This is a limitation of the immunostaining data. Primary antibodies were: polyclonal rabbit anti-FHR5 (Abnova #81494-D01P); monoclonal mouse anti-FHR1 (Abnova #3078-M01); monoclonal mouse anti-factor H, OX-24 (Abcam #118820); polyclonal rabbit anti-C3c (Dako #A0062); polyclonal rabbit anti-C3dg (Abcam #136916); monoclonal mouse anti-C5b9 (Dako #M0777); polyclonal rabbit anti-properdin (Biorbyt #2097); polyclonal rabbit anti-C4d (DB Biotech #107-01); monoclonal rabbit anti-C1q (Dako #A0136); and monoclonal mouse anti-CD68 (Dako #M0876). The antibodies used are summarised in Table 1. I graded glomerular antigen staining intensities from anonymised sections as 0 (absent), 0.5 (minimal), 1+, 2+ and 3+. Staining described as 'positive' includes 1+, 2+ and 3+. Staining described as 'negative' includes 0 and 0.5[267].

Grading was performed by me and Professor H. Terence Cook, Professor of Renal Pathology, Imperial College London. All cases were anonymised and graders were unable to determine the identity of cases. I calculated the inter-observer correlation coefficient for the grading of staining intensity using the 0 (absent), 0.5 (minimal), 1+, 2+ and 3+ scale for Professor Cook and me. We independently graded the DAB staining intensity of 26 anonymised cases of IgAN that had undergone detection of C3b/iC3b/C3c using the protocol detailed above. Correlation coefficient was calculated using Spearman's Rank correlation. The correlation (r) for glomerular staining intensity was 0.72, with a statistically significant correlation ($P<0.0001$). When graded as 'negative' or 'positive', the correlation coefficient improved to 0.80 ($P<0.0001$).

Antigen	Primary antibody	Concentration	Manufacturer and number	Secondary antibody	Concentration	Manufacturer and number
FHR5	Rabbit polyclonal anti-human CFHR5	1:400 in PBS	Abnova 81494-D01P	Swine anti-rabbit IgG peroxidase	1:2000 in PBS	Dako P0217
FHR1	Mouse monoclonal anti-human CFHR1	1:200 in PBS	Abnova 3078-M01	Rabbit anti-mouse IgG peroxidase	1:2000 in PBS	Dako P0260
FH	Mouse monoclonal anti-human FH, OX24	1:50 in PBS	Abcam ab118820	Rabbit anti-mouse IgG peroxidase	1:2000 in PBS	Dako P0260
C3b/iC3b /C3c	Rabbit polyclonal anti-human C3c	1:100,000 in PBS	Dako A0062	Swine anti-rabbit IgG peroxidase	1:2000 in PBS	Dako P0217
C3dg	Rabbit polyclonal anti-human C3dg	1:1000 in PBS	Abcam ab136916	Swine anti-rabbit IgG peroxidase	1:2000 in PBS	Dako P0217
C5b9	Mouse monoclonal anti-human C5b9	1:1000 in PBS	Dako M0777	Rabbit anti-mouse IgG peroxidase	1:2000 in PBS	Dako P0260
Properdin	Rabbit polyclonal anti-human properdin	1:50 in 30% NGS	Biorbyt Orb 2097	Swine anti-rabbit IgG peroxidase	1:2000 in PBS	Dako P0217
C4d	Rabbit monoclonal anti-human C4d	1:5000 in PBS	DB biotech DB-107-01	Swine anti-rabbit IgG peroxidase	1:2000 in PBS	Dako P0217
C1q	Rabbit monoclonal anti-human C1q	1:8000 in PBS	Dako A0136	Swine anti-rabbit IgG peroxidase	1:2000 in PBS	Dako P0217
CD68	Mouse monoclonal anti-human CD68	1:100 in 2% BSA/PBS	Dako M0876	Rabbit anti-mouse IgG peroxidase	1:2000 in PBS	Dako P0260

Table 1. Antibodies used for immunohistochemistry detection of complement proteins in formalin fixed and paraffin embedded renal biopsy tissue

PBS refers to phosphate buffered saline at 1x concentration in distilled water. NGS, normal goat serum in PBS (at 1x concentration). BSA/PBS, bovine serum albumin in PBS (at 1x concentration).

G. Double antigen immunofluorescence

Protocol

Double antigen IF staining was performed using two-to-four micron sections from FFPE renal tissue that were mounted on organo-silane coated slides, deparaffinised (xylene for 10 minutes followed by an ethanol gradient), washed in distilled water and subjected to antigen retrieval. With the exception of properdin with C3b/iC3b/C3c, immersion in sodium citrate tribasic buffer at 95°C for 30 minutes and incubation with HistoReveal (Abcam #103720) for 5 minutes at room temperature was the antigen retrieval method used for all double IF combinations. For properdin with C3b/iC3b/C3c double IF, the antigen retrieval was Tris-EDTA at pH 9 and heating in a pressure cooker for 30 minutes. Sections were blocked with 2% BSA (Sigma-Aldrich #A2058) for 60 minutes at room temperature and the first antibody applied overnight at 4°C. Sections were blocked again with 2% BSA at 4°C for eight hours followed by incubation of the second antibody overnight at 4°C. Sections were counterstained with 4',6-Diamidino-2-Phenylindole, Dilactate (DAPI, ThermoScientific #D3571) for 10 minutes and 0.1% Sudan Black B (Sigma #199664) in 70% ethanol and mounted using Mowial 4-88 (Calbiochem #475904).

Antibody combinations

The antibody combinations were as follows. Co-staining for C3c and C3dg: FITC-conjugated sheep polyclonal anti-human C3c antibody (Thermofisher #PA1-36179) and rabbit polyclonal anti-C3dg (Abcam #136916) with Alexafluor AF555-conjugated goat anti-rabbit IgG (Thermofisher #A-21429). Co-staining for C3c and FHR5: FITC-conjugated sheep polyclonal anti-human C3c antibody (Thermofisher #PA1-36179) and mouse monoclonal anti-human FHR5 antibody (Abnova #81494-B01P) with AF555-conjugated goat anti-mouse IgG (Abcam #150118). Co-staining for C3dg and FHR5: rabbit polyclonal anti-C3dg (Abcam #136916) with Alexafluor AF555-conjugated goat anti-rabbit IgG (Thermofisher #A-21429) and mouse monoclonal anti-human FHR5 antibody (Abnova #81494-B01P) with AF488-conjugated goat anti-mouse IgG (Thermofisher #A-11029). Co-staining for FHR5 and IgA: Mouse monoclonal anti-human FHR5 antibody (Abnova #81494-B01P) with AF488-conjugated goat anti-mouse IgG (Thermofisher #A-11029) and rabbit polyclonal anti IgA (Dako #A0262) with Alexafluor AF555-conjugated goat anti-rabbit IgG (Thermofisher #A-21429). Co-staining for C3b/iC3b/C3c

and properdin: FITC-conjugated sheep polyclonal anti-human C3c antibody (Thermofisher #PA1-36179) and rabbit polyclonal anti-properdin (Biorbyt #orb2097) with Alexafluor AF555-conjugated goat anti-rabbit IgG (Thermofisher #A-21429). Co-staining for C5b9 and FHR5: Mouse monoclonal anti-C5b9 (Dako #M0777) with AF488-conjugated goat anti-mouse IgG (Thermofisher #A-11029) and rabbit polyclonal anti-FHR5 (Abnova #81494-D01P) with Alexafluor AF555-conjugated goat anti-rabbit IgG (Thermofisher #A-21429). Antibody combinations are summarised in Table 2.

Antigen pair	Primary antibodies	Concentration	Manufacturer and number	Secondary antibody	Concentration	Manufacturer and number
C3b/iC3b/ C3c + C3dg	Sheep polyclonal anti-human C3c, FITC	1:50 in 2% BSA/PBS	ThermoFisher PA1-36179			
	Rabbit polyclonal anti-human C3dg	1:800 in 2% BSA/PBS	Abcam ab136916	Goat anti-rabbit IgG H&L, AF555 conjugated	1:1000 in 2% BSA/PBS	ThermoFisher A-21429
C3b/iC3b/ C3c + FHR5	Sheep polyclonal anti-human C3c, FITC	1:50 in 2% BSA/PBS	ThermoFisher PA1-36179			
	Mouse monoclonal anti-human CFHR5	1:50 in 2% BSA/PBS	Abnova 81494-B01P	Goat anti-mouse IgG H&L, AF555 conjugated	1:1000 in 2% BSA/PBS	Abcam ab150118
C3dg + FHR5	Rabbit polyclonal anti-human C3dg	1:800 in 2% BSA/PBS	Abcam ab136916	Goat anti-rabbit IgG H&L, AF555 conjugated	1:1000 in 2% BSA/PBS	ThermoFisher A-21429
	Mouse monoclonal anti-human CFHR5	1:50 in 2% BSA/PBS	Abnova 81494-B01P	Goat anti-mouse IgG H&L, AF488 conjugated	1:1000 in 2% BSA/PBS	ThermoFisher A-11029
FHR5 + IgA	Mouse monoclonal anti-human CFHR5	1:50 in 2% BSA/PBS	Abnova 81494-B01P	Goat anti-mouse IgG H&L, AF488 conjugated	1:1000 in 2% BSA/PBS	ThermoFisher A-11029
	Rabbit polyclonal anti-human IgA	1:100 in 2% BSA/PBS	Dako A0262	Goat anti-rabbit IgG H&L, AF555 conjugated	1:1000 in 2% BSA/PBS	ThermoFisher A-21429
C3b/iC3b/ C3c + Properdin	Sheep polyclonal anti-human C3c, FITC	1:50 in 2% BSA/PBS	ThermoFisher PA1-36179			
	Rabbit polyclonal anti-human properdin	1:50 in 2% BSA/PBS	Biorbyt Orb 2097	Goat anti-rabbit IgG H&L, AF555 conjugated	1:1000 in 2% BSA/PBS	ThermoFisher A-21429
C5b9 + FHR5	Mouse monoclonal anti-human C5b9	1:500 in 2% BSA/PBS	Dako M0777	Goat anti-mouse IgG H&L, AF488 conjugated	1:1000 in 2% BSA/PBS	ThermoFisher A-11029
	Rabbit polyclonal anti-human CFHR5	1:50 in 2% BSA/PBS	Abnova 81494-D01P	Goat anti-rabbit IgG H&L, AF555 conjugated	1:1000 in 2% BSA/PBS	ThermoFisher A-21429

Table 2. Antibodies used for double immunofluorescence analysis of formalin fixed and paraffin embedded renal biopsy tissue

PBS was at 1x concentration in distilled water. NGS, normal goat serum in PBS (at 1x concentration). BSA/PBS, BSA in PBS at 1x concentration. AF, Alexa Fluor.

H. Antigen deposition quantification from double immunofluorescence images

I used Image J software[273] to quantify the size of single antigens and double antigen co-localisation deposits. I followed standard and published Image J[259] methods. In brief, I first ensured the two images for comparison were taken under identical conditions, with standardised microscopy settings and no movement of the section between image recordings using light of different wavelengths. I checked the images were of identical sizes. I selected the glomerulus as the region of interest and used the Otsu[259] thresholding method and a standardised brightness threshold to record the number of pixels occupied by an antigen. For each antigen I analysed one representative glomerulus per biopsy section. I recorded the total number of positive pixels (Count) and the total area of antigen deposition.

I next calculated the size of glomerular deposition with two co-localised antigens. In addition to the brightness threshold, I standardised colour thresholds between red and green. This eliminated areas of pure red or green deposition and included pixels composed of a mixture of red and green. I recorded the number of pixels and the deposit sizes meeting these colour criteria within one representative glomerulus per biopsy section. Finally, I used the COLC2 plug-in[273] to measure the correlation of glomerular locations for two antigens in one representative glomerulus per biopsy section.

I. Purification of IgA1 from serum and plasma using Jacalin-Agarose.

I purified IgA1 from human plasma and serum by affinity chromatography using Jacalin-linked agarose (Vector Laboratories) under sterile conditions. I first used 50% ammonium sulphate (Sigma-Aldrich #A4418) to precipitate proteins from human serum or plasma samples. I mixed equal volumes of ammonium sulphate and sample at room temperature for one hour. I centrifuged the precipitate and resuspended the pellet in 0.175M Tris. After washing and suspending 5ml of Jacalin-Agarose in 0.175M Tris base (Sigma-Aldrich #A77861), I mixed the precipitated protein pellet with the 0.175M Tris base suspended Jacalin-Agarose for 90 minutes. I centrifuged the solution, stored the supernatant (supernatant A) and washed and resuspended the pellet in 0.175M Tris. I added an equal volume of 1M galactose to the Jacalin Agarose in 0.175M Tris and mixed the solution for two hours to elute the IgA from the Jacalin-Agarose. I spun the solution in a centrifuge and stored the supernatant (IgA eluted in galactose 1). To elute surplus IgA still bound to the Jacalin-Agarose, I

resuspended the pellet in galactose and mixed the suspension overnight. I separated the eluted IgA in galactose from the Jacalin-Agarose pellet by centrifugation and stored the supernatant (IgA eluted in galactose 2). Finally, I extracted remaining IgA from supernatant A by mixing it with the washed Jacalin-Agarose. After centrifugation and washing the pellet, IgA was eluted with 1M galactose, separated from the Jacalin-Agarose by centrifugation, and stored (IgA eluted in galactose 3).

I combined the 'IgA eluted in galactose' supernatants 1, 2 and 3 and dialysed them using Slide-A-Lyzer Dialysis Cassettes, 10K MWCO (Thermofisher 87733) overnight in 8L of sterile PBS. I concentrated the supernatant using Amicon Ultra-15 Centrifugal Filter Unit with Ultracel - 30K membrane (Merck Millipore UFC903024) to concentrations of 1-2mg/ml. I stored the concentrated IgA1 in 2ml aliquots in Lo-bind Eppendorf tubes (Sigma-Aldrich) at –20°C. Monomeric, dimeric and polymeric IgA1 fractions were isolated by fast protein liquid chromatography (FPLC) using a Superdex 200pg column (Amersham Biosciences). I concentrated the fractions with Amicon Ultra-4 Centrifugal Filter Units with Ultracel - 30K membrane concentrations of 1-2mg/ml. I stored the monomeric and dimer fractions in Lo-bind Eppendorf tubes at –20°C at the polymeric fractions in Lo-bind Eppendorf tubes at 4°C.

J. Western blot and Coomassie stains for IgA1

To confirm that the presence of IgA1 in IgA samples purified from human serum and plasma, I separated purified IgA by SDS-PAGE on a 10% gel under both non-reducing and reducing conditions. I prepared 1mm-thick 10% polyacrylamide resolving gels containing 33% Protogel (30% polyacrylamide, National Diagnostics EC890), 26% Protogel Resolving Buffer (National Diagnostics EC892), 0.15% ammonium persulfate (APS) and 0.15% N,N,N',N'-tetramethylethylenediamine (TEMED) in ddH_20. After the resolving gels had set, I poured 4% polyacrylamide sacking gel on top that contained 13% Protogel 30% solution, 25% Protogel Stacking Buffer (National Diagnostics EC893) 1% APS and 0.3% TEMED in ddH_2O and a 10-tooth comb was inserted. Once set, the gel was placed into electrophoresis buffer (25mM Tis hydroxymethylaminomethane, 192mM glycine, 0.1% sodium dodecyl sulphate (SDS, Sigma Aldrich EC893) and the comb removed. Thawed samples were diluted in NuPAGE lithium dodecyl sulfate (LDS) buffer x4 (Life Technologies NP007) with or without 5% 2-mercaptoethanol (MCE, Sigma –Aldrich 63689) for reducing and non-reducing conditions respectively. Proteins

were separated at 170mV constant voltage. The gel was separated from the glass plates in a container of transfer buffer (25mM Tis hydroxymethylaminomethane, 192 mM glycine, 20% methanol in ddH2O).

Electrophoresis bands of interest were visualised by staining with 0.1% Coomassie blue in fixer solution (45% methanol and 9% acetic acid in ddH20) for 60 minutes at room temperature. I confirmed the identification of these bands by Western blot. I repeated the separation of purified IgA by SDS-PAGE. Afer separating the gels from glass plates, I assembled a transfer cassette in transfer buffer. I performed elecrophoretic protein transfer to a 0.45um pore-sized polyvinylidene fluoride (PVDF) Immobilon-P membrane (Merck-Millipore, IPVH00010) a 100mV for 1 hour. I blocked non-specific binding with Carbo-Free blocking solution (Vector Laboratories) overnight. I incubated the membrane with a polyclonal sheep anti-human IgA1 antibody (Binding Site, AU087) followed by an anti-sheep IgG antibody (Sigma, A9452). After washing, the membrane was covered in Pierce enhanced chemiluminescence (ECL) western blot substrate A+B (Thermo Scientific) followed by dark room exposure using Amersham Hyperfilm ECL films (GE Healthcare). Subsequently, I will refer to the IgA samples purified by Jacalin-agarose from human samples as IgA1 preparations.

K. Western blot identification of complement proteins

I used western blot to detect complement proteins in IgA1 preparations purified from human serum and plasma. Using the method detailed above, I separated IgA1 preparations by SDS PAGE on a 10% gel under both non-reducing and reducing conditions and transferred he bands to a PVDF membrane. I blocked non-specific binding with 5% non-fat dried milk (NFDM, Marvel). Films were developed as above. The conditions and primary and secondary antibodies used to detect each antigen are detailed in Table 3.

Primary antibody	Concentration	Manufacturer and number	Secondary antibody	Concentration	Manufacturer and number
Mouse monoclonal anti-human CFHR5	1:2000	Abnova 81494-B01P	Rabbit anti-mouse IgG HRP	1:2000	Dako P0260
Rabbit polyclonal anti human CFHR5	1:1000	Abnova 81494-D01P	Swine anti-rabbit IgG peroxidase	1:2000	Dako P0217
Goat polyclonal anti-human FH	1:20000	Quidel A312	Mouse anti-goat/sheep IgG peroxidase	1:20000	Sigma A9452
HRP-conjugated goat polyclonal anti-human C3	1:3000	Cappel 55237	N/A		

Table 3. Antibodies used for the western blot detection of complement proteins in purified IgA1 preparations

L. Statistics

I used GraphPad Prism (version 6.0) for data analyses. Normally distributed continuous variables were tested with unpaired or paired t-tests and one-way ANOVA for multiple groups. Continuous variables with skewed distribution were tested using Mann-Whitney U tests and I used Kruskall-Wallis tests for multiple groups. I used Wilcoxon matched-pairs signed rank test for matched non-normally distributed variables. Differences in categorical data were tested by Chi-squared test, or Fisher's exact test if the sample cohort was less than ten. I used Pearson's or Spearman rank correlations to assess the correlations between variables. A value of $p<0.05$ was considered statistically significant. Confidence intervals were calculated using the Hodges-Lehmann method. When relevant, I adjusted for multiple analyses with the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli[281]. I have noted the points at which I applied the adjustments in the relevant results section.

Results

A. Patient cohorts

Introduction

I aimed to detect, quantify and analyse markers of complement deregulation in blood and renal tissue from patients with either IgAN or C3G. This necessitated the collection and then detailed phenotyping of a cohort of patients with IgAN and a cohort of patients with C3G. For controls, I also collected blood samples from healthy individuals and surplus renal tissue from individuals with other renal diseases. In this chapter I will describe the cohorts of patients and healthy individuals I collected. The subsequent Results chapters will detail the experiments performed on the samples. Because samples from the same individuals and cohorts have been used in a number of experiments and assays, to save repetition, I will describe each cohort in this chapter.

In general, my collection of detailed demographic, clinical and histology data from the IgAN and C3G cohorts was motivated by two aims. First, I wanted to compare the cohort of patients I enrolled with other published cohorts of patients. This would itself demonstrate whether my cohort was representative of other disease cohorts, and would potentially identify cohort differences that might explain or contribute to experimental results. Second, I aimed to correlate markers of FHR protein and complement activity with disease severity in IgAN and C3G. This necessitated detailed analysis of clinical features to stratify patients as having progressive or stable disease. Like all retrospective clinical data collections, data were limited by the quality, timing and availability of recorded information. As mentioned in the Methods chapter, the differences in timing between patient diagnostic biopsy and collection of serum and plasma samples at study enrolment is an important limitation of my methodology. Conversely, the long duration from diagnosis to enrolment and data collection allowed me to be confident in my definitions of disease severity, which can be difficult to predict in prospectively enrolled cohorts. I will next describe the recruitment, data collection and analysis of each cohort.

Cohorts of healthy individuals

Healthy control samples were obtained from three different groups. The first was healthy volunteers who consented to give serum, blood and DNA for analysis. The second group consisted of members of the TwinsUK cohort,[274] a cohort of twins of Caucasian ethnicity consented for their serum and DNA to be used in medical research. These samples were provided by Dr Mario Falchi (Kings College London, UK). We selected at random samples from one individual of each pair of twins. The third group was a cohort of healthy volunteers from Denmark collected by our collaborator, Professor Steffen Thiel (Aarhus University, Denmark). Demographic data and plasma and serum samples were available from all volunteers in this cohort. I will describe the control cohorts and the use of samples in more detail in the releveant Results chapter.

Transplant cohorts

We used two renal transplant recipient cohorts to delineate the relative contribution of disease activity and non-specific renal impairment to protein levels (see the relevant Results chapters). We calculated eGFR and measured serum levels of FHR1 and lectin complement proteins pre and post renal transplantation in a cohort of 28 transplant patients with biopsy-proven native IgAN and 37 transplant recipients with ADPKD. The results of these assays will be detailed in the relevant Results chapter. Because I measured serum FHR1 levels, we assessed *CFHR1* copy number. As described in the introduction, circulating FHR1 levels are influenced by the presence of a common deletion polymorphism of the *CFHR1* gene. The expected polymorphism allelic prevalence varies with ethnicity, but is estimated at 55% in individuals of African ethnicity, 0-5% in individuals of East Asian ethnicity, and has intermediate prevalences in individuals of European ancestry[2]. All patients had received their transplant and clinical follow-up at Imperial College Healthcare NHS Trust. All ADPKD patients had a radiological diagnosis. All transplant patients gave consent for the storage and use for research of serum and plasma samples surplus to clinical diagnostic requirements. I used blood samples that were taken within four weeks before, and between 12 and 16 weeks after transplantation. The precise timing of blood sampling was recorded and is shown in table 4. The cohorts had comparable pre-transplant characteristics (Table 4) and no patients had a clinical diagnosis of delayed graft function, transplant rejection or disease recurrence at the time serum samples were taken.

Characteristics	IgAN (n=28)	ADPKD (n=37)
Male (percentage)	21 (75.0)	26 (74.3)
Caucasian (percentage)	24 (68.6)	15 (53.6)
Median age (range) – years	46.9 (26.8-68.5)	53.7 (26.4-71.5)
Genotyping of rs6677604* GG / AG / AA – n (percentage)	25 (89.3) / 3 (10.7) / 0 (0)	23 (62.2) / 11 (29.7) / 3 (8.1)
Median time pre-transplant sample to transplant – days	7 (1-21)	5 (1-14)
Post transplant median eGFR – ml/min	45.7 (34.7-84.4)[1]	48.5 (33.1-64.1)[1]
Median time transplant to post-transplant sample – days	93 (84-112)	93 (87-101)

Table 4. Cohort characteristics for patients with IgAN or ADPKD who received a renal transplant.

If not otherwise stated, values within parentheses represent interquartile range; [1]P<0.001 compared to pre transplant levels. I include this analysis to demonstrate eGFR showed a significant improvement following transplantation. Although expected, this is not a universal outcome following transplantation. I felt it necessary to demonstrate this because this cohort will be used to estimate whether changes in circulating levels of complement proteins following transplantation might be explained by changes in eGFR
*rs6677604 tags the *CFHR3-1* deletion.

IgA nephropathy cohort

a. IgA nephropathy cohort design

I aimed to investigate the contribution of complement activity to IgAN and specifically to investigate whether imbalances of complement activity, deregulation, FH and FHR1 and FHR5 contributed to disease severity. I therefore needed to enrol, analyse and collect serum, plasma, DNA and stored biopsy samples from a large cohort of IgAN patients. I utilised a cohort collected through an Medical Research Council (MRC)-funded study entitled The Causes and Predictors of Outcome in IgA Nephropathy study. This was a retrospective cohort UK study of patients with biopsy-proven IgAN. The investigators on this study were my supervisors, Professors Matthew Pickering and Terry Cook, Dr Mario Falchi and Professor Charles Pusey. Sample collection was coordinated by Dr Lomax-Browne (Imperial College London, UK) and I performed the data collection, anonymization, data organisation and analysis of this study. The Causes and Predictors of Outcome in IgA Nephropathy study enrolled patients with biopsy-proven IgA nephropathy from 18 sites across the UK. Imperial College London was the study sponsor and Professor Matthew Pickering was the principal investigator. Ethical approval was granted by the UK National Research Ethics Service (RES) Committee, South East Coast and Surrey: reference 14/LO/0155. Enrolled patients consented to the use of stored clinical data, stored surplus renal biopsy tissue, and to provide samples of blood, serum, DNA and urine for research analysis. As we did not know the frequency of the outcome variables we aimed to measure, we did not use formal power calculations to decide on a target cohort size. However, other studies have identified significant associations between histology and complement markers and IgAN disease severity with patient cohorts of 270 patients[193, 253]. We therefore aimed to consent and enrol 300 patients. I would compare the patients to healthy controls and I stratify the patients to severe and stable disease phenotype cohorts, and compare these two disease cohorts.

The clinical information collected is detailed in Table 5 and included data from presentation and recruitment, previous and current treatment including immunosuppression, antihypertensive agents, dialysis and renal transplantation. The eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) Creatinine Equation and corrected for Body Surface Area, as calculated with the Mosteller equation, or the Boyd equation if the patient's height was not available, and to a maximum of 120ml/min. I reviewed and

recorded information from all biopsy reports. In addition to the Oxford Classification Scores, I recorded the number of glomeruli and the presence of cellular and fibrocellular crescents (Table 6).

In addition to collecting clinical and pathology data, Dr Hannah Lomax-Browne measured serum IgA1 and gd-IgA1 levels in all IgAN patients. The current theory of IgAN pathogenesis implicates gd-IgA1 levels in disease development. As described previously, other groups have reported higher circulating gd-IgA1 levels in IgAN patients[275]. We questioned whether our IgAN cohort would also show associations between higher gd-IgA1 levels and progressive disease. Serum IgA levels were measured by ELISA[219]. Serum gd-IgA1 was measured using a lectin-based ELISA[219]. The intra-class correlation coefficient (ICC) for the IgA assay was 0.74 (95% CI: 0.63-0.83) and the ICC for the gd-IgA1 assay was 0.89 (95% CI: 0.73-0.95).

Characteristic	All IgAN (n=323)	Progressive IgAN (n=191)	Stable IgAN (n=83)
Male/Female	214/109	127/62	51/32
Caucasian/Non-Caucasian	271/52	155/36	73/10
Median age (range) – years	48 (18-84)	48 (19-84)	48 (18-82)
Median eGFR – ml/min. NR >90ml/min	52.6 (28.7-81.8)	44.2 (17.7-71.3)[1]	77.3 (47.6-106)
Median urine PCR – mg/mmol. NR <30mg/mmol	44 (16-117, n=220)	46 (16-141.5, n=125)	40 (15-90, n=76)
Median anti-hypertensive drug classes per patient	1.6	1.6	1.4
Patients with ACEi/ARB, excluding dialysis patients - %	73.7 (n=199)	77.3 (n=176)	79.8 (n=83)
Median systolic/diastolic blood pressure – mmHg	134 (122-145) / 79 (70-88, n=294)	136 (124 - 146)[2] / 79 (70-88, n=181)	128 (116 - 140) / 77.5 (70-85, n=83)
Median duration biopsy to recruitment (months)	56.1 (22.8-104.7)	52.2 (23.3-94.8)	68.4 (22.8-171.3)
Reach ESRD - %	34.1	57.5[1]	0
History of macroscopic haematuria - %	27.8	20.1[1]	44.9
Diagnosis of Henoch-Schonlein purpura - %	6.4	8.9[1]	0
Median serum IgA – g/l. NR 0.7-4.0g/l	3.4 (2.9-4, n=293)	3.3 (2.7-3.8, n=178)[3]	3.7 (3.2-4.3, n=88)
Median serum gd-IgA1 – AU	0.50 (0.41-0.58, n=293)	0.52 (0.42-0.59, n=178)[4]	0.47 (0.37-0.55, n=88)

Table 5. IgA nephropathy patient cohort characteristics at diagnosis

Values within parentheses represent interquartile range and number analysed if less than the respective cohort numbers.

NR – normal range; ACEi - Angiotensin-converting enzyme inhibitor; ARB - Angiotensin receptor blocker; AU – arbitrary units; urine PCR – urine protein:creatinine ratio. [1]P <0.0001 vs stable IgAN, [2]P =0.0014 vs stable IgAN, [3]P =0.0007 vs. stable IgAN; [4]P =0.01 vs. stable IgAN. P-values shown have been adjusted for multiple analyses to minimise the false discovery rate.

Biopsy Characteristics		All IgAN (n=267)	Progressive IgAN (n=154)	Stable IgAN (n=76)
Median glomeruli per biopsy		14 (IQR 9-19)	11 (IQR 6-16)	10 (IQR 8-17)
Mesangial hypercellularity		221 (82.8%)	134 (87.0%)[2]	57 (75.0%)
Endocapillary hypercellularity		59 (22.2%)	51 (33.1%)[1]	3 (3.9%)
Segmental sclerosis		169 (63.5%)	111 (72.1%)[1]	33 (43.4%)
Tubular atrophy 0-25%		135 (50.6%)	58 (37.7%)[1]	61 (80.3%)
Tubular atrophy 26-50%		103 (38.7%)	73 (47.4%)[1]	14 (18.4%)
Tubular atrophy >50%		29 (11.7%)	23 (14.9%)[3]	1 (1.3%)
Cellular crescents in less than one fourth of glomeruli		49 (18.0%)	42 (27.3%)[1]	7 (9.2%)
Cellular crescents in at least one fourth of glomeruli		25 (8.6%)	24 (15.6%)[3]	1 (1.3%)
Glomerular deposition (at less intensity than IgA)	IgA	267 (100%)	154 (100%)	76 (100%)
	IgG	34 (13%)	22 (14.3%)	7 (9.2%)
	C3	205 (77%)	118 (76.6%)	56 (73.7%)
	C1q	61 (23%)	41 (34.7%)[4]	11 (14.5%)

Table 6. IgA nephropathy histology features at diagnostic biopsy

IQR, interquartile range; n, number analysed if less than the total cohort numbers. [1]P <0.0001, [2]P=0.026, [3]P <0.001 and [4]P=0.008 vs. stable IgAN. P-values shown have been adjusted for multiple analyses to minimise the false discovery rate.

b. Definitions of progressive and stable IgA nephropathy

My research aimed to determine whether complement deregulation influences the severity of glomerular inflammation in response to deposited IgA. Complement activity and imbalance would be predicted to potentially alter outcome and severity in cases with renal inflammation. I therefore aimed to identify and differentiate groups of IgAN patients by with and without evidence of glomerular inflammation. I wanted to compare patients who mounted an inflammatory response to deposited IgA with patients who apparently immunologically tolerated glomerular IgA deposition. I therefore designed criteria to divide my IgAN cohort into patients with 'progressive' and 'stable' disease. I aimed to capture patients with immunologically active glomerular inflammation in the progressive cohort, and patients who were relatively tolerant of deposited glomerular IgA in the stable cohort. I acknowledged that some patients may not meet either criteria. This does not mean these patients have IgAN of intermediate severity, but instead reflects the unavailability of information needed to accurately categorise these patients.

The design of these criteria was motivated by trying to demonstrate evidence of FH and FHR protein imbalance for the first time in IgAN. Due to the novel and exploratory nature of the research, I wanted to first test whether differences in FH regulation and complement activity could be seen in patients with and without objective evidence of glomerular inflammation. My aim was not initially to correlate complement activity with rates of disease progression in all IgAN patients, nor demonstrate FHR proteins are biomarkers of IgAN severity. My aim necessitated new criteria for patient selection. Traditional definitions of severe IgAN include progression to ESRD, 50% loss of eGFR, and ongoing significant proteinuria. However, these factors may capture patients who have non-inflammatory progressive chronic kidney disease due to a host of other factors such as glomerular sclerosis, tubular atrophy, interstitial fibrosis, microvessel renovascular disease, lower urinary tract outflow obstruction, concomitant use of nephrotoxic medications and recurrent urinary tract infections. Complement activity would not be predicted to drive severity in cases with these features.

Progressive disease was defined by the patient meeting at least one of the following criteria at any point during the follow-up period from diagnosis to study enrolment and data collection: (1) Progression to ESRD without histology evidence of a second pathology causing renal impairment; (2) Endocapillary hypercellularity on biopsy; (3) Cellular and/or fibrocellular crescents on biopsy; (4) Treatment with immunosuppression (including corticosteroids) for native IgAN; (5) Clinical HSP, unless spontaneous resolution and >20 years

follow-up with ‘stable’ criteria; (6) 50% loss of eGFR or average annual loss of eGFR of more than 5ml/min without evidence of a second pathology causing renal impairment. Stable disease was defined as the patient meeting none of the criteria for progressive IgA Nephropathy and meeting all of the following criteria: (1) Urine protein-creatinine ratio less than 100 units or daily proteinuria of less than 1g/24 hours; (2) Combined Oxford classification MEST score of less than 3; (3) Average annual loss of eGFR of less than 3ml/min/1.73m^2 (Table 7). Patients that met neither cohort criteria were not categorised as progressive nor stable.

In addition to comparing progressive and stable patients, I was also interested in analysing the cohort of IgAN patients who had received immunosuppression treatment. Immunosuppression options for IgAN include non-specific anti-inflammatory agents such as corticosteroids and mycophenolate mofetil. Therapeutic inhibitors of complement activity have not been routinely used to treat IgAN patients and none of the IgAN patients in our cohort received complement inhibition. I predicted patients who failed to show clinical improvement following non-complement targeting immunosuppression were more likely to have disease driven by complement activity. Those patients who responded to and improved following immunosuppression were more likely to have disease pathogenesis dependent on other inflammatory stimuli and may be less dependent on complement activity. I therefore applied the same progressive and stable criteria to all data available from patient follow-up since treatment with immunosuppressive agents, and compared cohorts who improved to meet stable criteria following immunosuppression with those who met criteria for progressive disease despite immunosuppression.

Progressive IgA Nephropathy	Patient meets any one of the following criteria: 1. Progression to end-stage renal failure (ESRF) without histology evidence of a second pathology causing renal impairment 2. Renal biopsy evidence of endocapillary hypercellularity 3. Renal biopsy evidence of cellular and/or fibrocellular crescents on biopsy 4. Treatment with immunosuppression (including corticosteroids) for native IgAN 5. Clinical henoch schonlein purpura, unless spontaneous resolution and >20 years follow-up with 'Stable' criteria 6. 50% loss of eGFR or average annual loss of eGFR of more than 5ml/min without evidence of a second pathology causing renal impairment
Stable IgA Nephropathy	Patient meets none of the criteria for progressive IgA Nephropathy and meets all of the following criteria: 1. Urine protein:creatinine ratio less than 100 units or daily proteinuria of less than 1g/24 hours 2. Combined Oxford classification MEST score of less than 3 3. Average annual loss of eGFR of less than 3ml/min

Table 7. Criteria for progressive and stable IgAN nephropathy

c. IgA nephropathy cohort: Results

We consented and recruited 334 patients from 18 sites across the UK. Twelve patients were excluded. Eight had an alternative diagnosis stated in the biopsy report; one biopsy reports described no tissue for immunostaining; and three biopsy reports could not be located. Consequently, 322 patients were included in the cohort for analysis. Of these, 120 were enrolled at Imperial College Healthcare NHS Trust. Using the criteria detailed in methods, 190 patients had progressive IgAN and 83 patients had stable IgAN. 49 patients fit neither the criteria for progressive nor stable IgAN (Figure 7). Of the progressive IgAN sub-cohort, 166 patients presented with native renal IgAN and 24 had data from post-transplant follow-up available only.

The cohort clinical data is summarised in table 5. At some point during follow-up (median follow-up 56.1 months, range 22.8-104.7 months), 110 patients (34.1%) reached ESRD secondary to IgAN and 84 (26%) patients received a renal transplant. Of the 166 patients who met my criteria for progressive IgAN, 61 (36.7%) were treated with immunosuppression; 23 patients (13.8% of patients with progressive disease and 37.7% of patients who received immunosuppression) continued to meet the criteria for progressive IgAN despite immunosuppression treatment; 38 patients who received immunosuppression (22.9% of progressive IgAN patients and 62.3% of patients who received immunosuppression) improved following immunosuppression treatment and subsequently met the criteria for stable IgAN.

The IgAN cohort we collected is similar to other reported cohorts in terms of the gender distribution and blood pressure[183, 276]. Our cohort had a higher average age, lower eGFR and less proteinuria on average than other IgAN cohorts[183, 276]. This may reflect the contemporaneous trend towards increasing proportions of elderly patients being diagnosed with glomerulopathies. About 16% (52 of 323 patients) of our IgAN cohort were non-Caucasian. This is a greater proportion than many published IgAN cohorts and probably reflects the demographics of the patients at Imperial College Healthcare NHS trust, from where a significant proportion of the cohort was enrolled. Our cohort showed a similar distribution of histopathology features as a cohort of over 1100 IgAN patients from European centres[276].

In order to estimate whether our IgAN patient cohort was representative of other published cohorts, I aimed to describe non-complement factors thought to be important to IgAN pathogenesis. The cohort of patients meeting the criteria for progressive IgAN showed lower eGFR, higher systolic blood pressure, a greater rate of annual loss of eGFR and a lower probability of survival without 50% reduction in eGFR or ESRD at five years

change (Table 5) than stable IgAN patients. Dr Hannah Lomax-Browne measured the serum IgA and gd-IgA1 levels in all patients (n=294) and healthy controls (n=161) with serum samples available at the time of assessment. Consistent with previous reports[207, 247, 275, 277], the median serum IgA and serum gd-IgA1 were higher in patients compared to controls. Median serum IgA was 3.4 g/L (inter-quartile range (IQR) 2.9-4.0 g/L) in patients and 2.8g/L (IQR 2.2-3.2 g/L) in healthy controls. Median gd-IgA1 was 0.50 arbitrary units (AU) of ELISA optical density (IQR 0.41-0.58 AU) in patients and 0.42 (IQR 0.33-0.55 AU) in healthy controls. The median serum IgA was similar in patients with progressive compared to stable IgAN, whereas gd-IgA1 levels were higher in the cohort with progressive disease (Table 5). This is similar to other cohorts[246]. Similarly, serum gd-IgA1 levels were higher in patients on immunosuppression who had progressive versus stable disease (0.54 AU vs 0.44 AU, p=0.04) (Figure 8A and 8B). Consistent with these data, we detected a negative correlation between serum gd-IgA1 levels and eGFR at the sampling time point (Figure 8C). These data suggested our cohort was comparable to other published cohorts. However, the differences in gd-IgA1 levels between the groups were small with significant overlap between the groups. This is addressed in the discussion section of this chapter. These data were generated by Dr Hannah Lomax-Browne.

Figure 7. IgA nephropathy patient enrolment

We consented and recruited 334 patients with IgAN from 18 sites to the Causes and Predictors of Outcome in IgA Nephropathy study. Twelve patients were excluded. Consequently 322 patients were analysed. At some point during follow-up (median follow-up 56.1 months, range 22.8-104.7 months), 110 patients (34%) reached ESRD secondary to IgAN and 84 (26%) patients received a renal transplant. Of the 166 patients who met my criteria for progressive IgAN, 61 (36.7%) were treated with immunosuppression; 23 patients (13.8% of patients with progressive disease and 37.7% of patients who received immunosuppression) continued to meet the criteria for progressive IgAN despite immunosuppression treatment; 38 patients who received immunosuppression (22.9% of progressive IgAN patients and 62.3% of patients who received immunosuppression) improved following immunosuppression treatment to then meet criteria for stable IgAN.

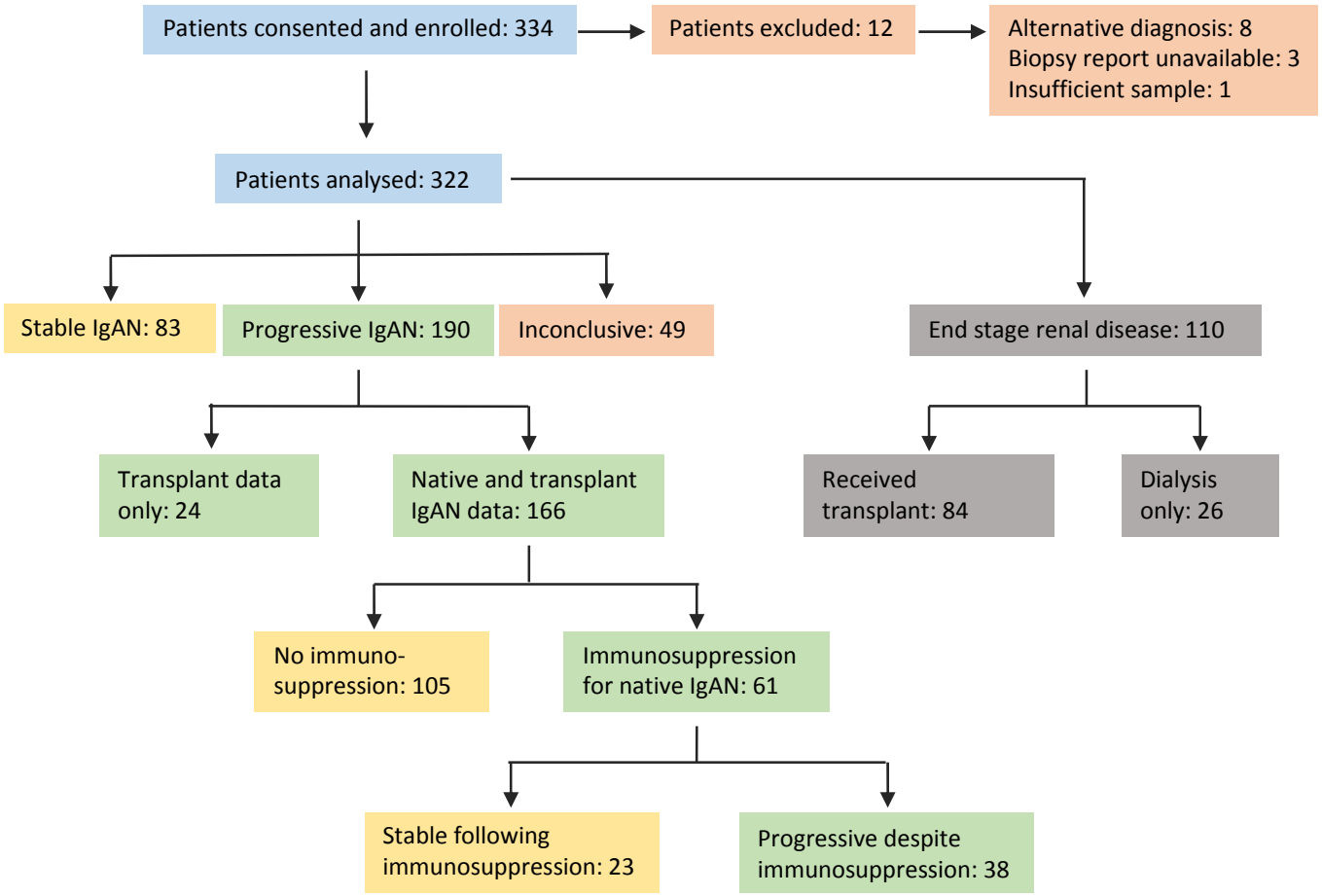
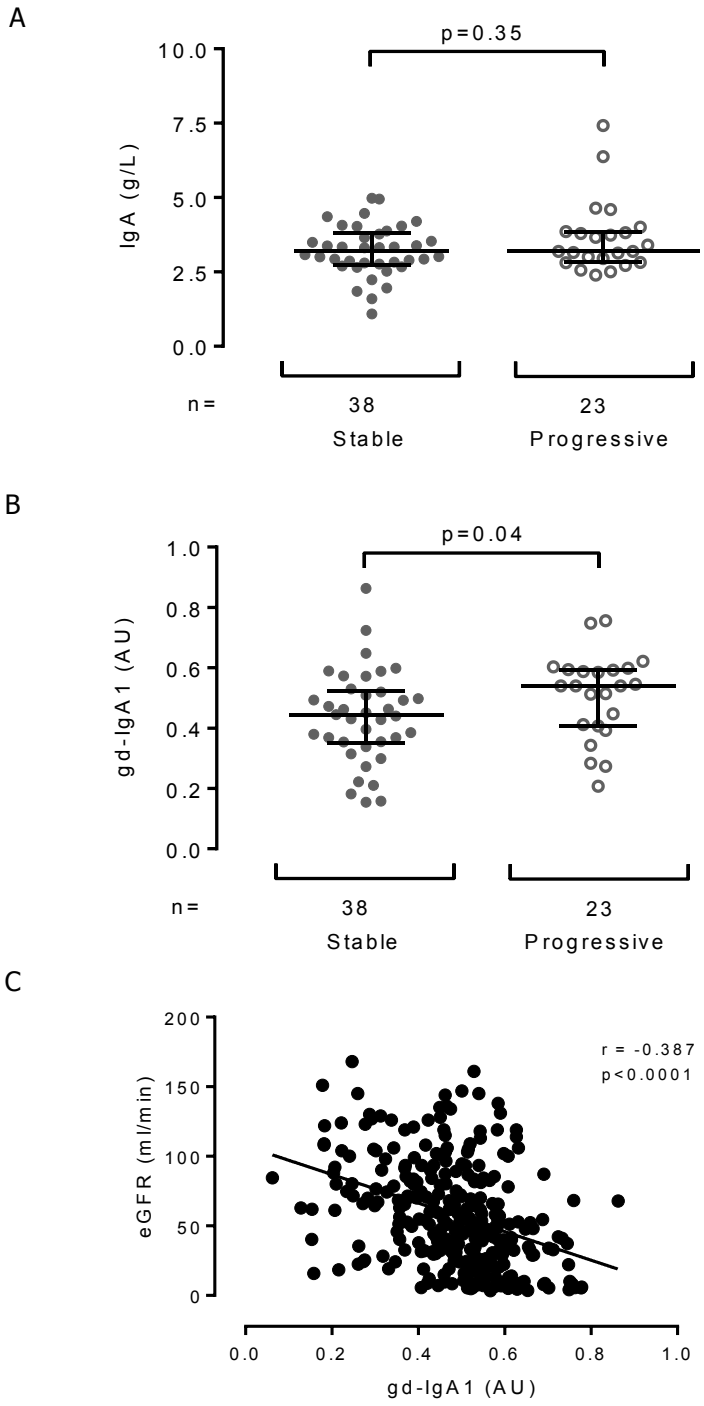


Figure 8. Serum IgA and galactose-deficient IgA1 levels in IgA nephropathy.

Serum IgA (A) and gd-IgA1 (B) concentration in all IgAN patients in our cohort who had received immunosuppression treatment for IgAN. Patients were divided on their clinical response to immunosuppression into those with stable (solid circles) and progressive (empty circles) disease after immunosuppression treatment. The progressive IgAN cohort shows higher median gd-IgA serum concentration. Lines represent median and interquartile range.
C: Correlation of eGFR and serum gd-IgA1 concentration in IgAN patients (n=293). Negative correlation was calculated with Spearman rank correlation and the black line represents the correlation equation.



d. IgA nephropathy cohort: Discussion

The IgAN cohort we collected and analysed is representative of other IgAN populations[183]. However, our cohort has a number of unique characteristics, which reflect in part the unique demographic of our study population. The progressive IgAN cohort was characterised by features known to correlate with poor prognosis in IgAN, such as reduced eGFR, more tubular atrophy at diagnostic biopsy and raised serum gd-IgA1 at presentation. However, it also included cases with histology features of active inflammation, such as patients with HSP and endocapillary hypercellularity. This suggests my classification captures patients with more severe IgAN and features of active glomerulonephritis. Analysis of the histology and immunostaining features at diagnostic biopsy demonstrated we had selected patients with histology features of inflammation in the progressive cohort. Despite this, the proportion of patients with detectable glomerular C3 was very similar in the stable and progressive IgAN cohorts. This emphasises the need for more precise immunohistochemic markers of complement activity for IgAN.

The differences in median gd-IgA1 levels was small and there was significant overlap between the compared cohorts. Furthermore, the quantification of gd-IgA1 is an arbitrary unit of relative ELISA optical density, and how this reflects biological abundance in vivo is difficult to predict. Based on the correlation between reducing eGFR and increased gd-IgA1 levels, the differences documented in our cohorts may be explained by an accumulation of circulating gd-IgA1 secondary to non-specific loss of renal function. A detailed analysis of gd-IgA1 levels in a separate cohort of 379 IgAN patients from the UK also found average gd-IgA1 levels were higher in progressive than stable IgAN patients and gd-IgA1 correlated with serum creatinine^{-1} [246]. This study demonstrated gd-IgA1 levels were not significantly higher in a cohort of 308 patients with membranous nephropathy (MN) compared to healthy controls, and gd-IgA1 did not correlate with serum creatinine^{-1} in the MN cohort[246], suggesting the correlations are specific to IgAN. Although we completed this assessment in order to establish whether our cohort showed similar characteristics to other published patient groups, the doubtful differences in gd-IgA1 levels between our sub-cohorts suggest two interpretations. First, differences we might detect in complement activity and FH deregulation between progressive and stable patient cohorts was unlikely to be significantly confounded or driven by differences in gd-IgA1 levels. And, secondly, the pathogenic relevance of gd-IgA1 levels in IgAN seems doubtful, strengthening my hypothesis that additional factors, such as complement deregulation, determine disease severity.

It is noteworthy that a number of biopsies did not have enough glomeruli to meet the criteria stipulated in the Oxford Classification of IgA nephropathy, which necessitates 8 glomeruli for assessment of mesangial hypercellularity, interstitial fibrosis and tubular atrophy[194]. This is an inherent limitation of using stored surplus diagnostic clinical biopsy tissue. The inclusion of cases with too few glomeruli could cause some progressive cases to be missed, as glomeruli with focal inflammatory lesions may not be sampled, or to be scored inaccurately as the denominator for percentage tubular atrophy and cellular crescent calculations may be too small. However, my presentation of the histology data in this way aims to allow qualitative comparison with other IgAN cohorts. I am not aiming to calculate alternative risk progression scores and therefore I believe the inclusion of cases with relatively few glomeruli is valid.

I did not use a single metric of renal filtration impairment or proteinuria to categorise disease severity because such biomarkers do not reliably categorise IgAN patients into progressive, active and stable, quiescent disease. This is because patients with chronic renal impairment of any cause will often cause significant proteinuria and progressive deterioration in glomerular filtration. Consequently, IgAN may not be the cause of renal impairment in a proportion of IgAN patients. For example, patients who present with established CKD and receive a biopsy diagnosis of IgAN may have had other medical insults that have contributed to irreversible kidney damage. Also, patients with IgAN may have additional co-morbidities, such as hypertension, diabetes, or significant cardiovascular disease that cause impaired renal function. In contrast, patients with relatively short follow-up may show no change in estimated glomerular filtration but may nevertheless have histology evidence of active glomerulonephritis. The criteria I designed therefore aimed to allow for these subtleties and differentiate patients with injurious, inflammatory IgAN, from those without IgAN-dependent renal deterioration. My definitions of stable and progressive disease have a number of limitations. The definitions capture a number of sub-cohorts of IgAN patients who would not traditionally be grouped together. Most obviously the definitions include patients who have progressed to significant loss of eGFR, ESRD and who are either on dialysis or have a renal transplant. The justification of this was that these cohort are likely to include patients with the most severe disease, and therefore could include patients with marked inflammation and complement activation. Clearly, the analysis of these groups could be limited and confounded by different encompassed clinical situations. Most obviously, the effect of maintenance haemodialysis and post-transplant immunosuppression on the measured markers is not known. Were I to detect differences in complement

activity and FH deregulation between these cohorts, I would plan to re-analyse the data based on more specific and traditional definitions of progressive disease, and I would analyse cohorts receiving dialysis or with a renal transplant at the time of study enrolment separately. Analysis of potential biomarker use obviously necessitates separate prospective analysis and comparison with traditional markers of disease severity, and is not the aim of this research.

C3 glomerulopathy cohort

a. C3 glomerulopathy cohort design

C3 glomerulopathy is rare but many C3G patients are referred to our Centre for expert review. My strategy for establishing a C3G patient cohort was to first identify all patients with C3G at Imperial College Healthcare NHS Trust. I reviewed the diagnosis of all kidney biopsies performed over a 20-year period (6592 native and 2577 transplant biopsies) to identify those with biopsy features of C3G. I derived clinical and pathology data from medical records. Ethical approval was through the Natural History Study of C3 Glomerulopathy: Discovery of Histological Predictors of Outcome study (UK National RES reference 16/WM/0497) and ICHTB. ICHTB is supported by the NIHR Biomedical Research Centre based at Imperial College Healthcare NHS Trust and Imperial College London. ICHTB is approved by UK National RES to release human material for research (12/WA/0196).

For the C3G cohort, the renal endpoints I assessed were loss of 50% of eGFR from presentation and ESRD, defined as commencing dialysis or receiving a renal transplant. Patients with less than 12 months follow-up were excluded from outcome analyses. eGFR was calculated using the CKD-EPI equation[278] for adults and the Schwartz formula[279] for children and corrected to a maximum of 120ml/min/1.73m2. Nephrotic syndrome was defined as a combination of serum albumin of less than 32g/L, oedema, and urine protein:creatinine ratio (UPCR) of at least 250mg/mmol or 24-hour urine proteinuria of at least 3g. C3 nephritic factor was detected with immunofixation electrophoresis (The Binding Site, UK) by the clinical pathology service at Imperial College Healthcare NHS trust.

b. C3 glomerulopathy cohort: Results.

In our C3G cohort (Tables 8, 9 and 10), a 50% loss of eGFR during follow up (median 70.2 months, range 18.6 to 143.1 months) was associated with an MPGN light microscopy pattern (P=0.02, n=25, odds ratio [OR] = 9.8, 95% confidence interval [CI] 1.5to 47.1, table 9) and the eGFR at time of biopsy (P=0.049, n=25,95% CI -0.2 to -76.6, table 8). Proteinuria at diagnostic biopsy did not associate significantly with a 50% loss of eGFR in our entire cohort but did show significant association with this outcome in the sub-cohort of C3G patients from whom surplus biopsy tissue was available for analysis (UPCR 430mg/mmol in cohort with and 70mg/mmol without 50% eGFR loss during follow-up. P=0.04, n=20, 95% CI 8 to 739mg/mmol). The serology and genetic complement analysis completed on our C3G cohort is shown in Table 11. Of 12 patients tested, four patients had an identified complement gene variant. Two of these four patients with an identified complement gene variant had the heterozygous *CFHR5* gene duplication mutation that defines CFHR5 nephropathy. Surplus tissue was available from 34 renal biopsies from 19 patients in our cohort of 27 individuals with biopsy-proven C3G. Nine of these were transplant biopsies from five patients. Two of the transplant biopsies with available tissue were surveillance biopsies indicated by protocols to monitor for sub-clinical transplant pathology.

c. C3 glomerulopathy cohort: Discussion

Although out cohort is relatively small, it is similar to other C3G cohorts in patient age at diagnosis, the wide spectrum of histopathology features captured by a diagnosis of C3G and the prevalence of significant renal impairment at diagnosis and progression to renal impairment[144, 148, 173]. Our C3G cohort is unique because of the availability of detailed clinicohistopathology data from patients with CFHR5 nephropathy, indolent transplant recurrent C3G, patients who underwent repeat biopsies, including transplant biopsies, and the availability of stored surplus unstained tissue for further immunohistochemic analysis. This study was a retrospective analysis of a relatively small C3G cohort. Nevertheless, I made a number of observations relevant to C3G pathogenesis. I identified an association between MPGN light microscopy pattern at diagnostic biopsy and a 50% loss of eGFR at an average of just over six years' follow-up. This was not demonstrated in a recent histopathology analysis of a large C3G cohort[148]. It is not clear whether this reflects differences in study design, local histopathology analysis and reporting or represents a novel finding in our cohort. The influence of histological morphology and ultrastructual deposit location on disease severity requires further investigation.

Characteristic	Number	Percentage	Median (range)
Biopsy at centre	27		
Age (years)			30.4 (9.4-73.8)
Male	14	51.9%	
Caucasian	16	59.3%	
eGFR (ml/min/1.73m^2)			74 (5-120)*
Urine protein:creatinine ratio (mg/mmol)			263 (0-1653)
Serum albumin (g/L)			29 (11-43)
Haematuria (macro- and microscopic)	16	59.3%	
Nephrotic syndrome	14	51.9%	
Paraproteinaemia (n=19)	3	17.6%	

Table 8. C3 glomerulopathy clinical features at diagnostic biopsy

* Associates with 50% loss of eGFR at follow-up. Of the 25 patients with follow-up data available, patients with 50% loss of eGFR during follow-up had median eGFR at diagnostic biopsy of 45.0 vs 117.7 ml/min/1.73m2 for those without (p=0.049, 95%CI -0.2 to -76.6).

Diagnostic biopsy features (n=27)	Number	Percentage
Dense deposit disease	6	22.2%
Light microscopy pattern:		
- Mesangial proliferative	11	40.7%
- Endocapillary proliferative	5	18.5%
- Membranoproliferative GN	16	59.3% **
- Other	2	7.4%
Cellular crescents	7	25.9%
Endocapillary hypercellularity	17	63.0%
Tubular atrophy:		
- None	5	18.5%
- 1-10%	9	33.3%
- 11-25%	8	29.6%
- 26-50%	5	18.5%

Table 9. C3 glomerulopathy histology features at diagnostic biopsy

** Associates with 50% loss of eGFR at follow-up. Of the 25 patients with follow-up data available, 78.6% of patients with 50% loss of eGFR during follow-up had MPGN on biopsy vs 27.3% of those without 50% eGFR loss (p=0.02, OR = 9.8, 95% CI 1.5 to 47.1).

Treatment after diagnostic biopsy (n=25)	Number	Percentage	Median (range)
ACEi/ARB	20	80.0%	
Any immunosuppression (including corticosteroids)	16	64.0%	
- Corticosteroids	14	56.0%	
- Mycophenolate mofetil	9	36.0%	
- Cyclophosphamide	7	28.0%	
Follow-up (n=25)			
Duration follow-up (months)			77.9 (31-155.7)
Loss of 50% eGFR at follow-up	14	56.0%	
Time diagnosis to 50% eGFR loss (months)			12.9 (0.1-50.8)
ESRD	12	48.0%	
Time diagnosis to ESRD (months)			40.0 (1-69.2)
Transplant	9	36.0%	
Transplant recurrence of C3G (n=9)	9	100.0%	
Time transplant to recurrence (n=9), months.			4.9 (1-26.8)
Recurrence within 6 months (n=9)	4	44.4%	
Recurrence within 18 months (n=9)	9	100%	

Table 10. Treatment and follow-up of C3 glomerulopathy

ACEi – Angiotensin-converting-enzyme inhibitor. ARB – angiotensin II receptor blocker.

Complement assessment at diagnostic biopsy	Number tested	Percentage of cohort	Number	Percentage	Median (range)
Serum C3, g/L (NR 0.7-1.7)	25	100%			0.77 (0.10-1.91)
Low serum C3	25	100%	11	44.0%	
Serum C4, g/L (NR 0.16-0.54)	25	100%			0.27 (0.14-0.4)
Low serum C4	25	100%	1	4.0%	
CH-50, %	22	88%			81 (20-130)
CH-50 <50%	22	88%	6	27.3%	
AP-50 (%)	22	88%			84.0 (20-115)
AP-50 <50%	22	88%	9	40.9%	
Anti-C1q antibody	10	40%	4	40.0%	
Serum factor H, mg/L	22	88%			597 (286-800)
Serum factor I, mg/L	22	88%			49 (26-76)
C3 nephritic factor	24	96%	2	8.3%	
Anti-FH antibody	22	88%	1	4.5%	
Complement gene variant*	12	48%	4	33.3%	

Table 11. C3 glomerulopathy cohort complement analysis

CH-50, 50% haemolytic complement activity of serum assay; AP-50, 50% alternative pathway haemolytic activity of serum assay; NR, normal range.

*heterozygous factor I mutation (n=1); heterozygous C3 mutation (n=1); CFHR5 nephropathy (n=2).

Conclusions

- I designed comprehensive criteria to categorise our IgAN study population into cohorts of either progressive disease or stable disease. These criteria aimed to select patients with (progressive) and without (stable) evidence of glomerular inflammation because I predict this stratification is most likely to reveal differences in complement activity. When considering subsequent results chapters, it should be remembered that my patient stratification is unvalidated and was biased to test whether evidence of FH deregulation could be detected in any IgAN patients. The aim was not to demonstrate utility of FH deregulation complement activity as clinical biomarkers.
- Although not a key aim of my research, analysis of serum gd-IgA1 levels in our IgAN cohort questions the pathogenic importance of gd-IgA1 in IgAN. Other factors, that might include complement deregulation, may be more important drivers of disease severity.
- Data from both the IgAN and C3G cohorts are limited by a number of factors. Importantly the cohorts include patients with CKD, renal transplants and regular immunosuppression and patients receiving maintenance haemodialysis. The potential contribution of these factors to subsequent findings must be remembered. Furthermore, there are significant time differences between diagnostic renal biopsy and patient trial enrolment, when serum and plasma samples were taken for my research assays. The effect of these on the results is not known.
- The cohorts allowed the collection of comprehensive sets of clinical and histopathology data over long periods of follow-up. They allow the direct analysis of patient samples and avoid the need for animal or *in vitro* disease models. Although analysis of clinical samples can be confounded by the above-mentioned clinical variables, the demonstration of significant associations despite the noise of inumerate conflicting clinical variables would allow the formation of hypotheses to test under more controlled experimental conditions.

B. Circulating FHR1 and FHR5 in IgA nephropathy

Introduction

In this chapter I will describe novel associations between circulating FHR1 and FHR5 levels, and markers of IgAN severity. Systemic and renal complement activation in IgAN is recognised. For example, evidence of complement alternative pathway activation has been identified in the plasma of 30-75% of adults with IgAN,[238, 239] and correlates with proteinuria and the rate of renal function loss in a cohort of 50 IgAN patients.[239] A link between serum and histology markers of complement activation in disease pathogenesis was suggested by the association of mesangial C3 deposition and decreased serum C3 levels with doubling of serum creatinine or reaching ESRD in a cohort of 343 IgAN patients.[240] C3 has also been identified with IgA1-containing immune complexes from IgAN patients. Glomerular C3 activation and deposition could follow local glomerular complement activation and C3 binding to IgA-containing immune complexes in circulation that subsequently deposit in glomeruli. IgA can activate complement *in vitro*, predominantly through the alternative pathway. IgA isolated from pooled human plasma triggers complement-dependent lysis of, and properdin deposition on, erythrocytes coated with mouse monoclonal anti-human IgA.[241] Despite these data, the contribution to and mechanistic role of complement activation in IgAN pathogenesis remains poorly defined.

Recent genetic studies implicate a role for the FHR proteins in IgAN.[2, 3, 245, 270] The deletion polymorphism del*CFHR3-1* is associated with protection from IgAN.[2, 3, 270] A meta-analysis of approximately 20,000 individuals of different ethnicities estimated inheritance of the minor A allele at SNP rs6677604, that tags the del*CFHR3-1* allele, reduced the risk of IgAN disease by 26% in heterozygosity and by 45% in homozygosity.[3] The del*CFHR3-1* frequency exhibits marked differences across worldwide populations in a pattern inverse to IgAN prevalence.[3] Association between the del*CFHR3-1* allele and histology markers of complement activation and IgAN severity have also been demonstrated.[245, 270].

Factor H is the major negative regulator of C3 activation via the complement alternative pathway. FHR1 is postulated to act as a deregulator by antagonising the effect of FH.[8, 9, 110, 151] *In vitro* data has shown that FHR1[9] and FHR5[8] compete with FH for binding to activated C3 (C3b). Unlike FH, interaction of C3b with either FHR1 or

FHR5 allows continued complement activation preventing the regulatory actions of FH[128], a phenomenon referred to as FH deregulation.

There are phenotypic similarities between IgAN and familial C3 glomerulopathy associated with mutant FHR5 proteins[5, 7]. In both conditions, the majority of patients have microscopic haematuria. Many patients with IgAN and CFHR5 nephropathy present with progressive proteinuric renal impairment accented with episodes of macroscopic haematuria and acute kidney injury at the time of infection[5, 7, 150]. In caucasians IgAN is more prevalent in males. CFHR5 nephropathy is characterised by a more severe clinical course in males; 78% of CFHR5 patients of Cypriot ethnicity who develop renal failure are male[150]. The similarities are exemplified by the observation that IgAN was considered the most likely diagnosis and listed as the indication for diagnostic biopsy for many patients subsequently diagnosed with CFHR5 nephropathy[5].

Hypothesis and aims

I predict FH deregulation influences disease severity in IgAN by enhancing complement activation in response to mesangial deposited gd-IgA1. Recent findings suggest FHR1 and FHR5 can deregulate FH in IgAN. Therefore, I hypothesised patients with more progressive IgAN would demonstrate higher circulating levels of FHR1 and FHR5 and lower levels of FH. In this chapter, I describe my assessment of circulating FH, FHR1 and FHR5 levels in a cohort of IgAN patients that were stratified into those with stable and those with progressive disease, and a cohort of healthy controls. Due to the influence of *CFHR1* copy number on circulating FHR1 levels, we also assessed *CFHR1* copy number in all patients and healthy controls.

Methods

We consented and recruited 334 patients. A total of 40 patients were excluded. In addition to the 12 patients excluded from the consented IgAN cohort described in the previous chapter, serum creatinine and patient samples were not available in 28 patients at the time of serology assay. Consequently, 294 patients were included in the analysis. The clinical characteristics of the patients are shown in Table 12.

Healthy control samples were obtained from healthy volunteer donors and from members of the TwinsUK cohort[274]. We randomly selected samples from only one individual of each pair of twins included in the cohort. I measured serum levels from samples taken pre- and post- renal transplantation in a cohort of 28 transplant recipients with biopsy-proven IgAN of their native kidneys and 37 with ADPKD.

Using techniques described in the Methods chapter, we assessed *CFHR1* copy number, plasma FH, FHR1 and serum FHR5 levels in IgAN patients and healthy controls. To test whether the concentrations differed between serum and plasma, I measured FHR1 levels in the same volume of both serum and plasma taken at the same time in a subset of the healthy volunteers. I measured serum FH, FHR1 and FHR5 levels in transplant patients. *CFHR1* copy number assessment was completed by Dr Hannah J. Lomax-Browne.

		IgA Nephropathy	
	All (n=294)	**Stable (n=89)**	**Progressive (n=179)**
Male/Female	195/99	53/36	120/59
Caucasian/Non-Caucasian	244/50	78/11	144/35
Median age (range) – years	48.2 (18-84)	47.5 (18-82)	48.3 (19-84)
Median eGFR – ml/min. NR >90ml/min	52.7 (28.7-82.7)	74.8 (47.7-106)	45.8 (17.4-70.9)[1]
Median urine PCR – mg/mmol. NR<30mg/mmol	44 (16-117)	39 (13.5-89)	50 (17-144.5)
Median anti-hypertensive drug classes per patient	1.6	1.4	1.6
Patients with ACEi/ARB at enrolment, excluding dialysis patients - % (n)	73.7 (n=199)	79.8 (n=71)	77.3 (n=109)
Median systolic/diastolic blood pressure – mmHg	134 (122-145) / 79 (70-88) (n=286)	128 (116 - 140) / 77.5 (70-85) (n=88)	136 (124 - 146)[2] / 79 (70-88) (n=174)
Median duration follow-up (months)	55.0 (22.5-100.8)	71.4 (22.9-177.6)	50.7 (22.2-91.6)
Reach ESRD - %	34.9 (n=103)	0	57.5[3] (n=103)
History of macroscopic haematuria - %	27.8 (n=82)	44.9 (n=40)	20.1[3] (n=36)
Henoch-Schonlein purpura - %	6.4 (n=19)	0	8.9[3] (n=16)

Table 12. Clinical characteristics of the IgA nephropathy cohort analysed for circulating factor H related protein levels

Values within parentheses represent interquartile range and number analysed if less than the respective cohort numbers [1]P<0.0001 vs. stable disease; [2]P=0.0014 vs. stable disease; [3]P<0.0001 vs. stable disease; [4]one patient had 3 copies of CFHR3 and was excluded from the analysis. P-values shown have been adjusted for multiple analyses to minimise the false discovery rate.

	IgA Nephropathy			Healthy Controls
	All (n=294)	Stable (n=89)	Progressive (n=179)	(n=161)
CFHR1 – 2 copies / 1 copy / no copies				
Patients (n)	183/ 101/ 9 (n=293)	58 / 28 / 3	111 / 63 / 4 (n=178)	85 / 45 / 3 (n=133)
Percentage (%)	62.4 / 34.5 / 3.1	65.9 / 31. 8 / 3. 4	62.4 / 35.4 / 2.2	63.9 / 33.8 / 2.3
CFHR3 – 2 copies / 1 copy / no copies				
Patients (n)	185/ 100/ 8 (n=293)	59 / 27 / 3	112 / 63 / 3 (n=178)	
Percentage (%)	63.1 / 34.1 / 2.7	67 / 30.7 / 3. 4	62.9 / 35.4 / 1.7	
Median plasma FHR1 – micrograms/ml	126.8 (86.4-158.6)	112.5 (74.7-149.3)	132.0 (88.6-162.8)	94.4 (70.5-119.6)[1]
Median plasma FHR1 – micromolar	3.37 (2.30-4.21)	2.95 (1.97-4.00)	3.49 (2.35-4.40)	2.51 (1.87-3.12)[2]
Median plasma FH – micrograms/ml	153.8 (130.6-187.6)	155.1 (137.4-187.8)	150.1 (126.0-186.9)	152.5 (122.9-189.8)
Median plasma FH – micromolar	0.99 (0.84-1.21)	1.00 (0.88-1.21)	0.97 (0.81-1.20)	0.98 (0.79-1.22)
Median plasma FHR1/FH ratio	0.85 (0.55-1.10)	0.77 (0.46-0.98)	0.89 (0.59-1.16)[4]	0.68 (0.40-0.86)[3]
Median plasma FHR1/FH molar ratio	3.24 (2.16-4.34)	3.1 (1.88-4.03)	3.59 (2.37-4.81)[6]	2.81 (1.63-3.54)[5]
Median serum FHR5 – micrograms/ml	2.74 (2.07-3.64)	2.80 (2.07-3.49)	2.79 (2.08-4.03)	2.46 (1.79-3.67)[7]
Median serum FHR5 – micromolar	0.043 (0.032-0.057)	2.80 (2.07-3.49)	2.79 (2.08-4.03)	0.038 (0.028-0.057)[8]

Table 13. Factor H and factor H related protein levels in IgA nephropathy

Values within parentheses represent interquartile range and number analysed if less than the respective cohort numbers. Molarity calculations are based on a molecular weight of 37651 daltons for FHR1 and 155000 daltons for FH.

[1]p<0.0001 vs. all IgAN cohort (difference between medians 32.4 micrograms/ml, 95%CI 19.9-37.6 micrograms/ml);

[2]p<0.0001 vs. all IgAN cohort (difference between medians 0.86, 95%CI 0.53-1.00);

[3]p<0.0001 vs. all IgAN cohort (difference between medians 0.17, 95%CI 0.12-0.24);

[4]p=0.019 vs. stable disease (difference between medians 0.12, 95%CI 0.02-0.2);

[5]p<0.0001 vs. all IgAN cohort (difference between medians 0.43, 95%CI 0.35-0.88);

[6]p=0.023 vs stable disease (difference between medians 0.49, 95%CI 0.06 to 0.84).

[7]p=0.041 vs. all IgAN cohort (difference between medians 0.28 micrograms/ml, 95%CI 0.01-0.48 micrograms/ml);

[8]p=0.041 vs. all IgAN cohort (difference between medians 0.004, 95%CI 0.0002-0.007);

Results

a. Plasma FHR1, and the FHR1/FH ratio were elevated in IgA nephropathy and associated with progressive disease.

The IgAN patient cohort included in the assessment of circulating FHR1, FHR1/FH and FHR5 levels was smaller than the entire IgAN cohort presented in a previous chapter. I have therefore detailed the characteristics of the cohort analysed in this chapter in Table 12. The median plasma FHR1 level was increased in IgAN patients compared to healthy controls, whereas plasma FH levels did not differ (Table 13). Because I hypothesised imbalances of FH deregulation would influence complement activation and disease severity, I next assessed whether the relative abundance of complement deregulator and regulator differed between patients and controls; the FHR1/FH ratio was significantly increased in IgAN patients (Table 13). I did not observe any difference in *CFHR3* and *CFHR1* copy number between patients with stable versus progressive disease (Table 13). The presence of the *CFHR3-1* deletion allele will influence circulating FHR1 levels. For example, the protein will be absent from individuals with the *CFHR3-1* deletion in homozygosity. I therefore stratified patients according to *CFHR1* gene copy number. Patients with two copies of the *CFHR1* gene had a higher FHR1 level than genotype-matched healthy controls (Figure 9A). This difference did not reach statistical significance in patients with one copy of FHR1 (Figure 9A). The FHR1/FH ratio was significantly elevated in patients compared to controls with the same *CFHR1* gene copy number (Figure 9B). FHR1 was undetectable in deletion homozygotes (n=12, including nine patients). Plasma FH levels remained similar between patients and controls when stratified according to *CFHR1* gene copy number (Figure 9C). As previously reported,[245, 280] FH levels were highest in *CFHR3-1* deletion homozygotes (Figure 9C)

I next examined whether FHR1 levels and the FHR1/FH ratio differed between patients with stable compared to progressive disease. In both cohorts with one and two copies of the CFHR1 gene, patients with progressive IgAN had significantly elevated serum FHR1 (Figure 10A) and FHR1/FH ratios (Figure 10B) compared with patients with stable IgAN. FH levels did not differ (Table 13). Elevated FHR1/FH ratio was also seen in IgAN patients who continued to meet my criteria for progressive IgAN despite immunosuppression compared with those who improved with immunosuppression treatment (Figure 10C).

b. Plasma FHR1 negatively correlated with eGFR but remained elevated in IgAN patients with normal eGFR.

Based on my observations of higher FHR levels in patients with progressive IgAN, I next questioned whether FHR1 levels were influenced by renal impairment. I stratified patients by *CFHR1* gene copy number and assessed the correlation between FHR1 levels and eGFR. I detected a negative correlation between plasma FHR1 levels and eGFR in patients with one (Figure 11A) and two (Figure 11B) copies of the *CFHR1* gene. When I stratified patients into those with an eGFR of <30 or >60 ml/min/1.73m^2, FHR1 levels were significantly higher in those with an eGFR of <30 ml/min/1.73m^2 (Figure 11C and 11D, one and two *CFHR1* copies respectively). To determine whether FHR1 levels were higher in IgAN patients prior to the development of renal impairment we compared FHR1 levels in IgAN patients with normal eGFR to healthy controls (Figure 12). Patients with two copies of the *CFHR1* gene and normal eGFR had a higher FHR1 level than CFHR1 copy number matched healthy controls (Figure 12). This difference was not seen in patients and healthy controls with one *CFHR1* (Figure 12).

Figure 9. Plasma FHR1, FHR1/FH ratio and FH in IgAN.

(A) Plasma FHR1 in healthy controls (HC, solid circles) and IgAN patients (IgAN, empty circles) stratified according to CFHR1 gene copy number.
(B) and (D) show the FHR1/fh ratio in healthy controls (solid circles) and IgAN patients (empty circles) stratified according to CFHR1 gene copy number. (B) displays the ratio of concentrations in micrograms/ml and (D) displays the molar ratio.
The presence of zero, one or two copies of the CHFR1 gene influenced plasma FHR1 and the FHR1/FH ratio; plasma FHR1 was undetectable in patients with 0 copies of the CFHR1 gene; in both the control and IgAN cohorts, individuals with 1 or 2 copies of the CFHR1 gene had significantly different plasma FHR1 levels and FHR1/FH ratios (p<0.001).
(C) Plasma factor H (FH) in healthy controls (solid circles) and IgAN patients (empty circles) stratified according to CFHR1 gene copy number.
Lines represent median value and interquartile range. P values derived from Mann Whitney test.

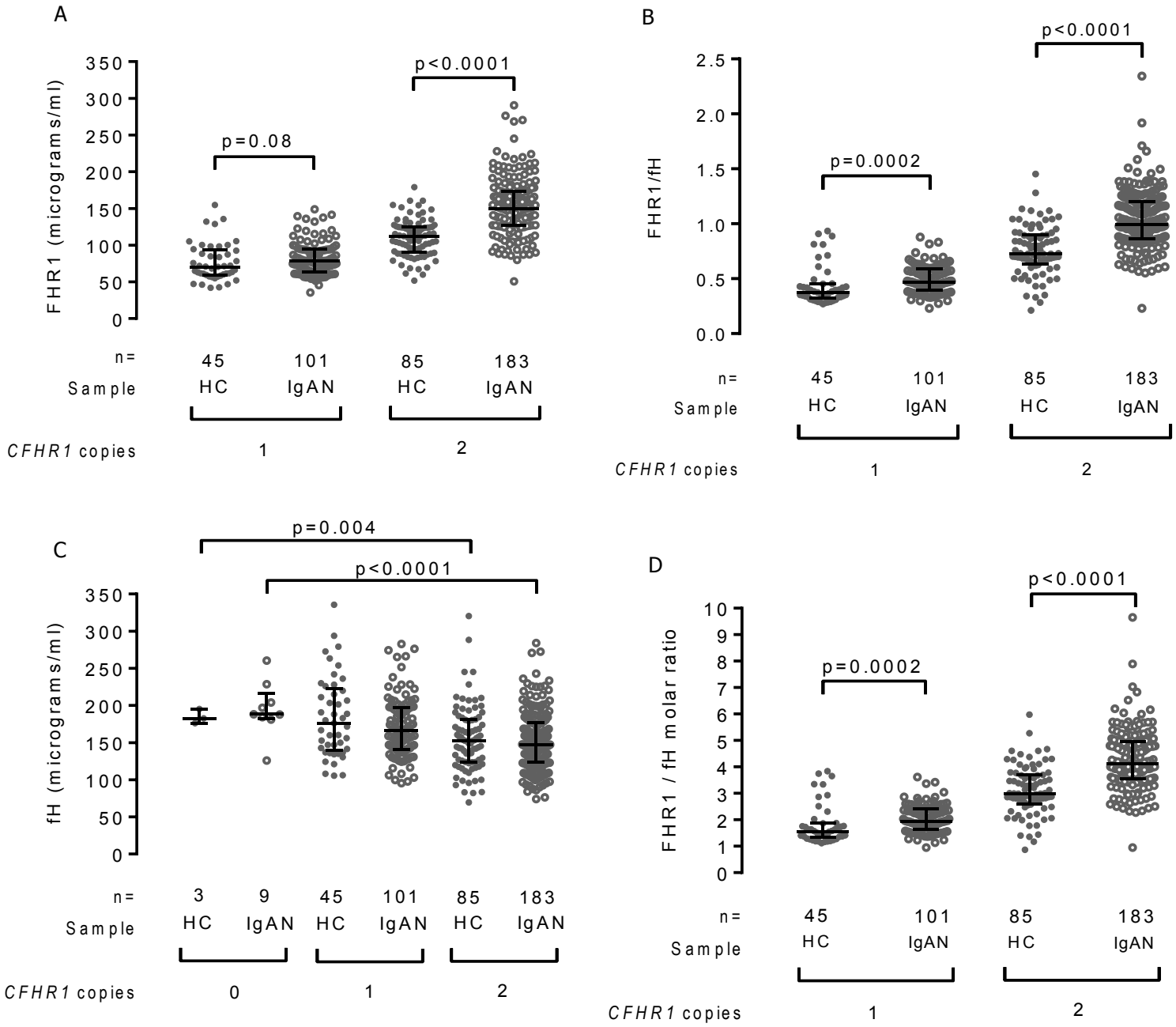


Figure 10. Plasma FHR1 and the FHR1/FH ratio associate with progressive IgA nephropathy

(A) Plasma FHR1 in stable (S, solid circles) and progressive (P, empty circles) IgAN stratified according to CFHR1 gene copy number.
(B) and (D) show plasma FHR1/fh ratio in stable (S, solid circles) vs. progressive (P, empty circles) IgAN stratified according to CFHR1 gene copy number. (B) displays the ratio of concentrations in micrograms/ml and (D) displays the molar ratio.
(C) FHR1/fh ratio in patients with stable (S, solid circles) vs. progressive (P, empty circles) IgAN after immunosuppression treatment.
Bars represents median value and interquartile range. P values derived from Mann Whitney test.

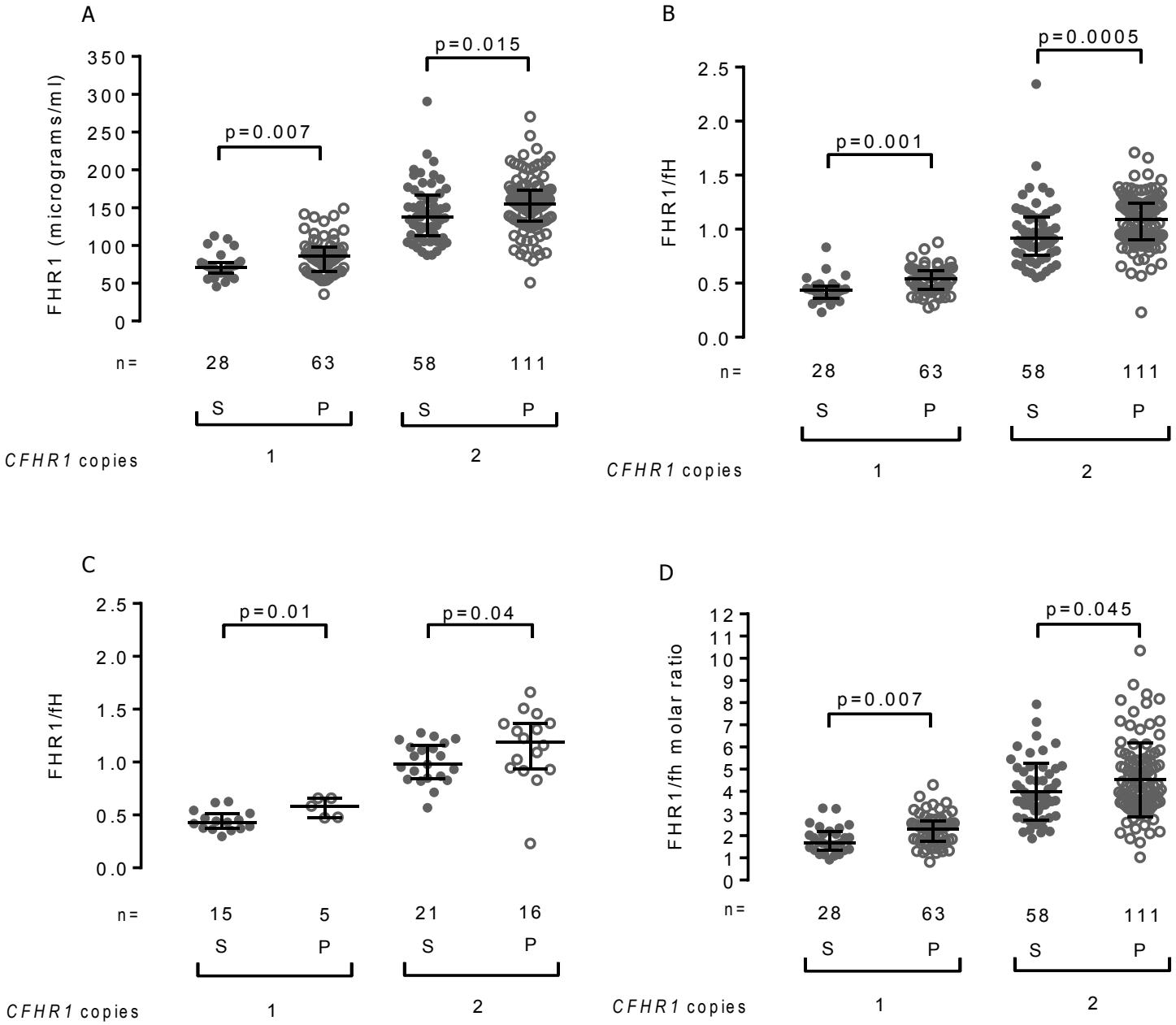


Figure 11. Plasma FHR1 correlates with estimated glomerular filtration rate in IgA nephropathy

Correlation between plasma FHR1 levels and eGFR after logarithmic transformation in IgAN patients with either one (A) or two (B) copies of the CFHR1 gene. P values derived from Spearman rank correlation.
Plasma FHR1 levels in IgAN patients with eGFR <30 (solid circles) or >60 (empty circles) ml/min stratified according to one (C) or two (D) copies of the CFHR1 gene.
Bar represents median value and interquartile range. P values derived from Mann Whitney test.

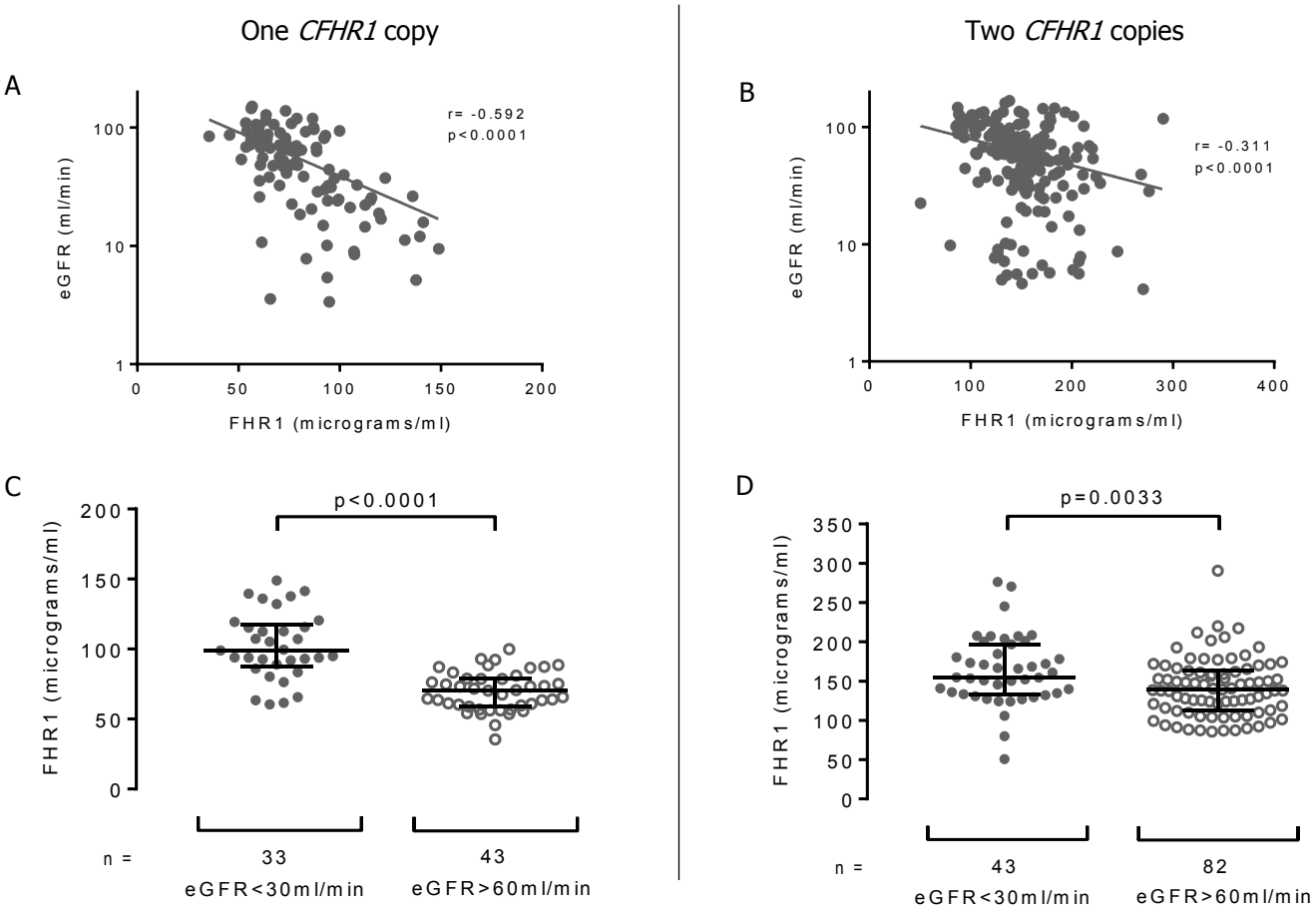
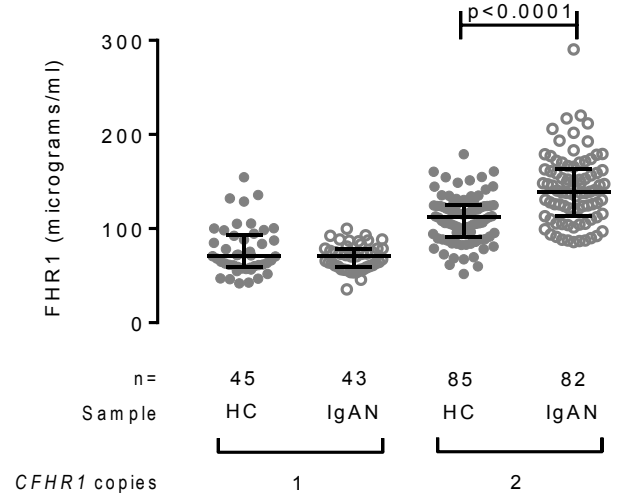


Figure 12. Plasma FHR1 in IgA nephropathy patients with preserved glomerular filtration



Plasma FHR1 levels in healthy controls (HC, solid circles) and IgAN patients (IgAN, empty circles) with eGFR >60ml/min, stratified according to CFHR1 gene copy number.
Bar represents median value, box represents interquartile range and whiskers represent range of values. P values derived from Mann Whitney test.

I next assessed FHR1 levels pre and post renal transplantation in patients with either biopsy-proven IgAN or ADPKD. Serum but not plasma samples were available from the transplant cohort. I established there was no significant difference in FHR1 and FH levels in plasma and serum samples from the same time point in healthy controls (Figure 13). I subsequently used serum samples from the transplant cohorts to quantify FHR1 and FH concentrations.

Two transplant patients with ADPKD, both of whom had the AA rs6677604 SNP genotype, had undetectable levels of FHR1 before and after transplantation. The cohorts had comparable pre-transplant characteristics (Table 4, p88) and no patients had a clinical diagnosis of delayed graft function, transplant rejection or disease recurrence at the sampling time. Both groups showed significant increase in eGFR and reduction in serum FHR1 levels following renal transplantation (Figure 14). Taken together, these data indicate that both the diagnosis of IgAN and eGFR are independently associated with higher FHR1 levels. Notably, there was no significant correlation between eGFR and plasma FH levels in our IgAN cohort (Figure 15).

Figure 13. FHR1 levels measured in serum and plasma

Paired FHR1 levels measured with the same ELISA technique from plasma (circles) and serum (squares) taken at the same time point from seven healthy individuals. There was no significant difference in the quantified FHR1 levels between serum and plasma samples.
P values derived from paired t-tests.

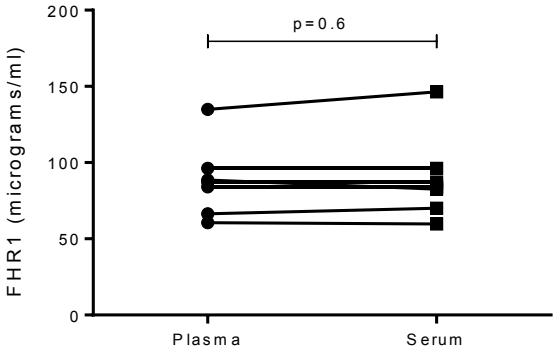


Figure 14. Serum FHR1 levels and change in renal function pre and post renal transplantation

eGFR (A) and paired FHR1 levels, (B) and (C) pre and post renal transplantation in a cohort of patients with ADPKD and (grey circles, n=25) and IgAN (white circles, n=23). (b) includes all transplant patients and (C) only includes FHR1 values from patients were homozygous for the major allele rs6677604, consistent with two copies of the CFHR1 gene. P values derived from paired t-tests.

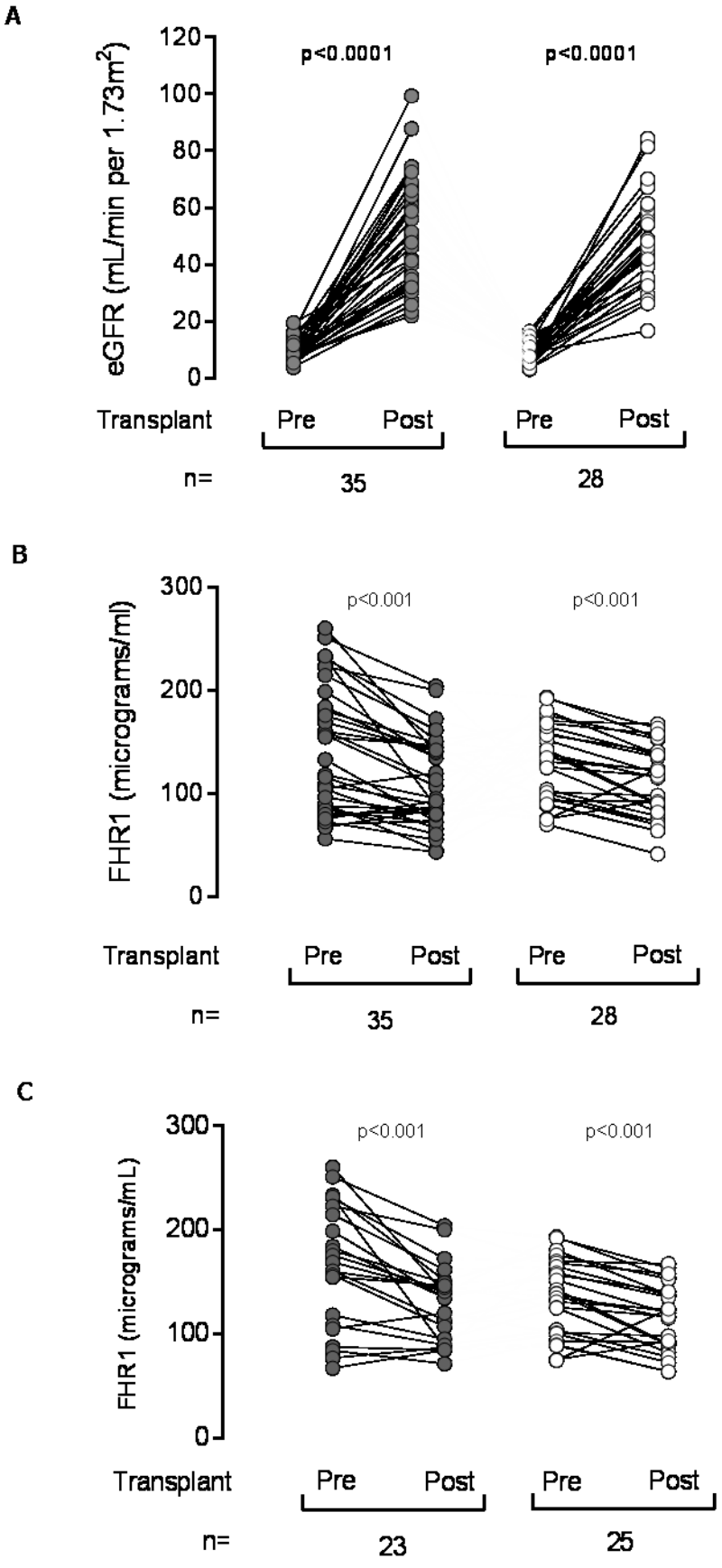
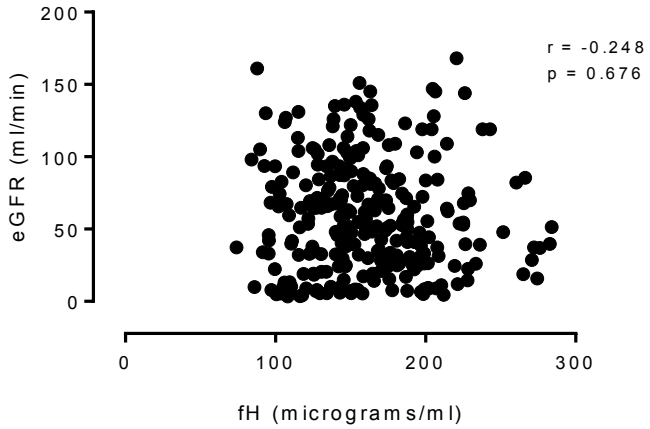


Figure 15. Plasma factor H levels and renal function in IgA nephropathy

Serum eGFR vs plasma FH at the same sampling point in IgAN patients (n=294) showed no significant correlation between eGFR and plasma FH concentration.
P value derived from Spearman rank correlation.



c. Serum FHR5 is elevated in IgAN and does not correlate with eGFR.

FHR5 is also able to deregulate FH *in vitro*.[8] I therefore measured serum FHR5 levels in our IgAN cohort. The median FHR5 level was increased in IgAN patients compared to healthy controls (Figure 16 and table 13). The magnitude of the difference was small: median FHR5 levels were 2.46 and 2.74 µg/ml in controls and IgAN patients respectively (difference between medians 0.28 micrograms/ml, 95% CI 0.01-0.48 micrograms/ml), and the differences were on a nanomolar scale.The biological significance of such small variations in circulating FHR5 concentrations is not clear. Furthermore, I did not detect any difference in FHR5 levels between stable and progressive IgAN patients (Figure 17 and table 13). However, patients with progressive disease following immunosuppressive treatment had significantly higher FHR-5 levels than patients who improved to meet stable IgAN criteria after immunosuppression (Figure 17). Unlike FHR1, I did not find an association between serum FHR5 concentration and eGFR (Figure 18A and 18B). I also found no significant difference in the FHR5/FH ratio between either patients versus healthy controls or progressive versus stable IgAN (Figure 19).

Figure 16. Plasma FHR5 is elevated in IgA nephropathy

Plasma FHR5 in healthy controls (solid circles) and IgAN patients (empty circles). Bar represents median value, and nterquartile range. P values derived from Mann Whitney test.

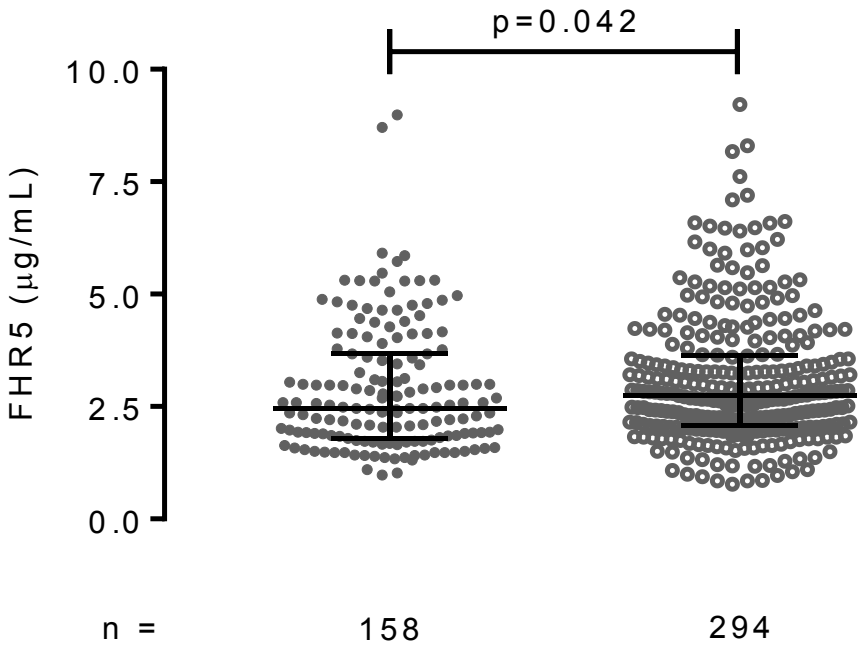


Figure 17. Serum FHR5 associates with IgA nephropathy severity following immunosuppression

Left panel: Serum FHR5 in stable (S, solid circles) vs. progressive (P, empty circles) IgAN.
Right panel: Serum FHR5 in patients with stable (S, solid circles) vs. progressive (P, empty circles) IgAN after immunosuppression treatment.
Bar represents median value and interquartile range. P values derived from Mann Whitney test.

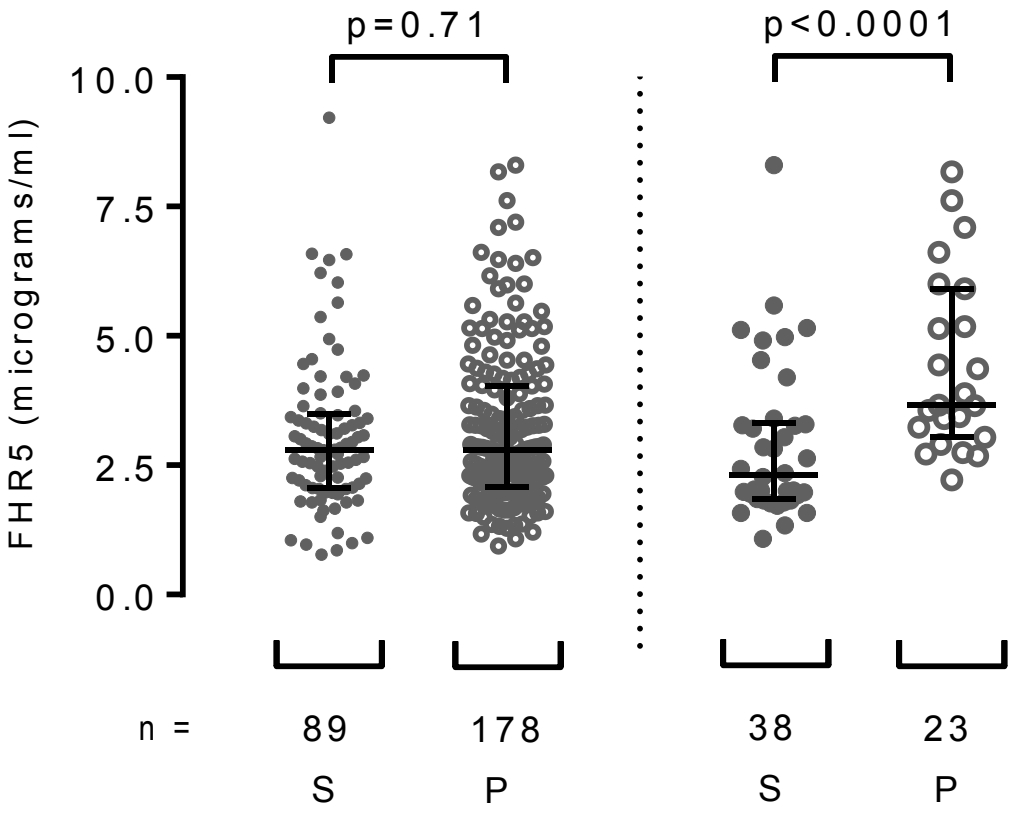


Figure 18. Serum FHR5 does not associate with estimated glomerular filtration rate in IgA nephropathy

(A) Correlation between serum FHR5 levels and eGFR in IGAN patients. P value derived from Spearman rank correlation.
(B) Serum FHR5 levels in IGAN patients with eGFR <30 (solid circles) or >60 (empty circles) mls/min.
Bar represents median value and interquartile range. P values derived from Mann Whitney test.

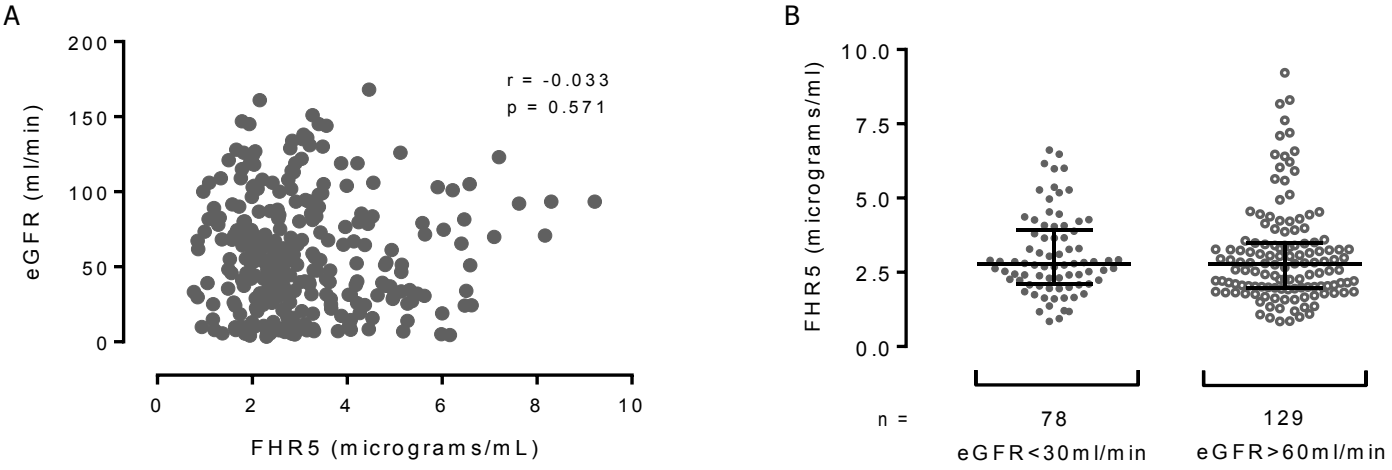
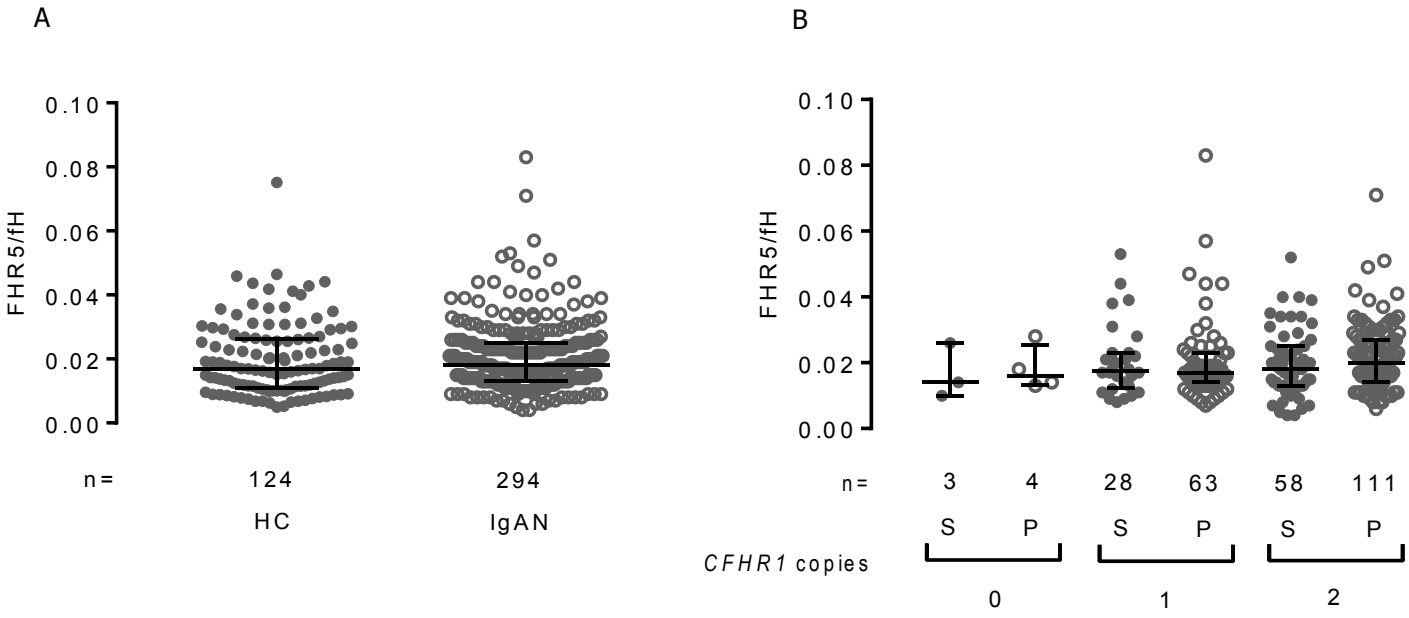


Figure 19. Plasma FHR5/FH ratio does not associate with IgA nephropathy

(A) FHR5/FH in healthy controls and IgAN patients. We did not identify a significant difference in plasma FHR5/FH between healthy controls (HC, solid circles) and IgAN patients (IgAN, empty circles box).
(B) FHR5/FH in stable and progressive IgAN. There was no significant difference in plasma FHR5/FH ratio between patients with stable (S, solid circles) and progressive (P, empty circles) IgAN at the same CFHR1 genotype.
Bar represents median value and interquartile range. P values derived from Mann Whitney test.

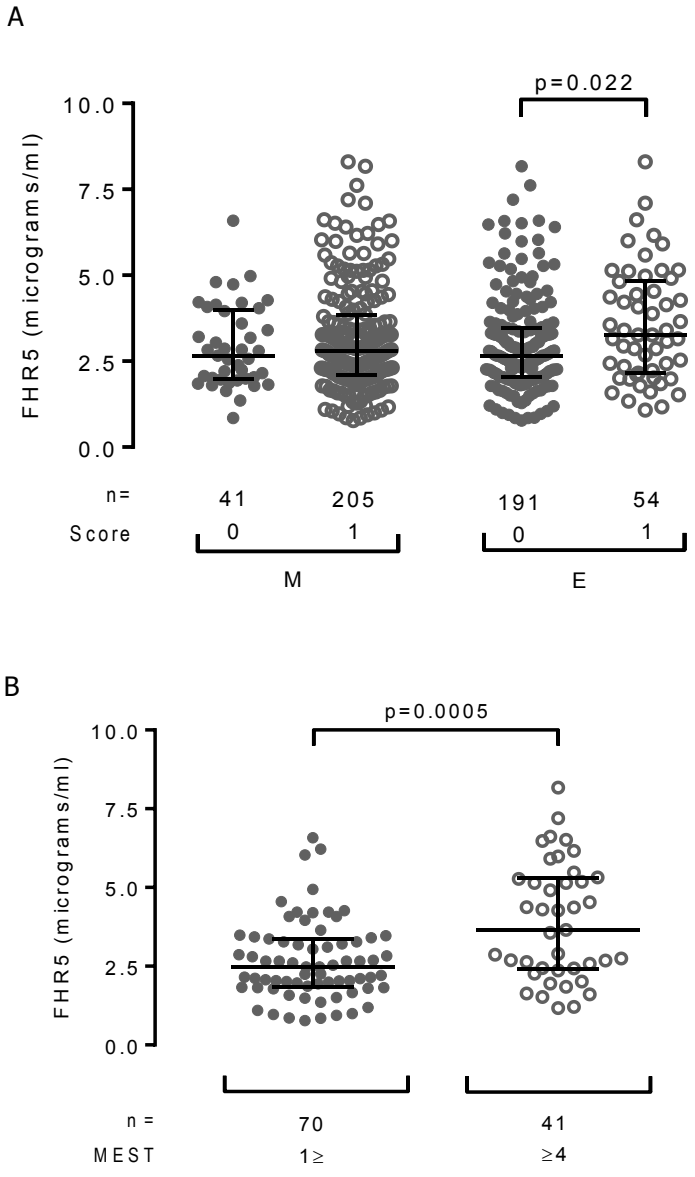


d. Serum FHR5 correlates with histological markers of renal injury

To explore the significance of the changes in serum FHR5, I correlated FHR5 levels with validated markers of histological injury in IgAN, the Oxford classification.[193] Patients with endocapillary hypercellularity score E1 at diagnosis had significantly higher serum FHR5 at study enrolment and plasma/serum sampling (Figure 20A, right panel) than the cohort with no endocapillary hypercellularity (E0). Serum FHR5 levels in IgAN patients with (M1) and without (M0) renal biopsy evidence of mesangial hypercellularity did not differ (Figure 20A, left panel). The total MEST score is calculated from the addition of the scores for mesangial hypercellularity (M), endocapillary hypercellularity (E), segmental sclerosis (S) and tubular atrophy (T). Serum FHR5 levels were higher in IgAN patients with MEST score of 4 compared to 1 (Figure 20B). Unlike FHR5, our data demonstrated that FHR1 levels were influenced by eGFR. Therefore I did not consider it valid to look for associations between histological markers and plasma FHR1 since eGFR would likely differ between the diagnostic renal biopsy and study plasma sampling in many patients.

Figure 20. Plasma FHR5 levels correlate with histology markers of IgA nephropathy severity

(A) Left panel: Serum FHR5 levels in IgAN patients without (M, 0, solid circles) and with (M, 1, empty circles) renal biopsy evidence of mesangial hypercellularity (denoted M); Right panel: Serum FHR5 levels in IgAN patients without (E, 0, solid circles) and with (E, 1, empty circles) biopsy evidence of endocapillary hypercellularity (denoted E).
(B) Serum FHR5 levels in IgAN patients with diagnostic renal biopsy MEST scores of either 1 or less (solic circles), or at least 4 (empty circles).
Bar represents median value and interquartile range. P values derived from Mann Whitney test.



Discussion

In this chapter, I first identified the associations between circulating levels of FHR1, which is predicted to deregulate and amplify the alternative complement pathway, and FH, an essential regulator of the alternative complement pathway, and the presence and severity of IgAN. Levels of FHR1, and importantly the ratio between FHR1 and FH, were increased in IgAN patients compared to healthy controls. In addition, when stratified by *CFHR1* gene copy number, patients with progressive IgAN had significantly elevated plasma FHR1/FH ratios compared to patients with stable disease. Inconsistent with this, I did not detect associations in the allelic frequency of del*CFHR3-1* and IgAN risk or disease severity. The explanation for this is not clear but, as discussed below, it may reflect the pathogenic redundancy of circulating FHR1 levels in IgAN.

These results are limited by number of important considerations. First, I categorised the patient cohort into those with progressive and those with stable IgAN. Our criteria aimed to separate patients with signs of immunologically active IgAN from those with inactive disease. Although this inevitably excluded a subset of patients (those we could not reliably categorise as either stable or progressive at study entry), our classification enabled us to detect differences in FHR1 and the FHR1/FH ratios. However, as previously discussed, the criteria have not been validated clinically and may have inappropriately cohorted patients with different IgAN subtypes, limiting interpretation of the results. In particular, patients with numerous clinical differences that could influence complement activity and circulating FHR1 and FHR5 levels, such as different levels of proteinuria, the use of chronic maintenance haemodialysis, or post kidney transplant immunosuppressants, are captured and analysed together. This strategy was adopted to maximise cohort size. Also, serum and plasma was sampled at a single time point at study recruitment. I have not been able to thoroughly investigate changes in circulating FHR1, FHR5 and FH levels with time and following clinical events including infection. Although this limits interpretation and application of the findings to clinical settings, the detection of associations despite the heterogeneity of the sample populations may reflect the importance of FHR proteins to disease pathogenesis. The associations of circulating FHR1 and FHR5 levels with IgAN risk and severity necessitate prospective analysis of serial levels and correlation with clinical events.

Additionally, FHR1 levels showed large overlap between the groups and biological significance of such modest differences in FHR1 levels, especially when considered on a molar scale, is unclear. In addition, our cohort contained nine individuals (three with stable, four with progressive, and two not fulfilling criteria) with

del*CFHR3-1* homozygosity demonstrating that even in the complete absence of FHR1 and FHR3, renal injury sufficient to require renal biopsy and hospital follow-up can occur. This is consistent with previous studies[270] and indicates that, although FHR-dependent FH deregulation may be important in determining IgAN severity in many patients, other factors also drive disease.

I found negative correlations between eGFR and plasma FHR1 levels measured at study recruitment. Both IgAN patients and ADPKD patients show a reduction in FHR1 levels coincidental with increased eGFR following renal transplantation. This implies FHR1 levels are predominantly influenced by changes in glomerular filtration rate and may neither reflect complement activity nor be specific to complement dependent kidney disease. However, when I compared patients with normal eGFR to healthy controls, patients with two copies of the *CFHR1* gene and normal eGFR had a higher FHR1 level than genotype-matched healthy controls. This indicated circulating FHR1 levels may have an independent effect on IgAN development. However, these data should be interpreted in caution because I did not know the exact eGFR of the control group and the difference was only apparent when comparing cohorts with two copies of the *CFHR3-1* gene. This itself may reflect higher circulating FHR1 levels in patients with two copies of the gene make cohort differences easier to detect and the larger number of individuals with two copies of the gene. However, the lack of difference between cohorts with one gene copy might represent biological unimportance of circulating FHR1 levels.

These data have been replicated in a separate IgAN cohort from Spain[254]. This study of 112 patients with IgAN, 46 patients with ADPKD and 76 control patients also demonstrated elevated FHR1 levels and FHR1/FH ratios in IgAN patients compared to healthy controls. FHR1 levels and FHR1/FH ratios were highest in IgAN patients with progressive disease and with ADPKD patients. Interesting, there were no significant differences in circulating levels between progressive IgAN and ADPKD patients, suggesting non-specific loss of renal function determines circulating FHR1 levels. However, in this cohort, del*CFHR3-1* allelic frequency was significantly decreased in IgAN patients compared to ADPKD patients and healthy controls, implying complement deregulation from FHR1 and FH imbalance may also influence renal injury. The authors theorised that imbalance FHR protein and FH ratios and deteriorating renal function could act synergistically to accelerate complement activation and renal injury[254].

I did not measure FHR3 levels due to lack of a reliable assay. FHR3 has not clearly been associated with FH deregulation and its role remains unclear. It is able to interact with the meningococcus FH-binding protein and,

through competition with FH, influence the complement-mediated clearance of meningococcal strains and hence meningococcal disease severity.[125] Through interaction with its ligand C3dg, FHR3 may influence B cell regulation through the B cell receptor complex (CD19/CD21/CD81) but how this might relate to IgAN is unclear.[281]

In the light of published data showing that FHR5 can mediate FH deregulation[8] and the strong association between FHR5 mutations and C3G,[5, 7, 122] I measured plasma FHR5 levels and the FHR5/FH ratio. In our IgAN cohort. I identified a small but significant increase in plasma FHR5 levels between IgAN patients and controls. It is difficult to draw conclusions on the influence of circulating FHR5 levels on IgAN risk from these data because the differences between the groups was extremely small and unlikely to be of biological significance. However, the finding was replicated in a large cohort of 1153 IgAN patients and 153 healthy controls from Beijing[282]. In this study, the patients had median circulating FHR5 level of 4.55 µg/ml compared to 3.19 µg/ml in healthy controls and higher FHR5 levels associated with markers of disease severity including lower eGFR, the presence of hypertension, tubular atrophy and glomerular crescents. High FHR5 levels also independently associated with risk of developing 30% drop in eGFR or ESRD[282]. Associations between circulating FHR5 levels and disease severity in my dataset were less convincing. I did not see any differences in FHR5 levels between patients with stable compared to progressive disease. And, I did not detect any correlation between the FHR5/FH ratio and disease outcome. Since eGFR did not influence plasma FHR5 levels, I looked for correlations between plasma FHR5 and validated histologic markers of renal injury in IgAN. Plasma FHR5 levels were associated with endocapillary hypercellularity independent of mesangial hypercellularity, a histology marker of active inflammation.[197] They were also associated with higher overall Oxford Classification of IgAN MEST scores[194]. These results are limited by the difference in timings between diagnostic renal biopsy, from which the histopathology data is harvested, and serum sampling, and the inumerate clinical events that may have ensued in the interim. However, the findings could also reflect a dominant role for FHR5 in glomeruli. Subsequently, small differences in circulating FHR5 may reflect pathologically important changes in glomerular FHR5.

The data in this chapter demonstrate circulating FHR1 and FHR5 levels associate with some markers of IgAN severity. When considered with data published from groups in Spain and Beijing, the findings support roles for FHR1 and FHR5 in IgAN pathogenesis. This could be consistent with my hypothesis that FHR1 and FHR5

dependent FH deregulation associate with IgAN severity. However, my results should be interpreted with caution as they are limited by inconsistencies in genotype frequencies and multiple clinical confounding factors that could influence the result. An alternative but plausible interpretation of these data is that circulating, systemic FHR1 and FHR5 levels do not influence disease risk nor severity. If FHR1 and FHR5 influence glomerular inflammation, it may be through local complement deregulation in the direct vicinity of surfaces where complement activation has been triggered, such as glomerular mesangium or capillary basement membranes. Therefore, I next questioned whether immunohistologic evidence of FHR-dependent FH deregulation and complement activation could be detected in progressive IgAN.

Conclusions

- Circulating levels of FHR1, and the ratio between FHR1 and FH, were increased in IgAN patients and associated with progressive compared to stable disease.
- But eGFR changes independently influenced plasma FHR1 levels.
- IgAN patients had more circulating FHR5 than healthy controls.
- Higher FHR5 associated with histology markers of IgAN severity.
- However, the differences in systemic FHR1, FHR1:FH and FHR5 were very small.
- These data are not, in themselves, conclusive and may reflect the unimportance of systemic FHR1, FHR5 and FH levels to IgAN pathogenesis.
- This necessitates investigating associations between glomerular FHR5 and FHR1, local complement activation and IgAN disease severity.

C. Glomerular FHR1 and FHR5 deposition in IgA nephropathy

Introduction

There is long-standing evidence for glomerular complement activation in IgAN[272]. For example, glomerular complement C3 is identified colocalised with IgA in 90% of cases[283] and mesangial C3 deposition[240] correlates with loss of renal function. Increased serum activated C3[239] and decreased serum intact C3[240] also associate with IgAN and may reflect local glomerular complement activation. Interestingly, hypocomplementaemia, as opposed to C3 levels at the lower end of the normal range, which is a surrogate for significant systemic complement activation and has not been established as a marker of IgAN severity[272]. These observations suggest glomerular complement activation, but not necessarily systemic complement activity, is important for IgAN pathogenesis.

I considered that using antibodies that can distinguish the different fragments of C3 (C3b, iC3b, C3c and C3dg) could provide information as to whether the detected glomerular C3 in IgAN was due to ongoing complement activation or was a consequence of previous complement activation. In immune complex experimental nephritis models in rats, glomerular C3c resolved within 24hours of stopping complement activation whereas glomerular C3dg remained unchanged. This is perhaps not surprising since C3dg is typically covalently bound to the activating surface [284]. Glomerular C3dg in the absence of glomerular C3c therefore indicates previous complement activation and is detectable in the absence of ongoing complement activation.

Antibodies to C5b9 detect the MAC, the deposited product of terminal complement pathway activation. C1q is a marker of classical complement pathway activation. C4d is deposited after classical or lectin complement pathway activation. Properdin deposition is indicative of alternative complement pathway convertase activity. Although the presence of many of these proteins has been established in IgAN, their association with disease severity and FHR deposition has not been investigated.

Glomerular FHR5 has been identified in the renal biopsies of IgAN and other glomerulopathies associated with complement activation, specifically membranous nephropathy, lupus nephritis and post-infectious nephritis[112]. In these cases, FHR5 was distributed in similar glomerular locations to C3[112]. It is not clear whether the antibody used in this study was specific for C3 fragments but given it was a sheep polyclonal anti-C3 antibody, it's unlikely to have differentiated C3b/iC3b/C3c and C3dg fragments. Nevertheless, these data indicate that

results from FHR5 immunostaining in IgAN cases with complement deregulation may be applicable to other glomerulopathies. Glomerular FHR1 deposition has not been extensively investigated.

Hypothesis and aims

I hypothesised that glomerular FHR1 and FHR5 deposition would correlate with IgAN disease severity. So I next aimed to characterise glomerular complement activation and FHR deposition in IgAN and then determine if there were differences in complement immunostaining between patients with progressive and stable IgAN.

Methods

I optimised IHC protocols for FFPE renal biopsy tissue. I found variation in glomerular FHR5 staining between non-sclerosed glomeruli from the same biopsy was minimal with bacterial enzyme antigen retrieval. Glomerular FHR5 staining was eliminated after pre-incubation of the primary antibody with purified FHR5 (Figure 6). Notably, when I used pressure cooker heating for antigen retrieval, I was able to detect tubulo-interstitial FHR5 staining in a number of glomerulopathies including C3G (Figure 6). However, unlike the glomerular FHR5 staining evident using enzyme antigen retrieval, tubulo-interstitial staining was not eliminated by pre-incubation of the primary antibody with purified FHR5, suggesting it was non-specific (Figure 6). The anti-C3c antibody cannot distinguish between C3c, C3b and iC3b so I refer to this staining as anti-C3b/iC3b/C3c. I tested anti-C3b/iC3b/C3c and anti-C3dg antibody specificity by ELISA as detailed in the methods chapter. I was interested in two similar research questions. First, I questioned whether the glomerular presence of FHR5 of any quantity associated with glomerular complement activity, inflammation and renal injury. Secondly, I questioned whether a quantifiable estimation of FHR5 deposition would correlate disease severity. I therefore analysed data based on a graded subjective scale of staining intensity (0, 0.5, 1+, 2+, and 3+) and presence (equating to staining intensity 1+, 2+, and 3+) and absence (0 and 0.5, because 0.5 is equivalent to a description of 'uncertain' deposition) of antibody staining. Example images of each glomerular intensity grade with the anti-C3b/iC3b/C3c antibody are shown (Figure 21).

As described in the Methods chapter, grading was performed by me and Professor H. Terence Cook, Professor of Renal Pathology, Imperial College London. All cases were anonymised and graders were unable to

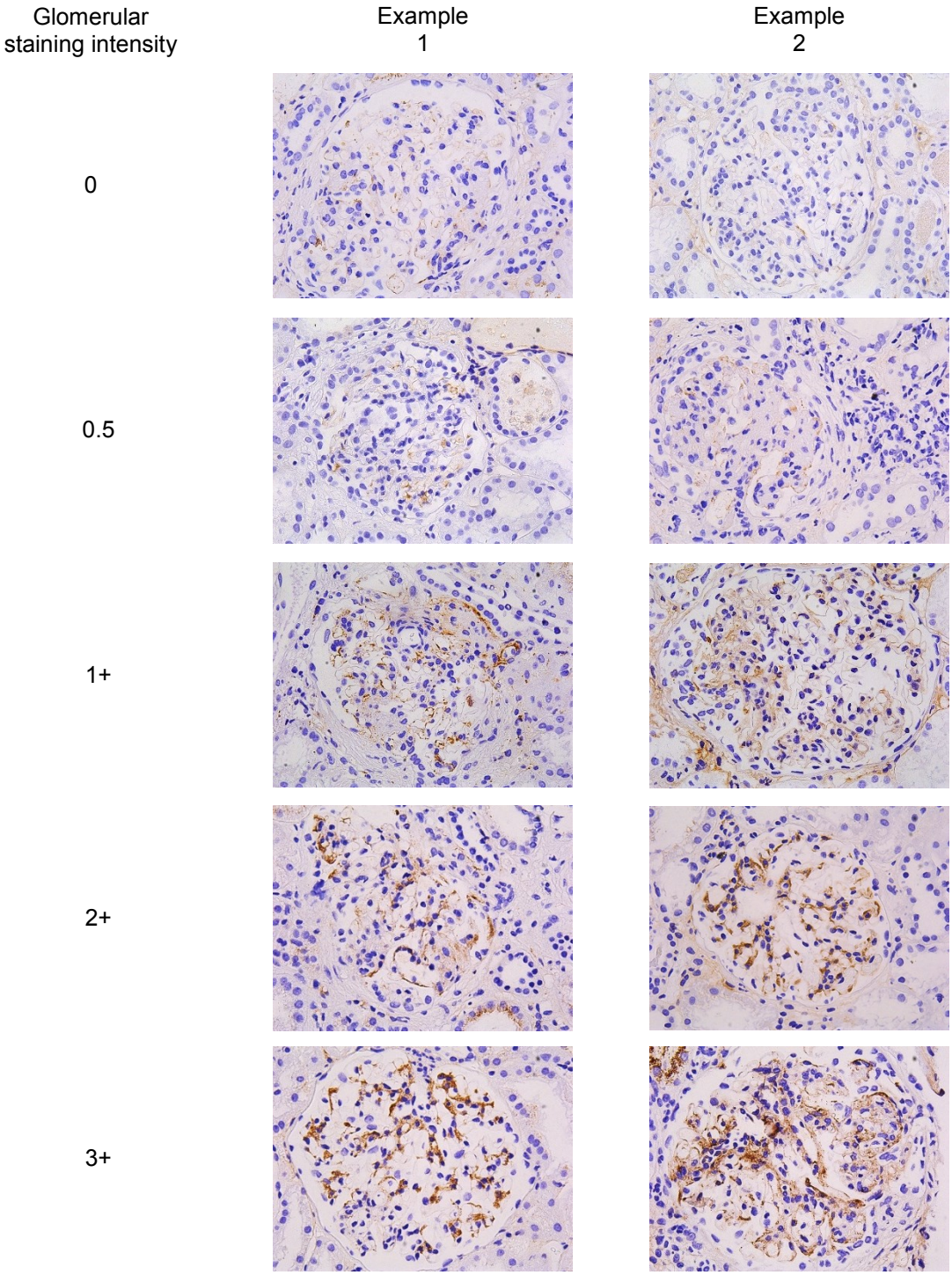
determine the identity of cases. I calculated the inter-observer correlation coefficient for the grading of staining intensity using the 0 (absent), 0.5 (minimal), 1+, 2+ and 3+ scale for Professor Cook and me. We independently graded the DAB staining intensity of 26 anonymised cases of IgAN that had undergone detection of C3b/iC3b/C3c using the protocol detailed above. Correlation coefficient was calculated using Spearman's Rank correlation. The correlation (r) for glomerular staining intensity was 0.72, with a statistically significant correlation ($P<0.0001$). When graded as 'negative' or 'positive', the correlation coefficient improved to 0.8 ($P<0.0001$).

I located stored surplus unstained tissue from diagnostic biopsies from 41 IgAN patients from my study cohort. The biopsies had median six glomeruli per section (range 2 to 16). Due to availability of stored surplus clinical biopsy material, it was rare to be able to analyse serial sections of the same glomerulus. This is a limitation of the immunostaining data. All biopsies had absent or non-significant C1q staining documented in clinical reports. I excluded sections that contained less than two non-sclerosed glomeruli. Depending on tissue availability, I stained and analysed one renal biopsy section from every case for each protein listed above. DAB reaction times were optimised to allow complete reaction with minimal background, non-specific staining and differed slightly for each antibody. DAB was applied for the same duration to all sections stained with the same antibody.

I compared clinical and biopsy features recorded at diagnosis, including histologic features defined by the Oxford classification of IgA nephropathy[194], and during follow-up in IgAN patients based on the presence or absence of glomerular FHR5.

Figure 21. Example images of renal immunohistochemic staining grades

Example images of glomerular intensity grades with the anti-C3c/C3b/iC3b antibody. A similar scale was used for other antibodies. Glomerular staining was graded by Professor H. Terence Cook.



Results

a. Antibody specificity

The anti-FHR5 antibody detected FHR5 incubated with surface bound C3c, iC3b and C3dg by ELISA. I detected minimal binding of the anti-FHR5 antibody to the complement proteins in the absence of FHR5 (Figure 22). The anti-C3b/iC3b/C3c antibody used for IHC staining detected surface bound C3c and iC3b by ELISA with significantly greater sensitivity than it detected C3dg or BSA. The antibody-antigen interactions were not affected by pre-incubation with FHR5. The anti-C3dg antibody used for IHC and IF detected surface-bound C3dg by ELISA with significantly greater sensitivity than it detected C3c, iC3b or BSA. The antibody-antigen interactions were not affected by pre-incubation with FHR5 (Figure 22).

b. Immunohistologic evidence of complement activation correlated with disease severity in IgAN.

I assessed complement deposition in stable and progressive cohorts (Figure 23). In progressive compared with stable disease, there was proportionately greater glomerular staining for C3c/C3b/iC3b (OR 5.66, 95% CI 1.49-23.39, p=0.02), C3dg (OR 17.6, 95% CI 3.01-89.97, p=0.001), C4d (OR 8.32, 95% CI 2.00-30.33, *P*=0.004) and C5b9 (OR 12.14, 95% CI 1.95-61.35, p=0.004). Glomerular C5b9 staining, a marker of complement terminal pathway activation, significantly correlated with both glomerular C3c/C3b/iC3b and C3dg but not C4d staining (Table 14). There was no correlation between glomerular C4d and either C3dg or C3c/C3b/iC3b.

Figure 22. Antibody specificity to purified human proteins by enzyme linked immunosorbent assay

(A) I demonstrated the anti-FHR5 antibody specifically detects FHR5 incubated with surface bound C3c, iC3b and C3dg (shown with a +) by ELISA. I detected minimal binding of the anti-FHR5 antibody to the complement proteins in the absence of FHR5 (shown with a -).

(B) The anti-C3b/iC3b/C3c antibody (Rabbit polyclonal anti-human C3c, Dako product number A0062) I used for immunohistochemistry staining detected surface bound C3c and iC3b by ELISA with significantly greater sensitivity than it detected C3dg or BSA, used as a negative control. The antibody-antigen interactions were not affected by pre-incubation with FHR5.

C: Similarly, the anti-C3dg antibody detected surface-bound C3dg by ELISA with significantly greater sensitivity than it detected C3c, iC3b or BSA.

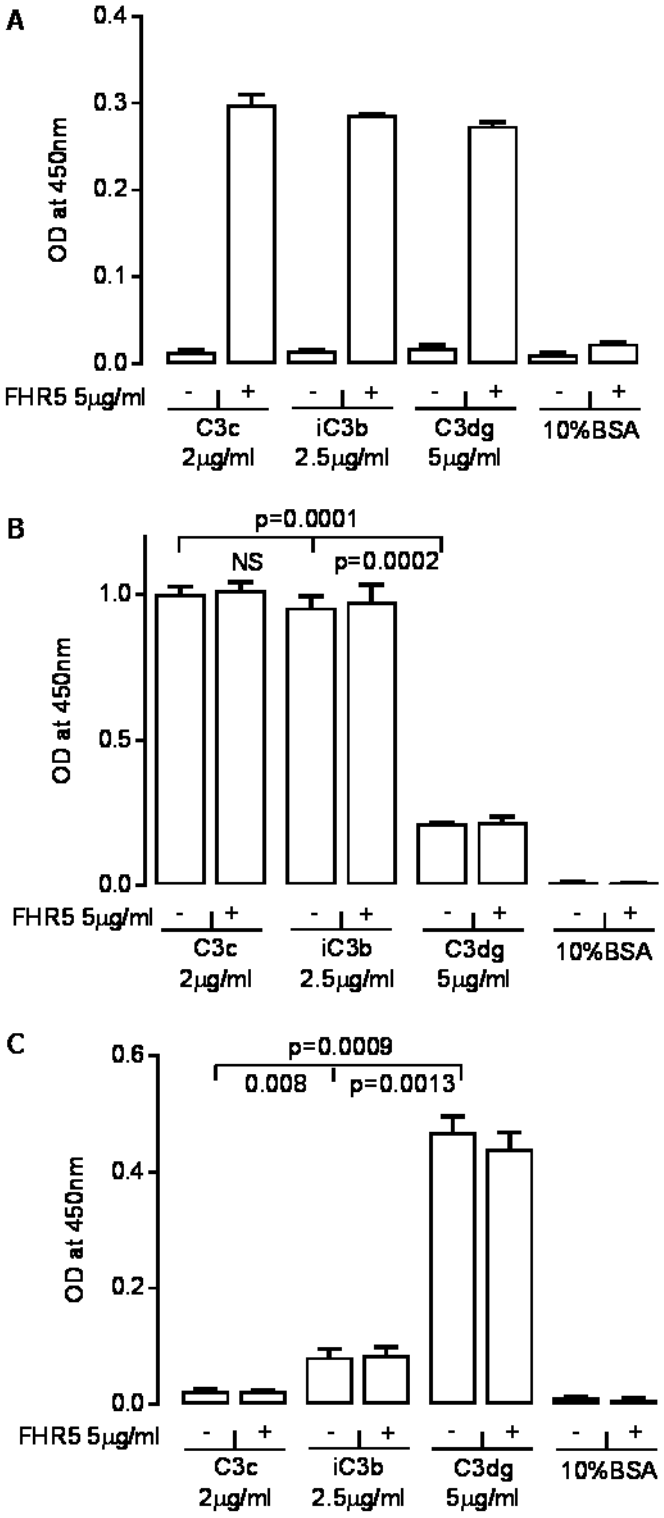
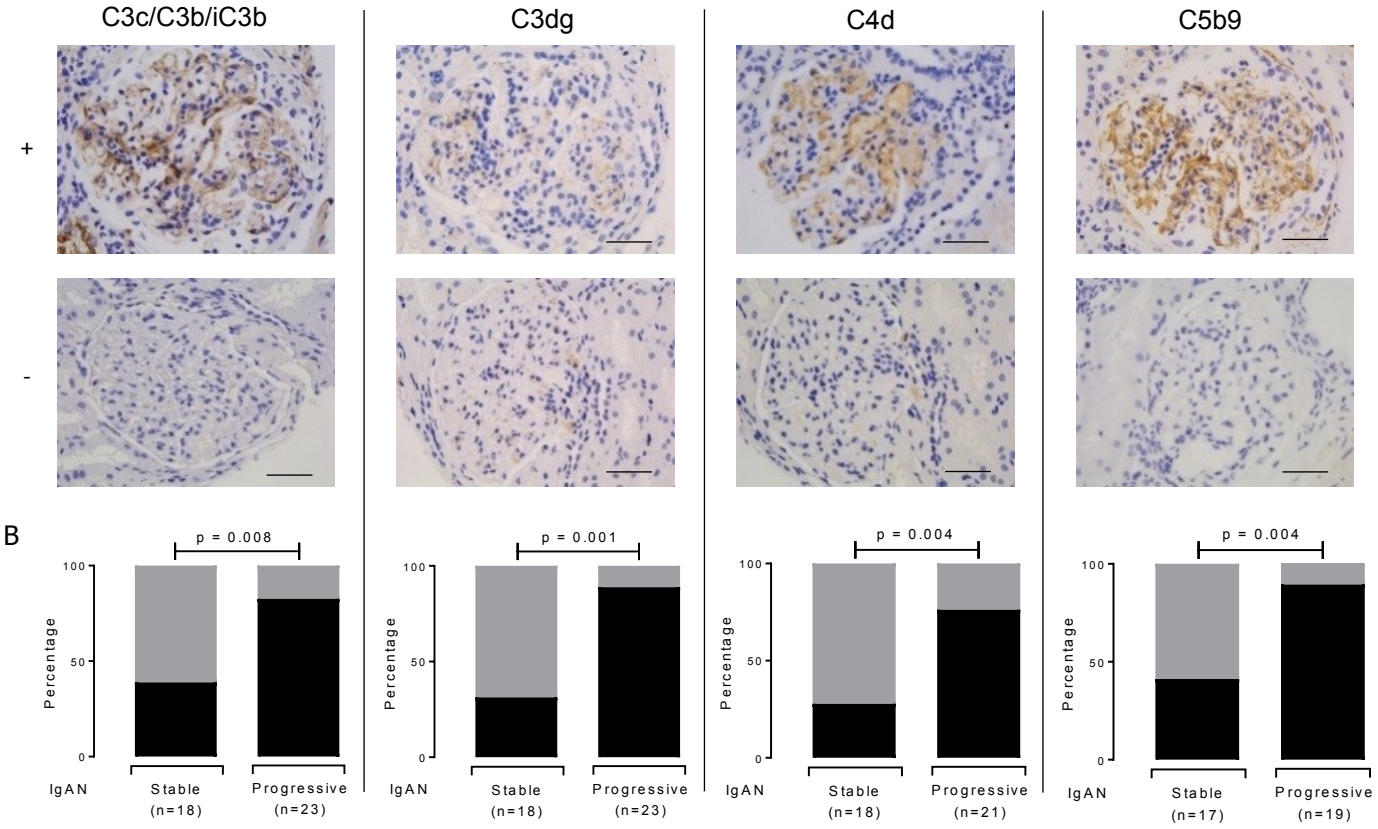


Figure 23. Glomerular complement deposition in progressive IgA nephropathy

(A) Representative images for complement C3b/iC3b/C3c, C3dg, C4d and C5b9. The top row represents present (+) and the bottom row represents negative (-) staining. Original magnification x400. Bars represents 100 µm.
(B) Proportion of cases with present (black) versus absent (grey) glomerular staining in stable and progressive IgAN. P values derived from Fisher's exact test. P-values shown have been adjusted for multiple analyses to minimise the false discovery rate.



Correlation coefficient R values	C3dg	C4d	C5b9	FH	FHR1	FHR5
C3b/iC3b/C3c	0.45 (p=0.01)	0.31 (p=0.09)	0.57 (p<0.001)	0.06 (p=0.52)	-0.28 (p=0.8)	0.64 (p<0.001)
C3dg	-	0.22 (p=0.20)	0.53 (p=0.003)	-0.29 (p=0.11)	-0.11 (p=0.42)	0.68 (p<0.001)
C4d		-	0.34 (p=0.08)	-0.22 (p=0.19)	0.08 (p=0.45)	0.27 (p=0.12)
C5b9			-	-0.26 (p=0.14)	-0.31 (p=0.09)	0.75 (p<0.001)
FH				-	-0.33 (p=0.09)	-0.16 (p=0.30)
FHR1					-	-0.31 (p=0.09)
FHR5						-

Table 14. Correlation of mesangial complement antigen intensity in IgA nephropathy native renal biopsies

A correlation of complement antigen staining intensities in sections from the same biopsy, using the 0, 0.5, 1+, 2+, 3+ scale. R values are calculated from Spearman's rank correlation. P-values shown have been adjusted for multiple analyses to minimise the false discovery rate.

c. Glomerular FHR5 deposition is associated with progressive IgAN and complement activation

Sufficient surplus tissue for FHR5 IHC was available from 36 IgAN cases. Of these, 24 (66.7%) had positive and 12 (33.3%) had negative glomerular FHR5 staining. One representative glomerulus from every IgAN case I analysed for FHR5 deposition is shown (Figure 24). Progressive IgAN associated with glomerular FHR5 staining (OR 13.4, 95% CI 2.2-66.9, p=0.002) and a trend for more FHR1 staining (Figure 25). In contrast, glomerular staining for FH was significantly reduced in progressive compared to stable disease (Figure 25 OR 0.10, 95% CI 0.008-0.87, p=0.04). Glomerular FHR5 deposition associated with a number of specific clinical markers of IgAN severity at the time of biopsy (Table 15 and figure 26). Positive glomerular FHR5 staining correlated with lower eGFR at biopsy (Figure 26, difference of median eGFR -26ml/min/1.73m2, 95% CI -50 to -3, p=0.03), more proteinuria (Figure 26. Difference of median UPCR 59mg/mmol, 95% CI 2 to 123, p=0.04), and lower circulating intact C3 levels (Figure 26. Difference of median eGFR -0.24g/l, 95% CI -0.46 to -0.05, p=0.02) at the time of biopsy. After a median of 51 months follow-up, the cohort of IgAN patients with positive glomerular FHR5 staining included a greater proportion of patients who had reached ESRD (<0.001) (Figure 26); none of the patients with negative FHR5 deposition at diagnostic biopsy (n=12) had reached ESRD at a median of 100 months follow-up (range 30-196 months). These differences are unlikely to be secondary to lead-time bias because the stable cohort had a significantly longer duration of follow-up than the cohort with progressive disease.

The presence of glomerular FHR5 also correlated with histology markers of disease severity and activity. At diagnostic biopsy, the cohort of IgAN patients with positive glomerular FHR5 staining had significantly more mesangial hypercellularity (OR 15, 95% CI 2.3 to 76, p=0.001) and endocapillary hypercellularity (OR 10, 95% CI 1.6 to 50.5, p=0.01), and a trend towards more cellular or fibrocellular crescents (p=0.08) (Figure 27).

Diagnostic biopsy	Glomerular FHR5		P-value	Difference	95% confidence interval
	Present (n=24)	Absent (n=13)			
Male	15 (62.5%	5 (38.5%)	NS		
Caucasian	13 (54.2%)	9 (69.2%)	NS		
Median eGFR, ml/min/1.73m^2	63.1 (39.7-106.9)	94.1 (27.5-117.6)	0.03	31.0	2.6 to 51.0
Median urine PCR – mg/mmol	144 (95-203, n=23)	85 (39-126, n=10)	0.04	-59	-123.0 to -2.0
Median systolic/diastolic blood pressure – mmHg	128/78	129/78	NS		
Serum C3 (g/L)	1.13 (0.94-1.27) (n=24)	1.40 (1.20-1.46) (n=7)	0.02	0.25	0.05 to 0.46
Median follow-up (months)	37.7 (10.0-57.5)	100.6 (85.7-201)	<0.001	62.9	40.9 to 128.3
Progressive IgAN at follow-up	17/24 (70.8%)	2/12 (16.7%)	0.002	OR = 12.1	2.2 to 66.9
ESRD at follow-up	4 (16.7%)	0 (0%)	NS		
Mesangial hypercellularity	18/24 (75.0%)	2/12 (16.7%)	0.001	OR = 15	2.3 to 76.0
Endocapillary hypercellularity	16/24 (66.7%)	2/12 (16.7%)	0.01	OR =10	1.6 to 50.5
Segmental sclerosis	20/24 (83.3%)	8/12 (66.7%)	NS		
Tubular atrophy >25%	12/24 (50.0%)	2/12 (16.7%)	0.08	OR = 5	0.8 to 25.5
Any cellular crescents	12/24 (50.0%)	2/12 (16.7%)	0.08	OR =5	0.8 to 25.5

Table 15. Clinical and histology features of severity at diagnostic biopsy associate with glomerular FHR5 in IgA nephropathy. Values within parentheses represent interquartile range unless otherwise stipulated.

Figure 24. Example images of glomerular FHR5 deposition in IgA nephropathy

An image of one representative glomerulus is shown from each of the cases with adequate tissue for FHR5 immunohistochemic staining. The cases we classified as showing positive FHR5 glomerular staining are shown in the left panel, and the cases with negative or absent glomerular FHR5 deposition are shown in the right panel.
All images at 400x magnification. Bars represents 100 µm.

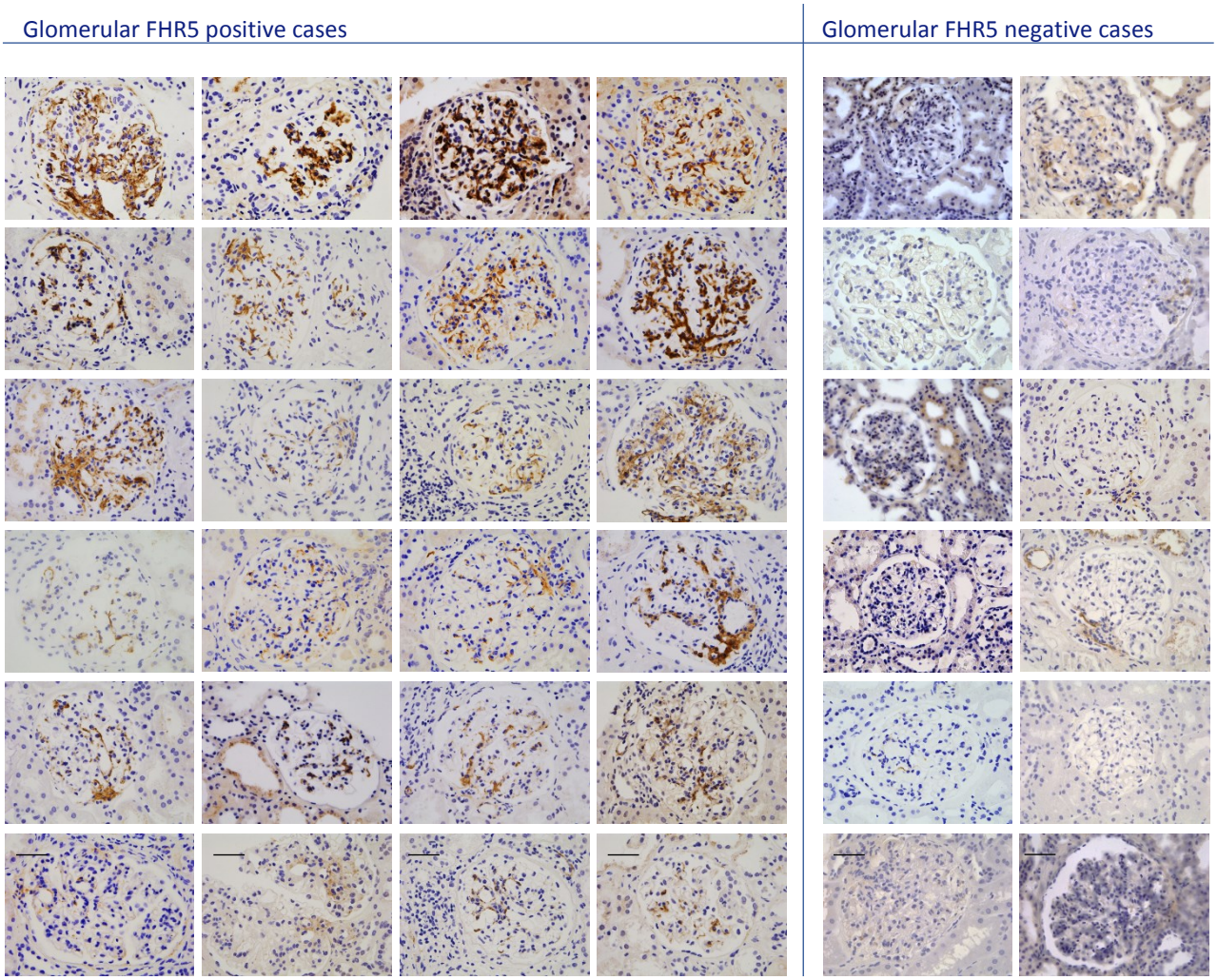


Figure 25. Glomerular alternative pathway regulator deposition in progressive IgA nephropathy

(A) Representative images for complement factor H (FH), factor H-related proteins 1 (FHR1) and 5 (FHR5) from six different IgAN cases. Top row represents positive staining (+) and bottom row represents negative staining (-). Original magnification x400. Bar represents 100 µm. Due to tissue availability, non-sequential sections were available only and the chort sizes differed for each antigen stain.
(B) Proportion of cases with positive (black) versus absent/minimal staining (grey) glomerular staining in stable and progressive IgAN.
P values derived from Fisher's exact test.

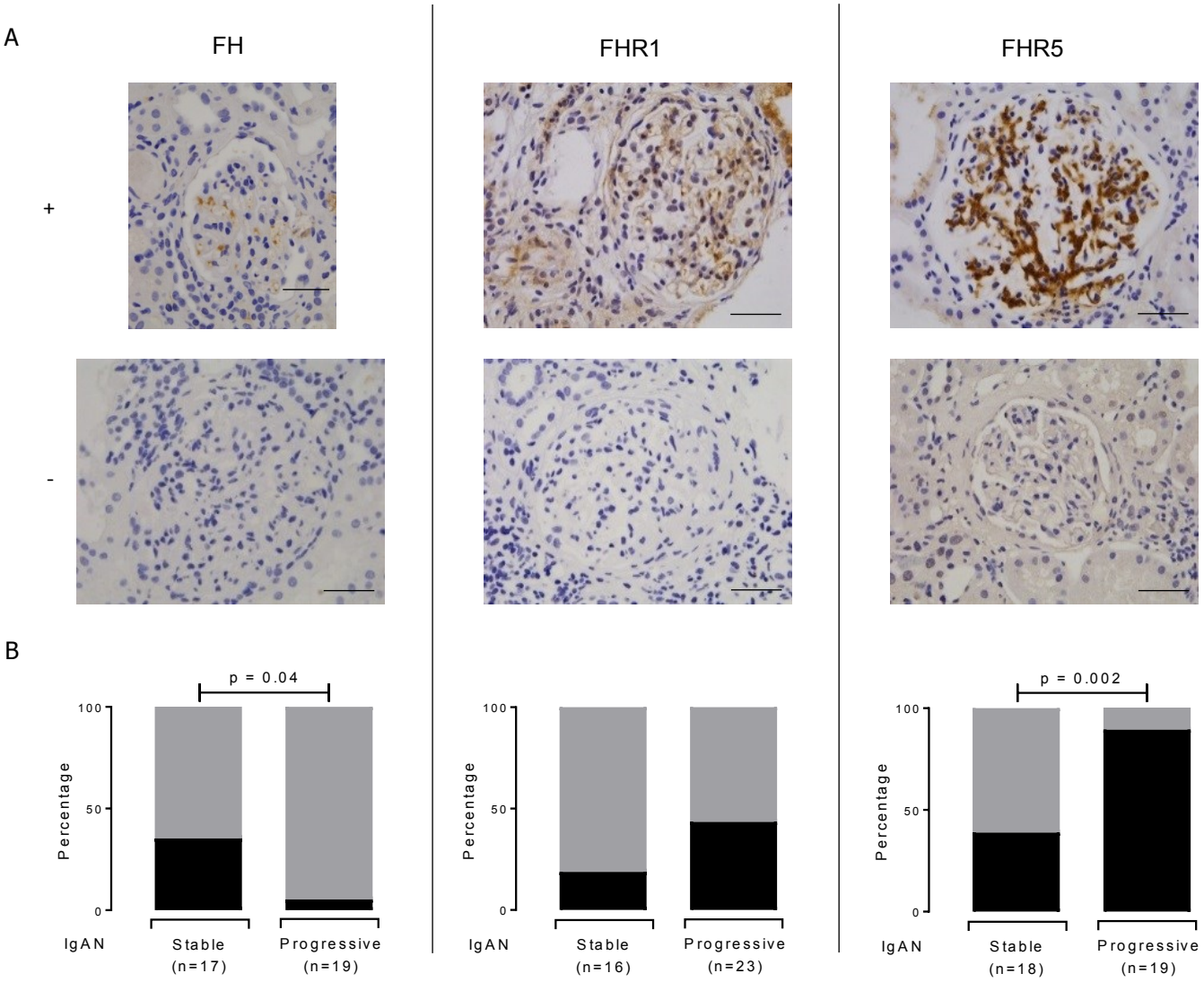


Figure 26. Glomerular FHR5 deposition associates with clinical markers of IgA nephropathy severity

IgAN cases with positive glomerular FHR5 deposition (FHR5+) showed (A) lower eGFR, (B) greater UPCR and (C) lower circulating C3 at biopsy.
Lines and whiskers represent median value and interquartile ranges

D: The proportion of IgAN patients who reached ESRD at a median of 51 months follow-up in cohorts with positive (FHR5+) and absent (FHR5-) glomerular FHR5 deposition.

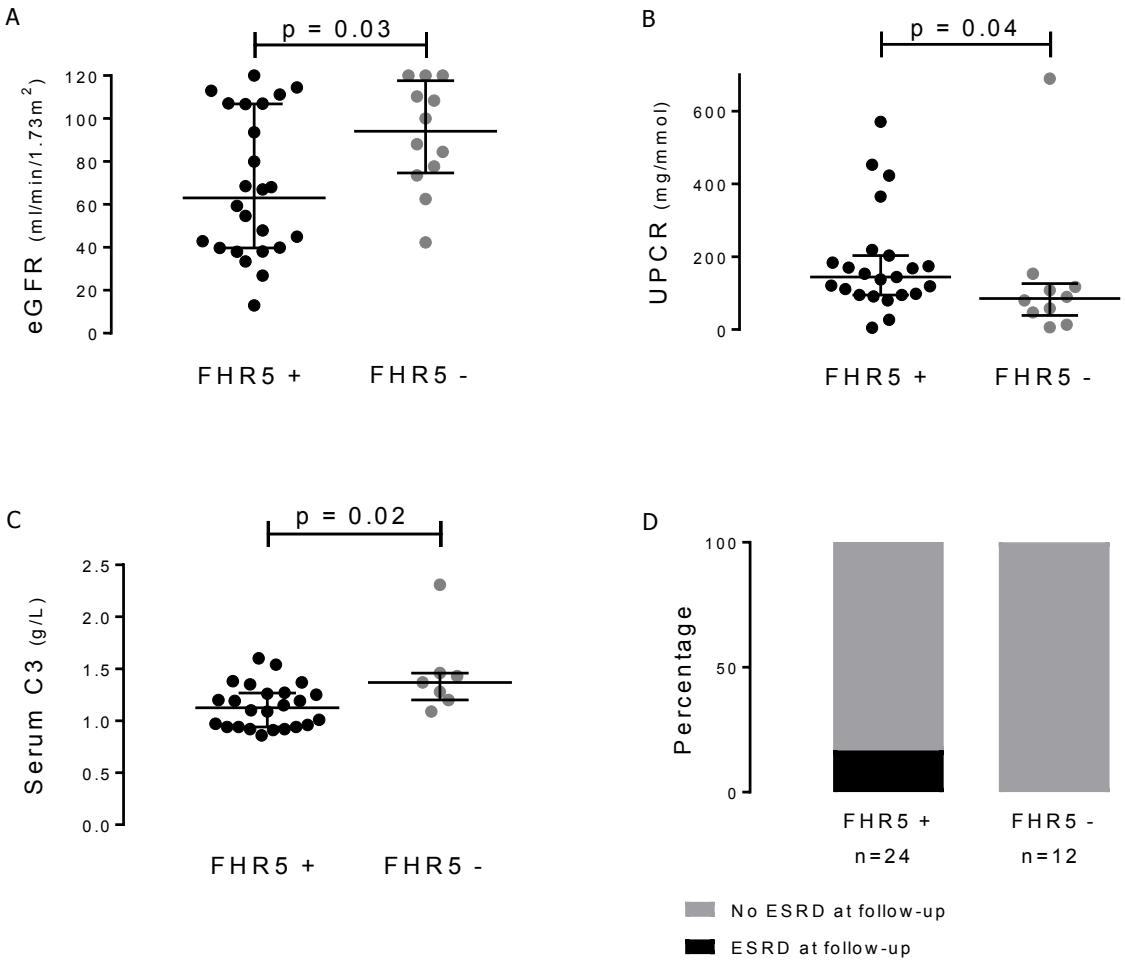
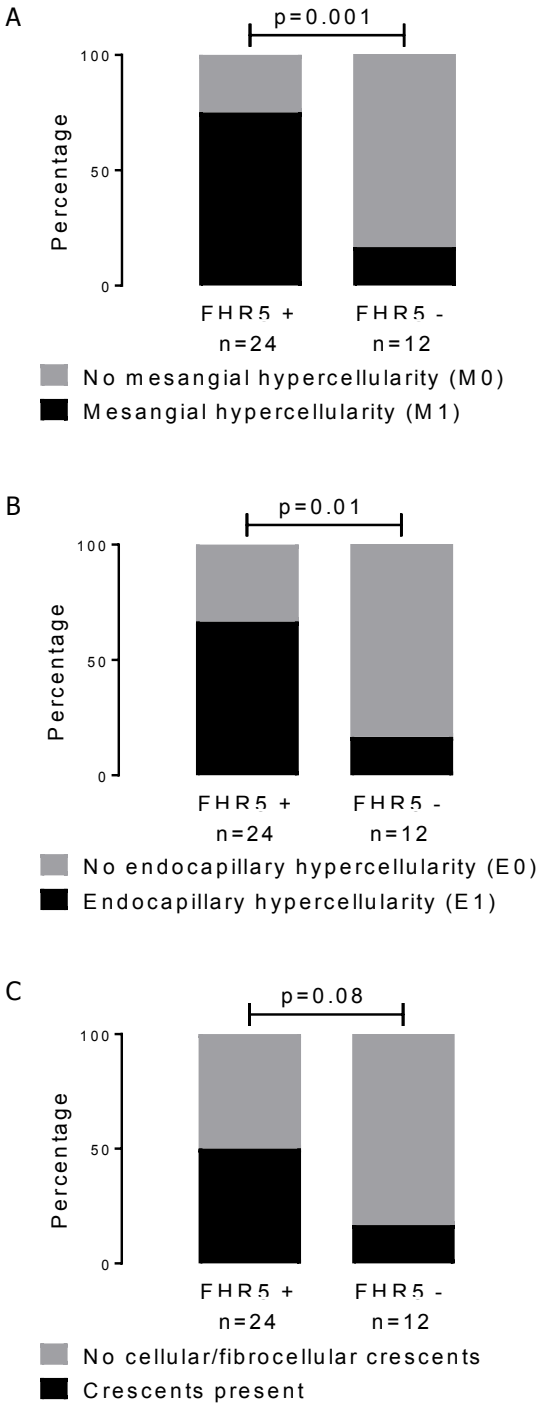


Figure 27. Glomerular FHR5 deposition associates with histology markers of IgA npehropathy severity

A greater proportion of IgAN cases with positive glomerular FHR5 deposition (FHR5+) showed (A) mesangial hypercellularity, (B) endocapillary hypercellularity, and (C) cellular or fibrocellular crescents than IgAN cases with absent FHR5 glomerular deposition (FHR5-).

P values derived from Fisher's exact test. P-values shown have been adjusted for multiple analyses to minimise the false discovery rate.



Glomerular FHR5 staining intensity correlated with the intensity of alternative and terminal complement activation markers, C3c/C3b/iC3b, C3dg and C5b9, but not C4d, a marker of lectin and classical complement activation (Table 14). In aggregate, a heat map of the glomerular staining data showed that the renal biopsies from patients with progressive disease had more staining for C3c/C3b/iC3b, C3dg, C4d, C5b9, and FHR5 than those with stable disease (Figure 28). Interestingly, the combination of positive glomerular staining for the complement deregulator FHR5 and negative staining for the regulator FH was significantly more common in patients with progressive (15/18, 88.2%) vs. stable (4/16, 25%) disease (OR 15, 95% CI 2.5-62.6, p=0.001). Furthermore, the combination of positive glomerular FHR1 and negative glomerular FH staining was more common in the progressive (9/19, 47.4%) than the stable (1/14, 6.7%) cohorts (OR 12.6, 95% CI 1.7 to 146.4. p=0.02). The cohorts included one patient with stable and one with progressive IgAN who had the deletion of the *CFHR3* and *CFHR1* genes in homozygosity. Glomerular FHR1 staining was negative in both cases but glomerular C3c/C3b/iC3b, C3dg, C4d, C5b9, and FHR5 was detectable (Figure 28). Surplus renal biopsy tissue was available from three patients with MBL deficiency, one with stable and two with progressive disease (Figure 28). Glomerular C4d deposition was present in the two patients with progressive disease but negative in the patient with stable disease. Glomerular C3c/C3b/iC3b was detectable in all three MBL-deficient IgAN patients.

Figure 28. Complement glomerular deposition intensity in IgAN: Heat map

Glomerular staining intensity scores from surplus native renal biopsy tissue from patients with either stable or progressive IgAN. Each row represents information from a single patient. Staining intensity was scored: 0 – absent, 0.5 – minimal; 1 – mild; 2 – moderate; 3 – strong. Filled cells indicate insufficient renal tissue to perform staining. Mannan-binding lectin (MBL) deficiency was defined as a plasma level of less than 100ng/ml. FH – complement factor H; FHR1 – factor H-related protein 1; FHR5 – factor H-related protein 5; MBL – mannan-binding lectin; CFHR3-1 – complement factor H-related 3 and 1 genes.

IgAN severity	*CFHR3-1* copies	MBL deficient	C3c/C3b/iC3b	C3dg	C4d	C5b9	fH	FHR1	FHR5
Stable	2	No	0	0	0	0.5	0	0	0
Stable	2	No	0	0.5	0.5	1	0.5	0.5	0
Stable	2	No	0.5	1	0	0	1		0.5
Stable	1	No	0.5	1	0	0	0	0.5	0
Stable	2	No	0.5	0.5	0	0.5	0.5	0	0.5
Stable	2	No	0.5		0.5	0		1	0
Stable	1	No	0.5	0.5	0.5	1	0	0.5	0.5
Stable	2	No	0.5	1	0.5	2	0	0.5	1
Stable	1	No	0.5	0.5	1	0.5	0.5	0.5	0.5
Stable	1	No	0.5	1	1	0.5	0	1	1
Stable	2	No	0.5	0		1		0.5	1
Stable	1	No	1	0	0	3	0.5	0	2
Stable	2	Yes	1		0.5		1	1	2
Stable	2	No	1	0.5	0.5	0.5	1		0.5
Stable	2	No	1	0.5	1	1	0	0.5	1
Stable	0	No	1	1	1	2	1	0	2
Stable	2	No	1	0	2	0	1	0.5	0
Stable	2	No	2	0.5	0.5	0	1	0	0.5
Stable	2	No			0		0.5		
Progressive	2	No	0.5					1	1
Progressive	2	No	0.5	1	1	1	0	2	1
Progressive	2	No	0.5	2	0.5	1	0	1	2
Progressive	2	No	0.5	1	1	2	0	0.5	2
Progressive	1	No	1		0.5			0	
Progressive	1	No	1	1				0	
Progressive	1	Yes	1	0.5	1	0.5	0	2	0.5
Progressive	2	No	1	0.5	1	0.5	0	2	
Progressive	2	Yes	1	1	1	1	0.5	1	1
Progressive	1	No	1		1	1		0.5	
Progressive	2	No	1	1	1	2	0	2	1
Progressive	2	No	1	1	1	2	0	0	1
Progressive	1	No	1	1	1	2	0.5	0	2
Progressive	0	No	1	1	2	2	0.5	0	2
Progressive	2	No	1	1	2	3	0	1	0
Progressive	1	No	2		0.5		0	1	2
Progressive	2	No	2		0.5	2	0	1	2
Progressive	1	No	2	2	1	2	0	0	3
Progressive	2	No	2	2	1	2	0	0.5	3
Progressive	2	No	2	2	1	3	0	0	3
Progressive	2	No	3	2	2	3	0	0	3
Progressive	2	No	3	1	1	3	0	0.5	3
Progressive	1	No	3	2	0.5	3	1	0	3

Discussion

I documented associations between IgAN severity and glomerular C3b/iC3b/C3c and C4d deposition. This replicates findings from other studies. For example, progressive renal disease associated with mesangial C3 deposition of 2+ and 3+ intensity in a cohort of 66 IgAN patients from Korea [240]. And mesangial C4d deposition was an independent predictor of ESRD in a cohort of 283 IgAN patients from Spain and correlated with proteinuria and mesangial proliferaiton glomerular sclerosis and interstitial infiltration in 60 Dutch IgAN patients[252, 253]. My data also identified associations between progressive IgAN and glomerular C3dg and C5b9 deposition. Glomerular C4d did not correlate with either C3b/iC3b/C3c or C5b9. This may reflect technical limitations such as our limited sample size, staining procedures, and limitations to the sensitivity of IHC techniques. However, it could also reflect the fact that the relative amount of deposited C4d, compared to C3 and C5b9, will be lower following complement amplification. Previous studies have identified glomerular C3 and C5b9 in the majority of both C4d positive and negative cases but did not record antigen staining intensity correlations[252, 253].

The correlation of glomerular FHR5 deposition with progressive IgAN is a key finding of my study. This was replicated by a recent proteomic analysis that showed glomerular FHR5 was 1.79 times more abundant in patients with progressive versus stable IgAN[285]. In our cohort, the presence of glomerular FHR5 correlated with a number of clinical markers of IgAN severity, including the development of ESRD at just over four years' follow-up, but also histologic markers of both IgAN severity and active glomerular inflammation. I also found glomerular FHR5 deposition correlated with markers of alternative complement pathway activity. *In vitro* data suggests FHR5 antagonises the ability of FH to negatively regulate C3 activation[8, 119]. It is interesting that patients with progressive disease had more glomerular FHR5 staining in the absence of FH. Notably, there are phenotypic similarities between IgAN and familial C3 glomerulopathy associated with mutant FHR5 proteins. The mutant FHR5 proteins show enhanced ability to compete with FH and deregulate complement activty[5, 7]. Together, these data suggest glomerular FHR5 is contributing to alternative pathway dependent complement activation and amplification, with subsequent glomerular inflammation and injury, and more severe IgAN.

Considering the genetic and serology associations between IgAN and FHR1[2, 245, 254, 286], it was surprising that glomerular FHR1 was absent in more than 50% of progressive IgAN biopsies. Unlike FHR5, glomerular FHR1 did not correlate with other complement antigens. This could be explained by differences in binding avidity of

FHR1 and FHR5 to C3b, iC3b and C3dg[8]. These differences may be exaggerated by the glomerular microenvironment. For example, glomerular complement activation would produce a nidus of surface bound activated C3 fragments. The glomerular basement membrane is absent of surface bound complement regulators and therefore dependent on FH for complement regulation. As FHR5 dimers can outcompete FH affinity and avidity, even small concentrations of FHR5 dimers could accelerate complete activity if surrounded by an abundance of C3 fragments. Theoretical mechanisms for this include preventing FH binding to C3b and C3 convertases, and binding and converting iC3b into a complement activating surface by recruiting C3b via the opposite dimer head. My data indicate a more prominent role for FHR5 than FHR1 in glomerular complement activation in IgAN. Notably, one patient with progressive IgAN had homozygous deletion of *CFHR1* and therefore no FHR1.

My complement staining data demonstrated the pathogenic heterogeneity of IgAN. For example, some patients exhibited co-deposition of FHR5, C3dg, C3b/iC3b/C3c and C5b9, in the absence of FH. This implies FHR5-dependent FH deregulation and alternative pathway activation. Other patients exhibited glomerular co-deposition of C4d with C3b/iC3b/C3c and C5b9 which is consistent with lectin pathway activation. Although FHR5 was commonly detected with C3b/iC3b/C3c, it could be detected in the absence of C3b/iC3b/C3c. However, in all cases but one, C3dg was detected in the FHR5 positive, C3b/iC3b/C3c negative cases. Whether FHR5 can be detected in the absence of C3 fragments requires further assessment, but in vitro data suggests it may be able to directly bind components of the glomerular basement membrane or mesangial extracellular matrix, such as laminins[121].

Interestingly, glomerular FHR5 staining was dependent on the method of antigen retrieval, demonstrating the importance of thorough IHC protocol development (Figure 6). Glomerular FHR5 staining was not detectable when pressure cooker heating was used instead of enzyme antigen retrieval. However, pressure cooker antigen retrieval revealed distinct tubular cell epithelial surface staining in all cases tested, including TBM (Figure 6). Tubular FHR5 staining after pressure cooker antigen retrieval did not associate with proteinuria and was not eliminated when the antibody was pre-incubated with purified human FHR5, which should eliminate antigen-antibody interactions (Figure 6 column of images labelled ‘Pre-incubation’). Pre-incubation of a TBM section with heparin that would limit non-canonical glycosaminoglycan interactions also had no effect on tubular staining. The tubular staining was reproduced in a case of IgAN when immunofluorescence was used

for primary antibody detection (Figure 6). The staining may be explained by Fc interactions but an antibody isotype control was unavailable for us to test this theory.

A number of limitations temper the robustness of these results. The most important is that due to tissue unavailability, sequential sections of the same glomeruli were not available to directly compare complement protein deposition in the same glomeruli. This is balanced against the benefit of a comprehensive immunostaining analysis of a large cohort of samples from patients with a range of disease severities. Secondly, the specificity of anti-C3dg antibody is unclear. Although differences in apparent C3b/iC3b/C3c and C3dg detection in some IgAN cases suggests the two antibodies a specific for different epitopes, the structural homology of the C3 fragments means antibodies that can differentiate specific to C3 fragments have been difficult to produce. I tried to estimate antibody specificity by comparing antibody binding to plate-bound purified C3 fragments. This is itself limited by possible differences in antigen and epitope conformation *in vivo* and *in vitro*. Furthermore, the concentrations used in the antibody analysis were not equimolar. At the concentrations used, the molarities of C3c and iC3b were similar at 14.5 nanomolar for C3c and 13.7 nanomolar for iC3b. The molarity of C3d was 111.1 nanomolar. This supports the specificity of the anti-C3b/iC3b/C3c antibody. However, the experiment needs to be repeated with equivalent C3d molarity to assess C3dg antibody specificity.

In summary, the data presented in this chapter indicate glomerular FHR5 staining of diagnostic biopsies can identify patients with severe disease who are at risk of progression to renal impairment. Although these data suggest FHR5 might be a useful immunohistochemic marker of complement activity and disease severity, this was not an *a priori* aim requires prospective assessment. As glomerular C3 can be detected in about 90% of IgAN cases, the ability to determine which cases a driven by alternative complement pathway would be clinically useful, especially with the increasing availability of therapeutic complement inhibitors. I next questioned whether the IHC associations also reflected a pathogenic role for FHR5 in alternative complement pathway activation and glomerular injury in IgAN. In the next chapter I will describe my subsequent work looking for glomerular FHR5 in a renal condition dependent on alternative complement pathway activity, C3 glomerulopathy.

Conclusions

- Glomerular FHR5 deposition associated with clinical and histologic markers of disease severity in IgAN.
- Glomerular FHR5 deposition correlated with glomerular C3c/C3b/iC3b, C3dg and C5b9 but not C4d.
- Glomerular FHR5 staining of diagnostic biopsies can identify patients with IgAN who are most at risk of progression to renal impairment.

D. Glomerular factor H related protein deposition and complement in C3 glomerulopathy

Introduction

C3 glomerulopathy is a rare group of kidney diseases caused by uncontrolled complement activity[137, 287, 288]. C3G provides opportunity to interrogate complement dependent glomerular injury. C3G is associated with significant morbidity; up to fifty percent of C3G patients progress to ESRD within five years of diagnosis[144, 148] and recurrent C3G frequently causes transplant dysfunction[173, 177]. However, the pathogenesis of the majority of C3G cases is unknown. We do not understand why C3G includes a wide range of clinical and histopathology features[139, 289]. Furthermore, glomerular complement composition in C3G, including changes following clinical events such as treatment, has not been thoroughly documented[141]. It is unclear whether glomerular deposition of specific complement proteins predicts C3G prognosis and therapeutic response.

The FHR proteins are structurally similar to FH, an essential regulator of the alternative complement pathway[34] but lack its complement-regulatory domains[290]. Current evidence suggests dimeric FHR1 and FHR5, that circulates as an obligate homodimer *in vivo*[109], compete with FH binding to activated complement C3, impair FH-dependent regulation and magnify complement activation[8, 9]. Glomerular FHR5 has been documented in a range of glomerulopathies in similar distributions to C3[112]. FHR5 also shows affinity for C3dg and iC3b *in vitro*[8]. CFHR5 nephropathy, a subtype of C3G associated with a gain-of-function mutation to FHR5 in heterozygosity[5], demonstrates the potential of FHR5 to cause complement-dependent glomerular injury. A number of other hybrid and mutant FHR proteins have been identified in specific C3G cases[6, 7, 122, 291, 292]. In previous chapters, I demonstrated circulating FHR5 levels[282, 286] and the intensity of glomerular deposition of FHR5[293] associated with the severity of IgAN. However, the pathogenicity of non-mutant FHR5 is yet to be established.

Hypothesis and aims

The research I present in this chapter aimed to investigate the pathogenesis of FHR5 in complement-dependent glomerular injury. I first aimed to determine whether glomerular FHR5 staining associated with the severity of C3G, which is pathogenically associated with complement activation. I next aimed to identify glomerular complement ligands for FHR5 in C3G. I therefore undertook detailed FHR5 immunostaining in C3G. I hypothesised FHR5 glomerular staining would associate with features of C3G severity, complement activation

and glomerular deposition of proteins representative of ongoing complement activation. I hoped this would determine whether FHR5 may be a useful biomarker and pathogenically important in C3G, with potential relevance to other renal conditions characterised by glomerular C3 deposition, including IgAN.

Methods

I identified 34 biopsies from 19 C3G patients with available, unstained FFPE tissue. Nine of these were transplant biopsies from five patients. All patients were part of the C3G cohort described previously. I first optimised staining protocols using FFPE sections from patients with complement (C3G, IgAN and SLE) and non-complement (thin basement membrane disease) associated renal conditions, as previously described. I completed approximately half of the IHC of C3G patient sections. The other half was completed by Hilary (Moffitt) McPhail. We applied IHC protocols, as detailed in the Methods chapter, for the following proteins: FHR5, FHR1, FH, C3b/iC3b/C3c, C3dg, C5b9, properdin, C4d, C1q and CD68. This was dependent on the availability of tissue for each case.

To identify the glomerular ligand for FHR5, Hilary (Moffitt) McPhail and I performed double IF staining for FHR5, C3b/iC3b/C3c and C3dg in three cases where sufficient surplus tissue was available. In each case, we also performed double staining for C3b/iC3b/C3c and C3dg. Before performing double IF, Dr Hannah J. Lomax Browne tested the antibodies for binding specificity to purified proteins by ELISA, with and without pre-incubation with FHR5.

I used Image J software and the COLOC2 plug-in[273] to calculate antigen location correlations. This method compares identical pixels in two versions of the same image filtered for different colours, and counts the pixels that are stained with each colour above the pre-specified brightness threshold. I ensured IF images were captured with identical microscope settings, images were of identical size, and the brightness threshold at which monochromatic (red or green) fluorescence was counted as positive deposition was standardised. A representative and non-sclerosed glomerulus from each section was selected as the region of interest for analysis. I calculated Pearson's correlation coefficients for the glomerular location of each antigen combination in all available glomeruli. I calculated median correlation coefficients for each antigen combination.

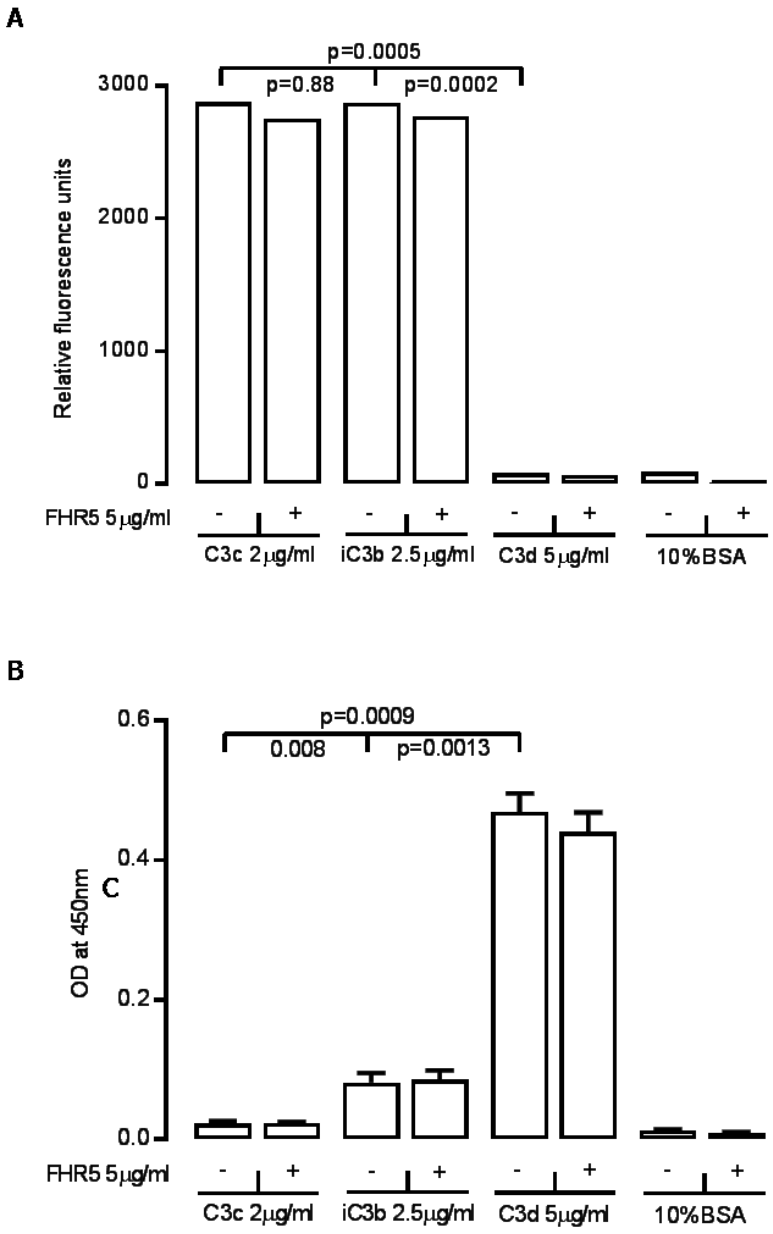
Results

a. Antibody specificity

By ELISA, the anti-C3b/iC3b/C3c and anti-C3dg antibodies used for IF analysis showed preferential binding for their canonical C3 fragments irrespective of the presence of FHR5 (Figure 29, data courtesy of Dr Hannah J. Lomax-Browne). I have previously shown the antibodies used for IHC demonstrated adequate antibody specificity for their ligands in the form of purified recombinant human proteins immobilised on ELISA plates. As mentioned in the previous chapter, this estimation of antibody specificity is limited by possible differences in antigen and epitope conformation *in vivo* and *in vitro*. Furthermore, the concentrations used in the antibody analysis were not equimolar. At the concentrations used, the molarities of C3c and iC3b were similar at 14.5 nanomolar for C3c and 13.7 nanomolar for iC3b. The molarity of C3d was 111.1 nanomolar. This supports the specificity of the anti-C3b/iC3b/C3c antibody. However, the experiment needs to be repeated with equivalent C3d molarity to assess C3dg antibody specificity.

Figure 29. Antibody specificity to purified human proteins by enzyme linked immune-sorbent assay (ELISA)

(A) Dr Hannah J. Lomax-Browne established the anti-C3b/iC3b/C3c antibody (sheep anti-human C3c-FITC (ThermoFisher PA1-36179) I used for immunofluorescence (IF) staining detected surface bound C3c and iC3b by ELISA with significantly greater sensitivity than it detected C3dg or bovine serum albumin (BSA), used as a negative control. The antibody-antigen interactions were not affected by pre-incubation with FHR5.
(B) Similarly, the anti-C3dg antibody detected surface-bound C3dg by ELISA with significantly greater sensitivity than it detected C3c, iC3b or BSA.



b. Glomerular FHR5 is highly prevalent in C3G

FHR5 was detected in all the transplant biopsies (n=9 from 5 patients) and all but one of the native biopsies (n=25 from 16 patients). FHR5 was the most prevalent glomerular complement protein detected at 3+ intensity (Figure 30). Two biopsies were from patients with CFHR5 nephropathy, one with and one without evidence of renal disease (Figure 31). The individual with renal disease had intense glomerular FHR5 and C5b9 staining. Despite normal glomeruli by light microscopy, the patient without renal disease had prominent C3b/iC3b/C3c staining. Glomerular FHR5 and C5b9 were also detectable in the case with normal renal function and in the absence of light microscopy evidence of glomerular inflammation, but at less intensity than the patient with renal impairment. Therefore, abnormal complement deposition in CFHR5 nephropathy can occur without histology signs of glomerular inflammation. FHR5 and C5b9 IHC may provide both sensitive detection of complement dysregulation and, unlike C3b/iC3b/C3c IHC, proportionately reflect injurious complement deposition.

Two of the renal biopsies from the five transplant patients were indicated by routine surveillance programs, which provided another opportunity to characterise sub-clinical C3G (Figure 32). In one of these surveillance biopsies (six months post-transplant), although the glomeruli appeared normal by light microscopy and showed no glomerular staining for C3, C5b9 and CD68, there was low intensity glomerular FHR5 staining. Nine months later the patient developed biopsy-proven C3G recurrence. Unfortunately, no surplus tissue was available from the second transplant biopsy. In the other three cases, glomerular staining for both FHR5 and C3b/iC3b/C3c was evident, two with and one without glomerular C5b9 staining.

One patient with recurrent C3G in the transplant kidney underwent four renal biopsies and treatment for crescentic recurrent C3G that included C5 inhibition with eculizumab. This enabled me to examine changes in glomerular FHR5 over time and in response to terminal complement pathway blockade. FHR5 and other markers of complement activation were identified in subclinical recurrent C3G despite absent proteinuria (Figure 33). Crescentic recurrent C3GN development was mirrored by increases in FHR5, C3b/iC3b/C3c, C5b9, and C3dg staining intensities (Figure 33). Eculizumab treatment coincided with improved endocapillary hypercellularity, reduced CD68-positive cell infiltration, eGFR stabilisation and proteinuria improvement. Eculizumab would not be predicted to affect alternative complement pathway activation and, consistently, glomerular FHR5, C3b/iC3b/C3c and C3dg deposition showed no improvement at eculizimab course

completion (Figure 33, third column of images). Three months after stopping eculizumab, graft function deteriorated and a fourth transplant biopsy showed recurrent crescentic C3GN. Rapid clinical improvement was seen after reintroducing eculizumab treatment. Subsequently proteinuria increased and transplant function deteriorated despite ongoing eculizumab therapy.

Figure 30. Glomerular complement protein staining intensity in native and transplant C3 glomerulopathy biopsies

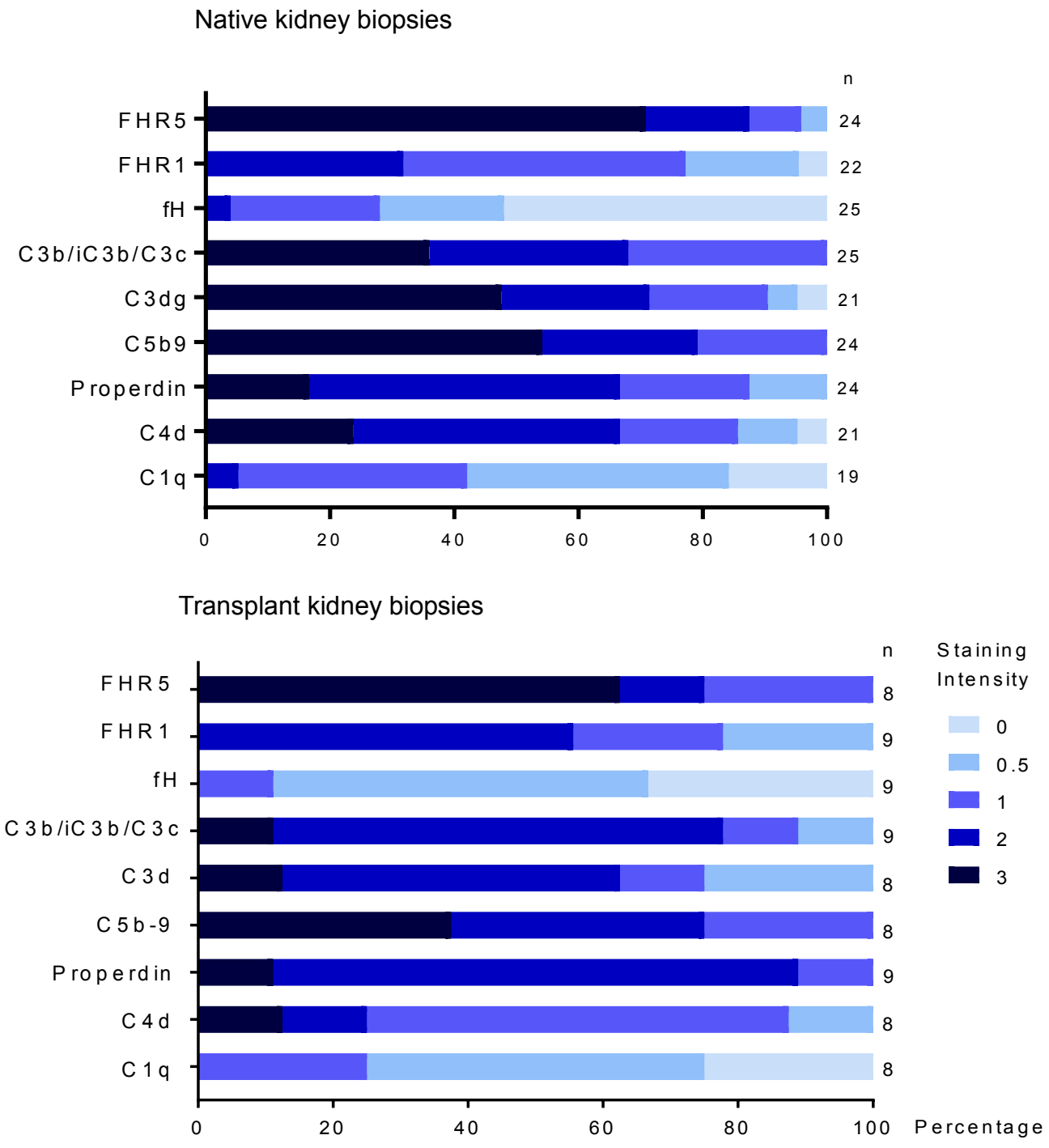


Figure 31. Representative images of complement staining in the native kidney

For each set of images the biopsy indication together with the eGFR and proteinuria at the time of biopsy and the appearances of the glomeruli by light microscopy are indicated. Sequential sections of the same glomeruli were not available.
EH - endocapillary hypercellularity; GN - glomerulonephritis; FHR5 mutation – the mutation described in CFHR5 nephropathy; NS - Nephrotic syndrome. Potential donor – individual underwent assessment for living kidney donation.
Original magnification 400x. Bars represent 100 µm.

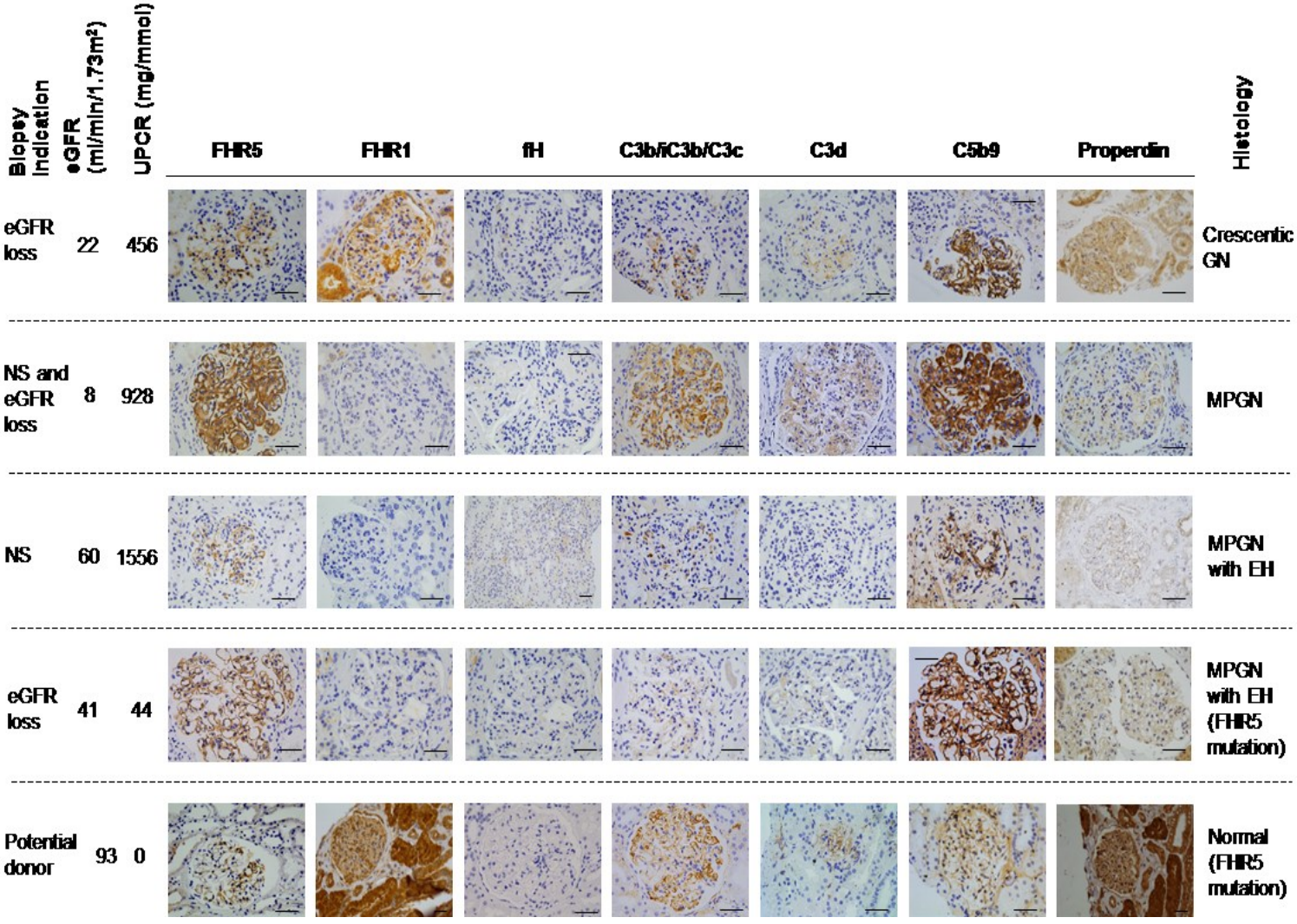


Figure 32. Representative images of complement staining in transplant kidneys

For each set of images the biopsy indication (either protocol or due to clinical changes i.e. indication biopsy), time post-transplant together with the eGFR and proteinuria at the time of biopsy are indicated. The histology including the presence of absence of rejection and the histology in later biopsies is also shown.
Original magnification 400x. Bars represent 100 µm.

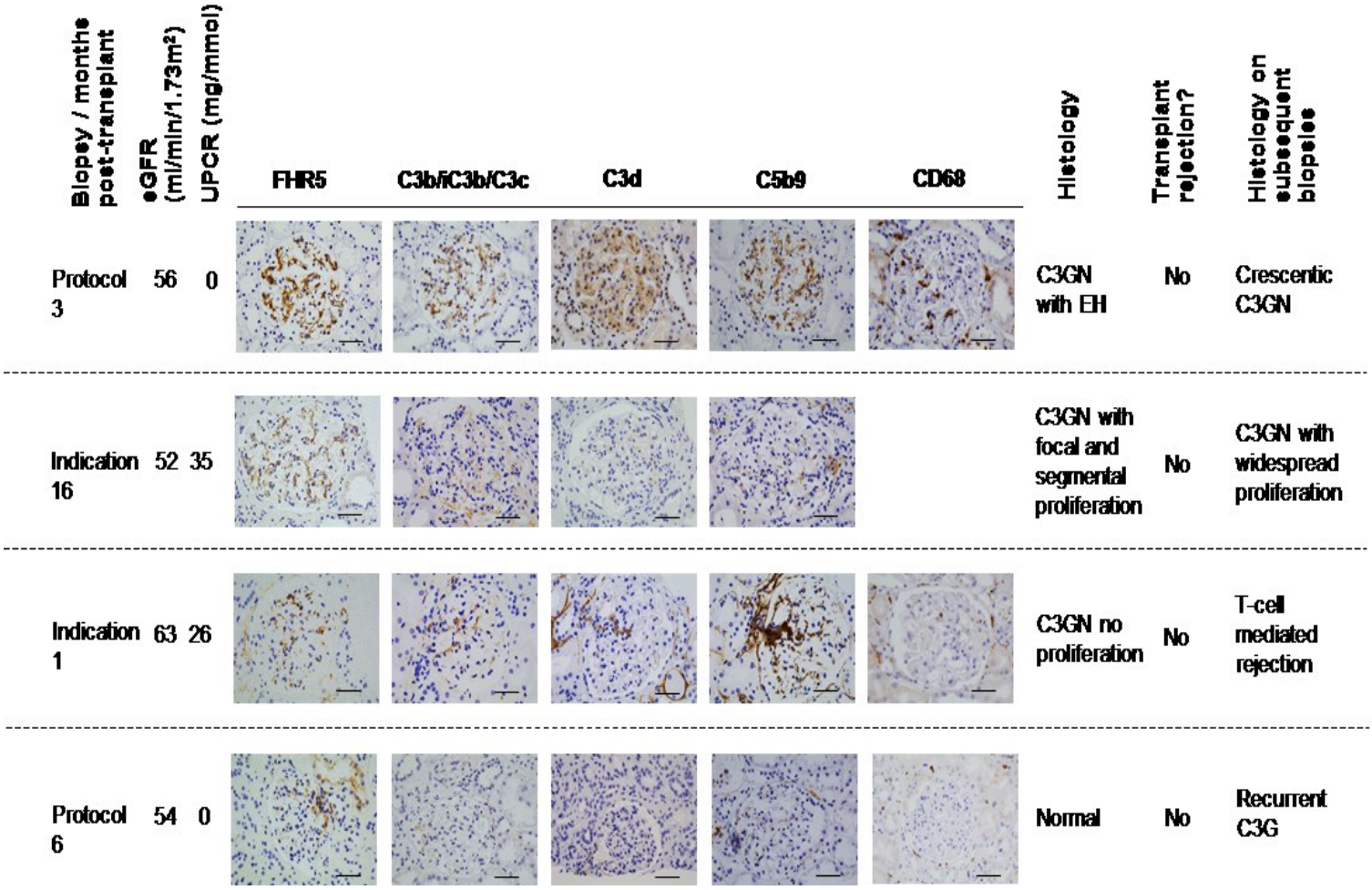
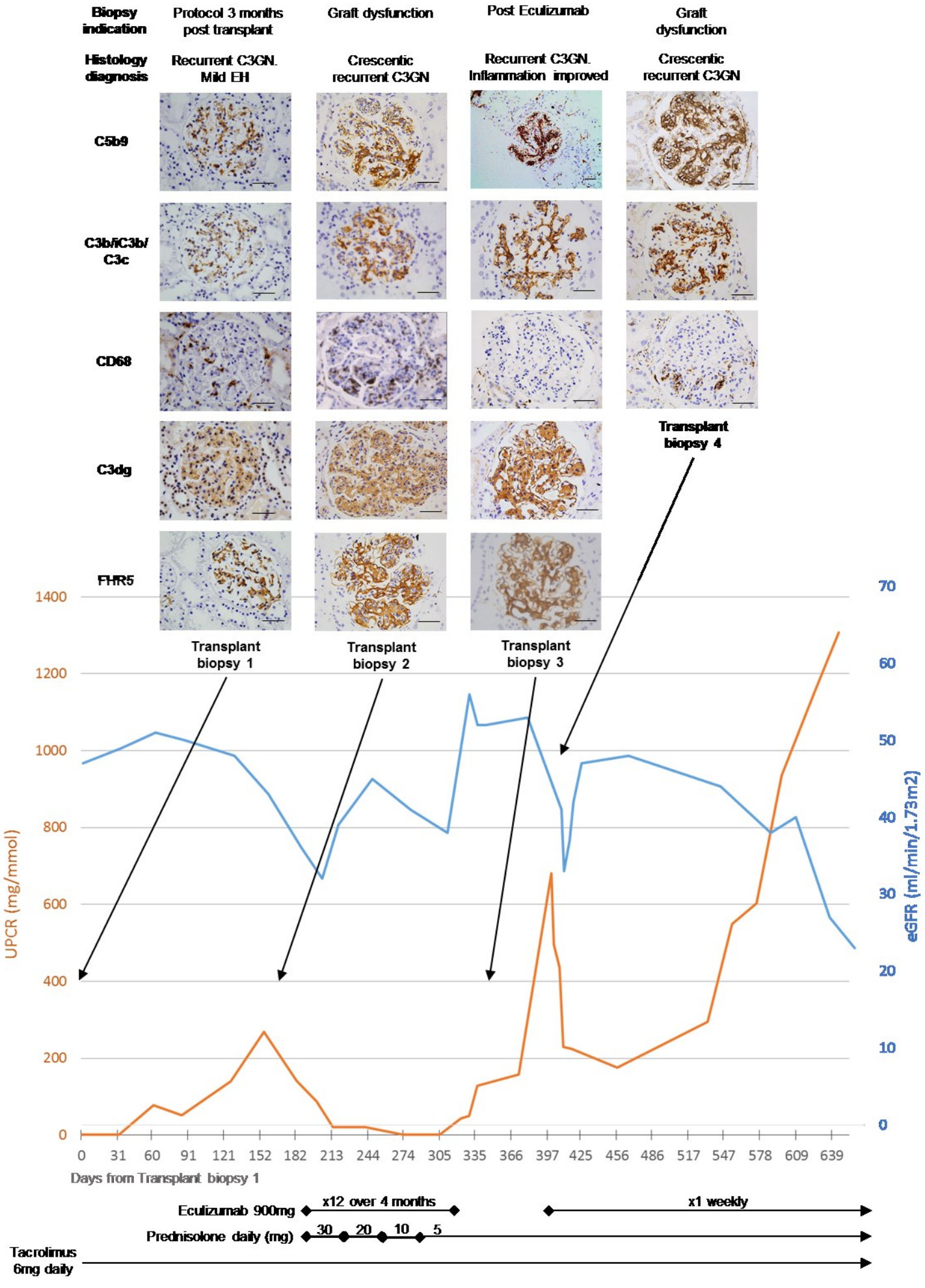


Figure 33. Sequential glomerular FHR5 staining in a single case of C3G transplant recurrence

Images and information from the same biopsies are organised in columns. A protocol surveillance biopsy performed 3 months after transplantation (Transplant biopsy 1) showed recurrent C3GN with mild EH. Glomerular FHR5, C5b9, C3b/iC3b/C3c, and C3dg were detected. The eGFR was stable and there was no significant proteinuria. Approximately 6 months post-transplant the patient developed proteinuria and a fall in eGFR. Biopsy showed crescentic C3GN and increased glomerular CD68 positive cells (Transplant biopsy 2). Glomerular FHR5, C5b9, C3b/iC3b/C3c, and C3dg were detected but at increased staining intensity compared to the first transplant biopsy. The eGFR and proteinuria improved with eculizumab and prednisolone treatment. After 4 months of eculizumab treatment, renal biopsy (Transplant biopsy 3) showed resolution of glomerular CD68 staining but glomerular staining for FHR5, C5b9, C3b/iC3b/C3c, and C3dg remained unchanged. After a further 3 months, proteinuria increased and biopsy showed crescentic C3GN and recurrence of glomerular CD68 positive cells (Transplant biopsy 4). Proteinuria improved with re-introduction of eculizumab. However, after about three months of weekly eculizumab treatment, significant proteinuria recurred and transplant function, as evidenced by eGFR, deteriorated.
Original magnification 200x or 400x magnification. Bars represent 100 µm.


Biopsy indication
Protocol 3 months post transplant
Graft dysfunction
Post Eculizumab
Graft dysfunction
Histology diagnosis
Recurrent C3GN. Mild EH
Crescentic recurrent C3GN
Recurrent C3GN. Inflammation improved
Crescentic recurrent C3GN
C5b9
C3b/iC3b/ C3c
CD68
C3dg
FHR5
Transplant biopsy 1
Transplant biopsy 2
Transplant biopsy 3
Transplant biopsy 4
UPCR (mg/mmol)
eGFR (ml/min/1.73m2)
Days from Transplant biopsy 1
Eculizumab 900mg
x12 over 4 months
x1 weekly
Prednisolone daily (mg)
30
20
10
5
Tacrolimus 6mg daily

c. Glomerular FHR5 associates with C3G severity

I next examined whether or not glomerular FHR5 correlated with either renal impairment or histological changes. Glomerular FHR5 of 3+ intensity correlated with renal impairment at the time of biopsy (Figure 34) and was the single complement staining metric associated with an MPGN pattern at diagnostic biopsy (90% patients with vs 33.3% without MPGN pattern, P=0.049. OR 18, 95% CI 1.6 to 201). Notably, glomerular C5b9 also correlated with renal impairment at the time of biopsy (Figure 34) indicating a significant contribution of C5 activation to renal impairment in this setting. Glomerular FHR1, C3b/iC3b/C3c, C3dg and C4d did not correlate with renal impairment (Figure 34).

Figure 34. Glomerular FHR5 and C5b9 staining intensity associated with lower estimated glomerular filtration rate at biopsy in C3 glomerulopathy

The eGFR at the time of biopsy was significantly lower in biopsies that had maximal staining intensities for FHR5 (p=0.04, difference of medians 19.7 ml/min/1.73m2; 95% CI 1.1 to 43.0) and C5b9 (p=0.03, difference of medians 14.86 ml/min/1.73m2; 95% CI 3.8 to 46.6). This was not seen for glomerular FHR1, C3b/iC3b/C3c, C3dg and C4d. The cohort size differences are explained by the availability of surplus stored renal biopsy tissue for analysis and the prevalence of antigen staining at 3+ intensity. Bars represent median values and interquartile ranges.

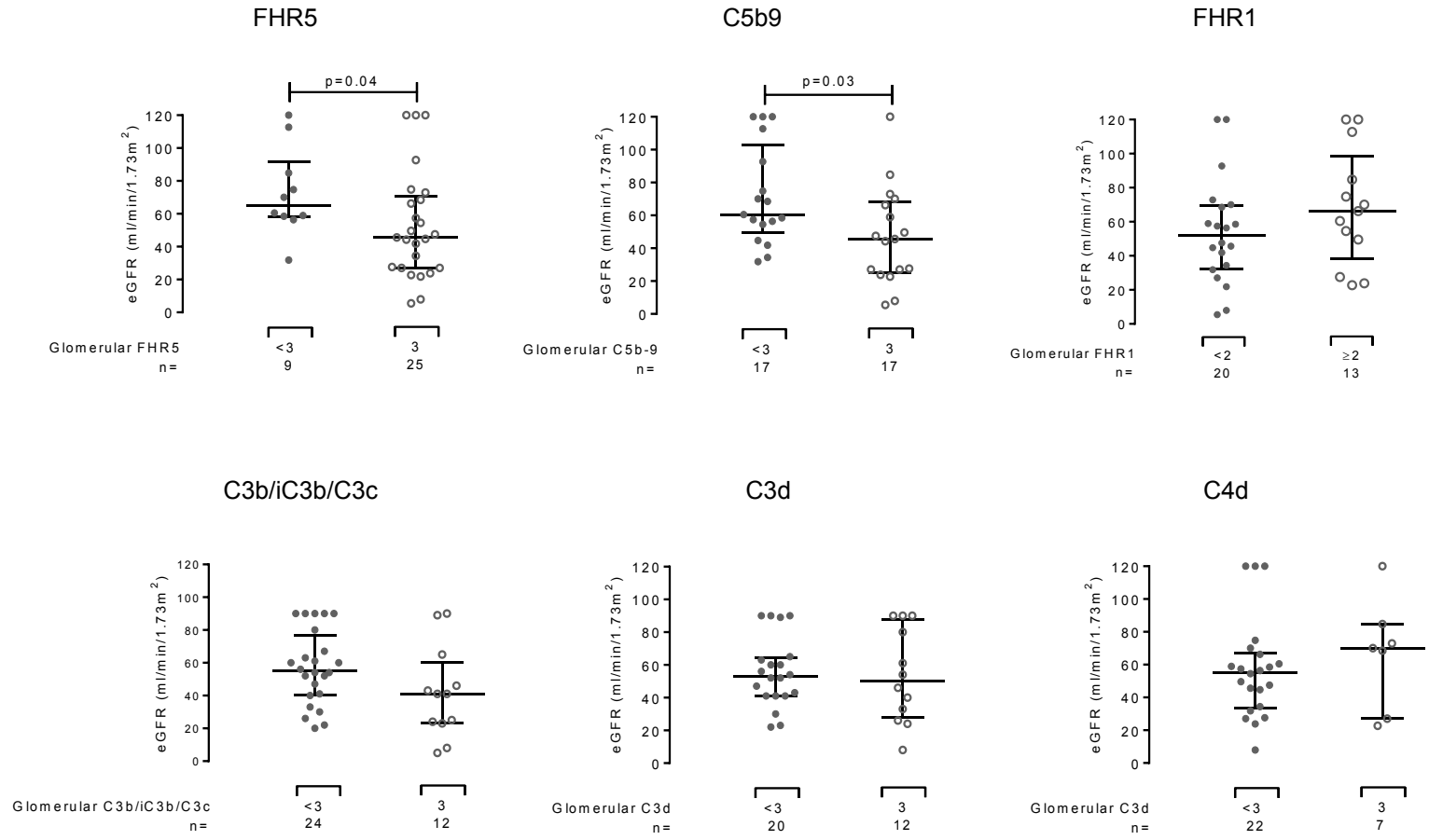


Figure 35. Glomerular FHR5 staining intensity positively correlated with C3b/iC3b/C3c, C3dg and C5b9

R values are calculated from Spearman's rank correlation and P-values adjusted for multiple comparisons. P-values shown have been adjusted for multiple analyses to minimise the false discovery rate.

Correlation coefficient R values	FHR5	C3b/iC3b/C3c	C5b-9
C3b/iC3b/C3c	0.61 (p=0.0008)		
C5b9	0.52 (p=0.02)	0.65 (p=0.0004)	
C3dg	0.41 (p=0.02)	0.40 (p=0.02)	0.42 (p=0.02)

d. Glomerular FHR5 co-localises with glomerular C3 in C3G

Glomerular FHR5 staining intensity positively correlated with the glomerular C3b/iC3b/C3c, C5b9 and C3dg deposition (Figure 35). Glomerular C3b/iC3b/C3c and C3dg positively correlated with glomerular C5b9. Glomerular C3b/iC3b/C3c and C3dg intensities also correlated significantly. Notably, in our cohort glomerular C5b9 was prevalent in both native and transplant biopsies (Figure 30).

Double immunofluorescence for FHR5, C3b/iC3b/C3c and C3dg in three cases of C3GN demonstrated both areas of glomerular co-deposition and areas of single antibody binding (Figure 36), although the relative degree of antigen co-deposition varied (Figure 36).

At the time of biopsy serum C3 was normal in case 1 and low in cases 2 and 3. We noted areas of C3b/iC3b/C3c and C3dg co-deposition (Figure 36, right-hand column, arrows), and areas staining for either C3b/iC3b/C3c (Figure 36, right-hand column, stars) or C3dg (Figure 36, right-hand column, triangles) alone. Capillary wall and mesangial FHR5 co-localised with C3b/iC3b/C3c (Figure 36, left-hand column, arrows). But there were also glomerular areas of C3b/iC3b/C3c without FHR5 (Figure 36, left-hand column, stars) and FHR5 without C3b/iC3b/C3c (Figure 36, left-hand column, triangles). Glomerular C3dg deposition was detected predominantly along capillary walls in cases 2 and 3 but was less marked and mesangial in location in case 1 (Figure 36). C3dg was found co-localised with FHR5 (Figure 36, middle column, arrows) and we observed FHR5 without C3dg deposition in cases 1 and 2 (Figure 36, middle column, triangles).

Using Image J software and the COLOC2 plug-in, I calculated whether the image pixels were inhabited by single or double antigens and whether antigen glomerular locations correlated on a pixel-by-pixel basis. Antigen glomerular locations correlated positively in all glomeruli (Figure 37). The median correlations (r) for antigen locations were 0.72 for C3b/iC3b/C3c with FHR5 (8 glomeruli from 3 cases. 95% CI 0.61 to 0.79), 0.75 for FHR5 with C3dg (5 glomeruli from 3 cases. 95% CI 0.5 to 0.91), and 0.74 for C3b/iC3b/C3c with C3dg (6 glomeruli from 3 cases. 95% CI 0.57 to 0.82).

Figure 36. Glomerular FHR5 co-localises with glomerular C3 in C3 glomerulopathy

Representative images of combined immunofluorescence staining in three C3G cases for glomerular FHR5 with either C3b/iC3b/C3c or C3dg, and C3b/iC3b/C3c with C3dg. Renal biopsies in all three cases showed C3-dominant membranoproliferative glomerulonephritis and the biopsy indications together with the UPCR, eGFR and serum C3 levels at time of biopsy are listed. The staining patterns for C3b/iC3b/C3c and C3dg (right-hand column of images) showed areas of co-localisation (arrows), areas of C3b/iC3b/C3c alone (stars), and areas of C3dg alone (triangles). The staining pattern for C3b/iC3b/C3c and FHR5 (left-hand column of images) showed areas of co-localisation (arrows), areas of C3b/iC3b/C3c alone (stars), and areas of FHR5 alone (triangles). The staining pattern for C3dg and FHR5 (middle column of images) showed areas of co-localisation, particularly along capillary walls in cases 2 and 3 (arrows), areas of C3b/iC3b/C3c alone (stars), and areas of FHR5 alone in cases 1 and 2 (triangles). Notably I did not detect areas of C3dg without FHR5 staining. MMF - mycophenolate mofetil. Original magnification 400x. Bars represent 100 µm.

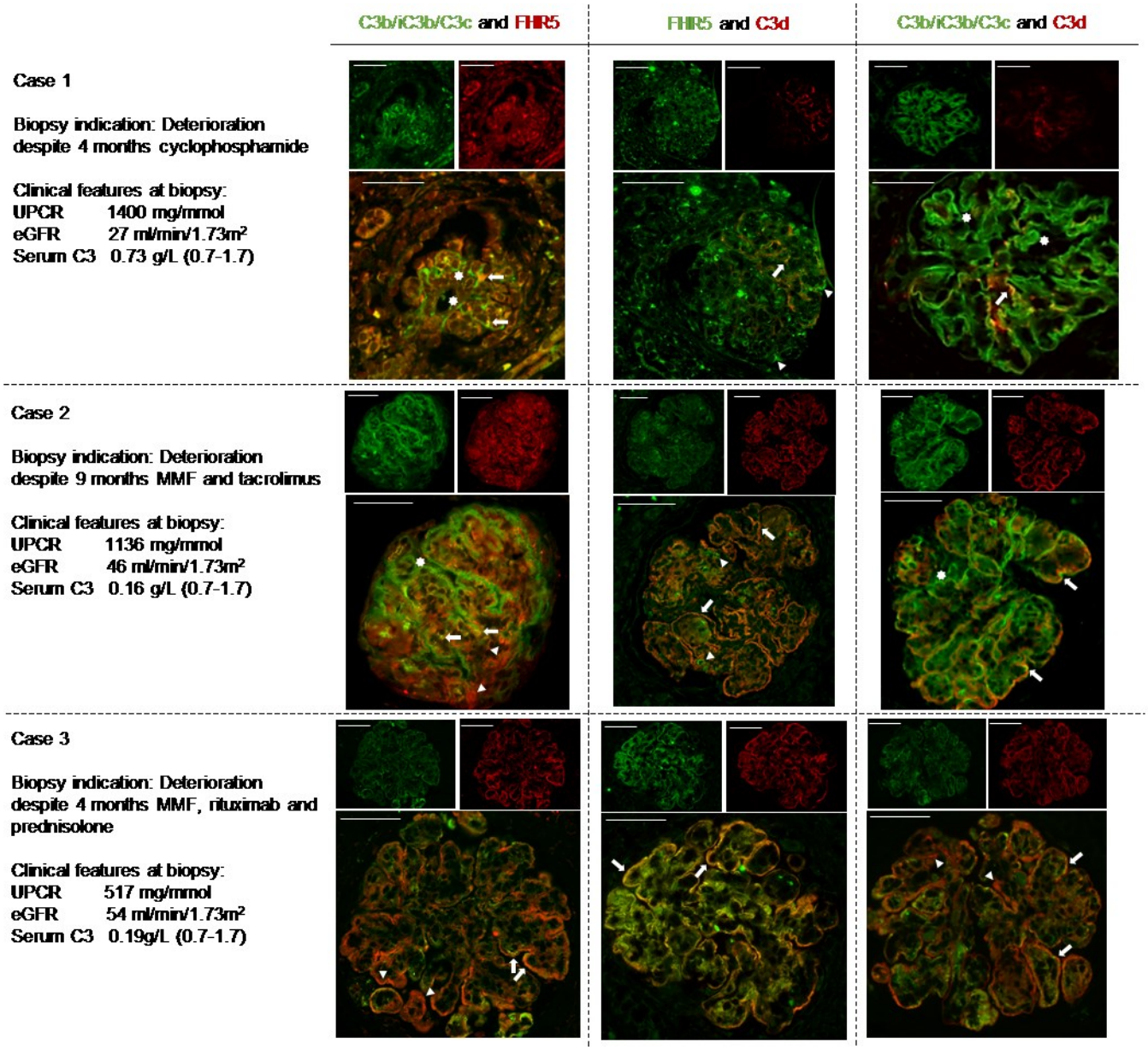
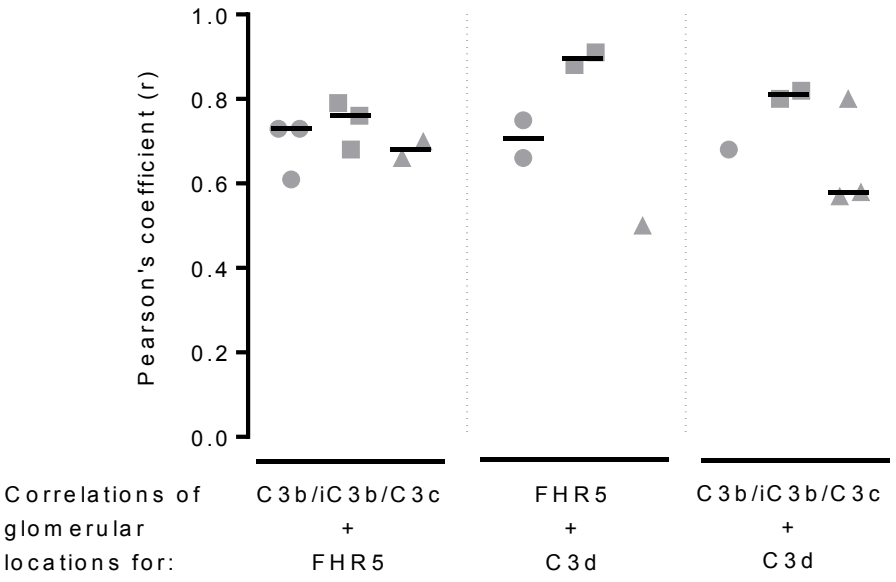


Figure 37. FHR5, C3b/iC3b/C3c and C3dg glomerular locations correlate in C3G

I calculated the correlation of glomerular antigen locations in all available glomeruli from the three C3G cases. The Pearson's correlation coefficient for all glomeruli from cases 1 (circles), 2 (squares), and 3 (triangles), and the median correlation for each case (horizontal lines) are shown. The median correlation coefficients (r) for each case were: C3b/iC3b/C3c with FHR5; 0.73 (case 1), 0.76 (case 2), and 0.68 (case 3). FHR5 with C3dg; 0.71 (case 1), 0.90 (case 2), and 0.5 (case 3). C3b/iC3b/C3c with FHR5; 0.68 (case 1), 0.81 (case 2), and 0.58 (case 3).



e. Tubular cells show FHR1 and properdin deposition

I identified minimal tubular cell FHR5 deposition (Figure 38). However, we observed tubular cell FHR1 and properdin staining in our C3G cohort (Figure 38). This was evident in native and transplant biopsies and did not correlate with proteinuria, renal function, tubular atrophy, loss of 50% eGFR or ESRD (data not shown). There was also no correlation with tubular C3b/iC3b/C3c, C5b9 or FHR5 deposition (Figure 38). Tubular FHR1 staining was detected despite changing the antigen retrieval technique (Figure 39) and in non-C3G cases (Figure 39). FHR1 deposition was absent in IgA nephropathy with the FHR1 gene (*CFHR1*) deletion polymorphism in homozygosity (Figure 39).

Figure 38. Tubulo-interstitial staining for complement proteins in C3 glomerulopathy

Representative images of staining for FHR5, FHR1, FH, properdin and C3b/iC3b/C3c. No tubulo-interstitial staining for FHR5 or FH was evident but there was strong tubulo-interstitial staining for both properdin and FHR1.
Original magnification 200x. Bars represent 100 µm.

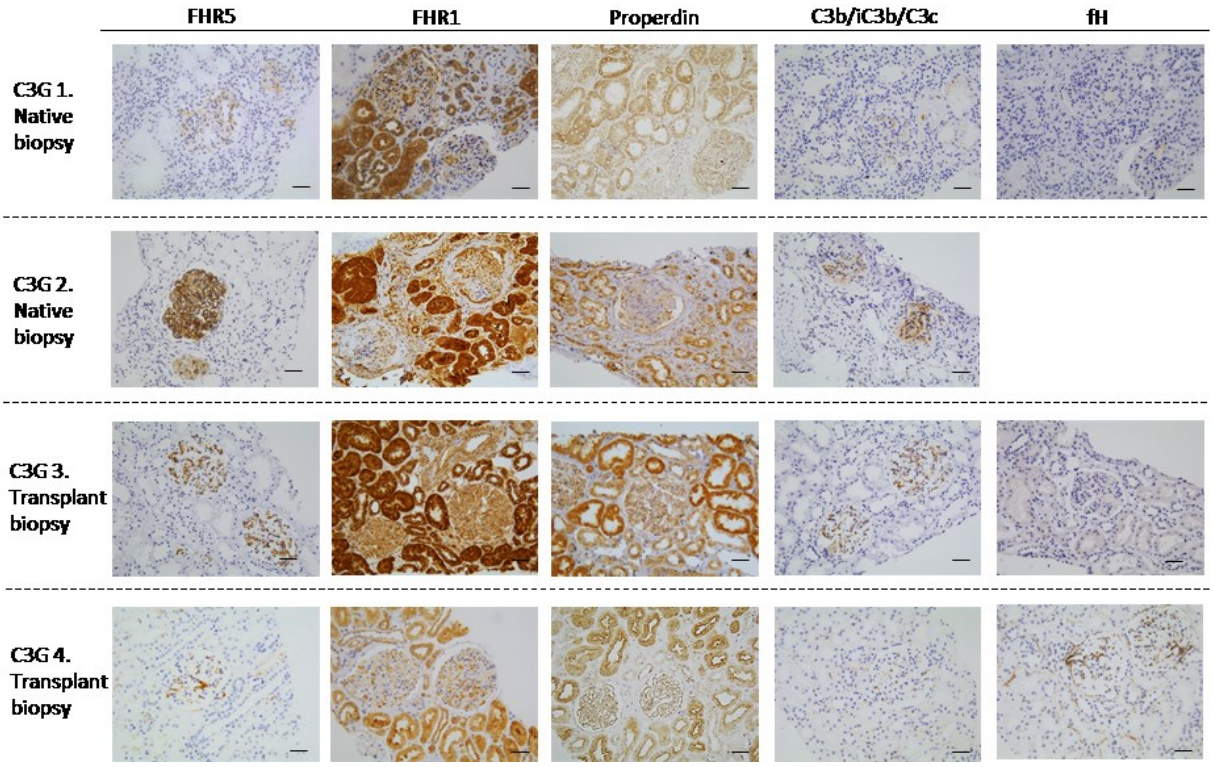
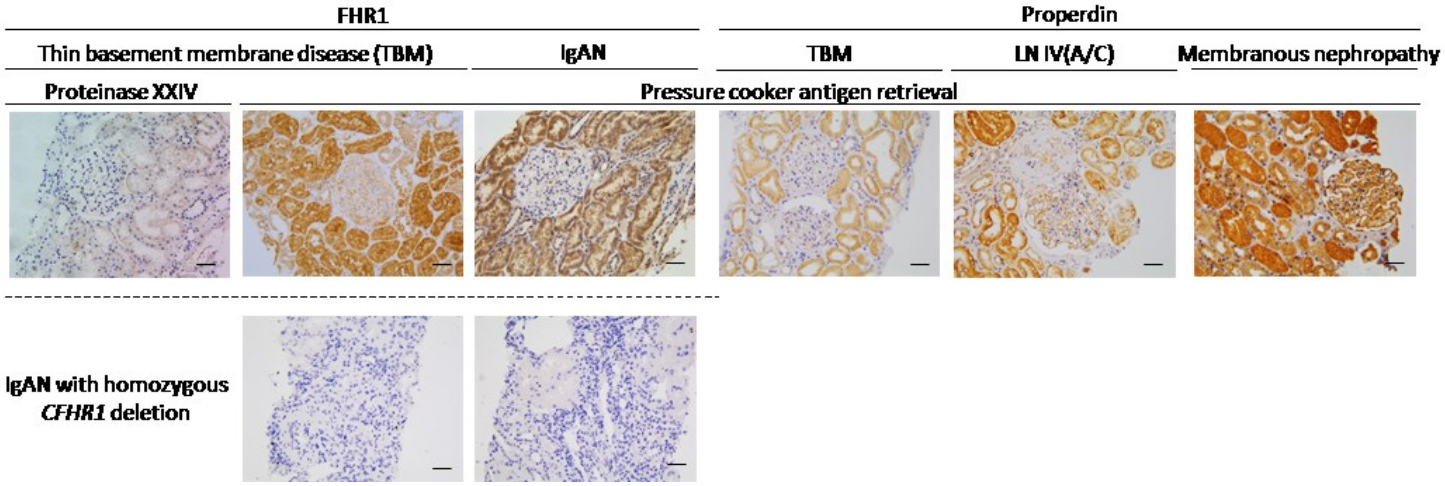


Figure 39. Tubulo-interstitial staining for properdin and FHR1 in glomerular conditions with different antigen retrieval techniques

Tubulo-interstitial staining for properdin was also seen in other renal diseases (TBM, LN and membranous nephropathy). Tubulo-interstitial staining for FHR1 was demonstrable in biopsies from patients with TBM and IgAN. Tubulo-interstitial FHR1 staining in TBM was still detectable but less intense when proteinase XXIV enzyme was used instead of pressure cooker antigen retrieval. Glomerular and tubulo-interstitial FHR1 was absent in renal tissue from a patient with IgAN and FHR1 deficiency. Original magnification 200x. Bars represent 100 µm.



Discussion

I analysed glomerular FHR5 deposition in a cohort of patients with C3G, a condition pathogenically dependent on alternative complement pathway deregulation and C3 deposition. I also aimed to identify the glomerular ligands for FHR5 in C3G. I hypothesised this would demonstrate the potential utilisation as an immunohistochemic biomarker and pathogenicity of FHR5 in C3G, with relevance to other glomerulopathies associated with C3 deposition.

I found glomerular FHR5 was the most prevalent protein in native and transplant renal biopsies and, in transplants, could be identified even in the absence of C3b/iC3b/C3c. FHR5 staining intensity associated with eGFR at the time of biopsy and correlated with the intensities of glomerular C3b/iC3b/C3c, C3dg and C5b9 deposition. Transplant FHR5 deposition seemed to sensitively detect C3G recurrence and may reflect recurrence severity. These findings are evidence of the pathogenic importance of FHR5 to complement-dependent glomerular injury. I also identified variability in C3b/iC3b/C3c glomerular staining in C3G, the technique employed by most diagnostic services. The variability between cases did not correlate with markers of disease severity, such as eGFR or histology evidence of glomerular inflammation. FHR5 IHC may therefore add sensitivity and information on disease severity to C3G diagnoses.

Our C3G cohort demonstrated an association between MPGN light microscopy pattern at diagnostic biopsy and a 50% loss of eGFR at 70 months follow-up. Intense glomerular FHR5 deposition also associated with a MPGN pattern in our cohort. The interaction of glomerular FHR5 deposition, MPGN morphology, and C3G natural history requires further investigation. I identified C3G cases with prominent glomerular complement protein deposition but absent clinical signs of glomerulopathy. One case of CFHR5 nephropathy showed complement deposition in the absence of glomerular inflammation. This demonstrates the importance of developing novel biomarkers for C3G and highlights our inadequate understanding of C3G pathogenesis.

FHR5 and C5b9 were the only glomerular complement antigens to associate with renal impairment. Based on our research in IgAN, the association of reduced eGFR and FHR5 deposition is unlikely to be an artefact of impaired glomerular filtration[286]. However, associations between glomerular C5b9 and renal impairment may reflect both terminal complement pathway activation and C5b9 surface-binding kinetics. C5b9 has been shown to persist in lupus nephritis following complement activity cessation[294]. This may bias its relative glomerular abundance compared to antigens with more rapid elimination. C4d was also prevalent in native and transplant

biopsies, suggesting the use of glomerular C4d to exclude a diagnosis of C3G is unreliable. Glomerular C4d in C3G requires further investigation. C4d deposition is the result of classical or lectin pathway activity, as indicated by the presence or absence respectively of C1q, and may identify triggers of C3G exacerbations. Similar to C5b9, its prevalence may also be explained by its covalent binding to surfaces and subsequent persistence despite a cessation to ongoing lectin and classical pathway activation.

I made a number of interesting observations from individual transplant cases. Glomerular FHR5 deposition was identified in the absence of and predated detectable C3b/iC3b/C3c deposition and glomerular inflammation in one transplant patient. Whether this implicates FHR5 in early C3G pathogenicity or represents the sensitivity and affinity of FHR5 for otherwise indolent and undetectable C3 fragments requires further investigation. Serial transplant biopsies during recurrent crescentic C3G showed FHR5 deposition correlated with initial disease activity but was unaltered by C5 inhibition. This is consistent with a role for FHR5 in alternative but not terminal complement pathway activity. As evidenced by improved CD68-positive cell infiltration, clinical improvement following eculizumab treatment is likely secondary to reduced C5a production and inflammatory cell recruitment. Whether continued terminal pathway blockade would cause long-term disease improvement is unclear. The case of transplant recurrent C3G and eculizumab treatment progressed to increased proteinuria and transplant dysfunction despite terminal pathway inhibition, suggesting the alternative complement pathway should be targeted to provide long-term benefit. Evidence from C3G animal models suggests alternative pathway activity in the absence of C5 leads to glomerulopathy[172]. The role of C5 inhibition in treating acute exacerbations of disease and its ability to modify long term outcomes requires further study.

Double IF examination of three C3G cases demonstrated areas of FHR5, C3b/iC3b/C3c and C3dg co-deposition and dominant individual antigen staining. I noted variation between cases and within glomeruli. This may reflect the complexity of local glomerular complement activation in C3G. Our IF protocols demonstrated FHR5 deposition correlated closely with ongoing and previous alternative pathway activation, providing further evidence that FHR5 likely exacerbates complement mediated glomerular disease. Furthermore, the co-deposition and prevalence of glomerular FHR5 to C3 fragments suggests FHR5 could provide novel therapeutic strategies for glomerulopathies with pathogenic C3 deposition. FHR5 binding could be exploited to target therapeutic complement inhibitors to areas of complement activation. The lack of association of FHR5 with other human diseases suggests this approach could target glomerular C3 fragments and limit the sequelae of

systemic complement inhibition. However, enthusiasm for these findings must be balanced by the preliminary nature of these data and the need for comprehensive analysis of FHR5 binding in prospective cohorts, following non-disease specific events, such as infections and immunosuppressant treatments. Importantly it is essential to elucidate the mechanism of FHR5 contribution to glomerular complement activation, inflammation and kidney injury and ensure FHR5 is more than simply are sensitive marker of complement activation.

These data also suggest FHR5 may be a useful immunopathogenic marker as it seems to identify alternative complement activation with improved sensitivity and disease severity correlation than current anti-C3b/iC3b/C3c based protocols. Comparing FHR5 and C3dg immunostaining could sensitively identify cases with dominant ongoing (C3dg-absent FHR5) or previous (C3dg-co-deposited FHR5) alternative complement pathway activation (Figure 40). Sensitive complement activation detection would be of obvious benefit. Identifying previous complement activation could evidence effective complement inhibition and provide diagnostic insight to cases of glomerular scarring with unknown aetiology. The addition of anti-C5b9 and CD68 protocols could also identify acute terminal complement pathway activity; the presence of both these markers indicates ongoing terminal pathway activity with C5a production, but C5b9 in the absence of CD68 implies previous terminal pathway activity only. The presence of CD68 cells with C3dg-absent FHR5 would indicate alternative complement pathway activity is also contributing to inflammatory cell infiltration, perhaps through the release of C3a.

Because FHR5 binds all immobilised C3 fragments *in vitro*, the identification of glomerular C3b/iC3b/C3c deposits without FHR5 was unexpected (Figure 40). The structural conformation of C3c *in vivo* may hide the epitope that FHR5 binds *in vitro*. Alternatively, we speculate glomerular C3b/iC3b/C3c staining without FHR5 is C3c released from extra-renal complement activation that deposits in glomeruli but does not participate in complement activation[295] and therefore does not interact significantly with FHR5. In this scenario, comparing C3b/iC3b/C3c and FHR5 deposition could differentiate local from extra-glomerular complement activation.

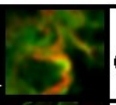
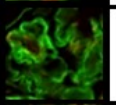
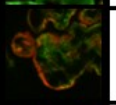
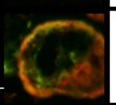
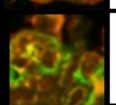
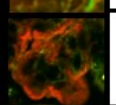
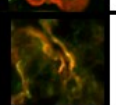
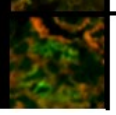
Figure 40. Complement factor H related protein 5 IHC protocol optimisation

Glomerular C3c is known to be cleared quickly in experimental nephritis so its detection in human glomeruli most likely indicates ongoing or recent complement activation. Conversely, glomerular C3dg is cleared slowly in experimental models so its presence in the absence of C3c in human glomeruli most likely indicates previous complement activation. We hypothesized that the combination of FHR5, C3b/iC3b/C3c and C3dg staining patterns could provide information on the nature of the glomerular complement C3 deposition: ongoing vs previous activation; and local (i.e. glomerular activation) vs systemic activation with subsequent glomerular deposition of complement fragments.

In this figure, I show magnified images of glomerular areas exemplifying each antigen combination and our interpretation of the staining.

I have show that FHR5 interacted with iC3b, C3c and C3dg in vitro. Consistent with this all areas that stained with the anti-C3dg antibody also stained with the anti-FHR5 antibody. When these areas were not stained with the anti-C3b/iC3b/C3c antibody we concluded that they represented C3dg bound to FHR5. Areas of FHR5 staining without C3dg reactivity most likely represent FHR5 bound to C3b/iC3b. Notable, I did detect glomerular areas that stained with the anti-C3b/iC3b/C3c antibody but not with anti-FHR5 antibodies. I consider these to represent C3c that could derive from either complement activation in the circulation or released from complement activation within glomeruli. This interpretation depends on FHR5 interacting poorly with C3c in vivo.

Bars represent 50µm.

Immunofluorescence combination			Example	Consistent with antigen	Interpretation
C3b/iC3b/C3c	C3d	FHR5			
+	+		 C3b/iC3b/C3c C3d	C3b/iC3b/C3c and C3d	Ongoing and previous local C3 activation AND/OR C3c from local and/or systemic complement activation
+	-		 C3b/iC3b/C3c C3d	C3b/iC3b/C3c	Ongoing local C3 activation AND/OR C3c from local and/or systemic complement activation
-	+		 C3b/iC3b/C3c C3d	C3d	Previous local activation
+		+	 C3b/iC3b/C3c FHR5	C3b/iC3b (or local C3c)	Ongoing local C3 activation
+		-	 C3b/iC3b/C3c FHR5	C3c	Systemic complement activation
-		+	 C3b/iC3b/C3c FHR5	C3d	Previous local activation
	+	+	 FHR5 C3d	C3d	Previous local activation
	-	+	 FHR5 C3d	C3b/iC3b (or local C3c)	Ongoing local C3 activation

Intra-tubular complement activation is thought to associate with proteinuria[296-298] and renal insufficiency[299]. However, I identified tubular FHR1 and properdin deposition in cases without these features. The absence of FHR1 staining in a case with the *CFHR1* gene deletion polymorphism in homozygosity and the replication of tubular FHR1 and properdin staining in other renal conditions[293, 300, 301], suggest these findings are neither artefactual nor secondary to non-specific antibody binding. FHR1 and properdin could have roles in tubulointerstitial pathology that my analysis was not been designed to detect. The findings could also document the physiological tubular absorption of proteins filtered through abnormal glomeruli[302]. The identification of non-specific FHR5 staining following pressure cooker antigen retrieval emphasises the importance of thorough immunostaining protocol development. Tubular complement activation and its impact on glomerulopathy pathogenesis requires further research.

Our IF images did not reproduce the capillary wall double linear "railroad track" or "mesangial ring" deposits previously described in C3G[303]. Our antibody combinations may have bound deposited proteins sufficiently to eliminate these appearances. Microscopy resolution was inadequate to confirm this and the use of confocal microscopy by Hilary (Moffitt) McPhail did not improve antigen localisation.

Our data do not illustrate the mechanisms linking glomerular FHR5 deposition and complement-dependent injury. Our results could be consistent with FHR5 competing with the regulatory binding of FH to C3b. Also, C3dg-bound FHR5 dimers could bind C3b and facilitate C3 convertase formation and activation[119] on surfaces that would not otherwise activate complement. FHR5 dimers could also bind non-complement glomerular components, such as laminin-521 or -211[121], and foundation complement opsonisation and activation. In all scenarios, we predict FHR5 inhibition would regulate complement and improve glomerular inflammation and injury. Furthermore, utilising FHR5 C3 binding characteristics could target complement inhibitors to alternative complement pathway activity regardless of the triggering mechanism.

Conclusions

- Glomerular FHR5 deposition associates with C3G severity
- FHR5 co-localises with C3 fragments *in* vivo

- FHR5 is an important glomerular marker and potential therapeutic target in glomerular diseases associated with C3 deposition
- The demonstration of similar co-localisation of FHR5 with C3 fragments in IgAN suggests the mechanism of FHR5 driving alternative complement deregulation and glomerular injury is similar in IgAN and C3G.

E. Glomerular FHR5, IgA and complement deposition and co-localisation in IgA nephropathy

Introduction

I predict alternative complement pathway deregulation drives IgAN pathogenesis by determining the degree of glomerular inflammation and injury in response to mesangial deposited IgA1-complexes in IgAN. The data I have presented in previous chapters describes multiple associations between FHR5 and IgAN severity. C3G pathogenesis is dependent on alternative complement pathway deregulation and activation. In the previous chapter, I identified the co-deposition of FHR5 with markers of ongoing and previous alternative complement pathway activity in C3G. Glomerular FHR5 deposition also correlated with markers of disease severity. This suggests FHR5 is important in C3G pathogenesis and thereby implicates FHR5 in alternative complement pathway dependent pathogenesis. The mechanisms of complement activation contributing to glomerular inflammation and injury in IgAN have not been defined. The demonstration in IgAN of FHR5 co-deposition with markers of previous and ongoing alternative complement pathway activation, as seen in C3G, would be further evidence of FHR5 dependent alternative complement activation in IgAN pathogenesis. I therefore questioned whether FHR5 co-deposited with markers of complement activation and deposited IgA in IgAN, the results of which I present in this chapter.

Hypothesis and aims

I aimed to apply the double IF protocols to a case of proteinuric IgAN with light microscopy evidence of inflammatory disease. If successful, I then aimed to extend the staining protocols to a cohort of ten IgAN patients with stored surplus FFPE diagnostic biopsy material available who had been consented and enrolled into The Causes and Predictors of Outcome in IgA Nephropathy. I aimed to quantify antigen deposition and co-deposition and to compare antibody staining and co-deposition and correlate with disease severity. I hypothesised that FHR5 would co-localise with mesangial IgA and markers of alternative complement pathway activation in IgAN. I also hypothesised patients with progressive, severe IgAN would demonstrate quantifiably more FHR5 and C3b/iC3b/C3c deposition, and co-deposition of these two antigens, than patients with stable disease.

Methods

Hilary (Moffitt) McPhail and I applied double IF protocols as described in the Methods chapter to FFPE biopsy sections from a case of IgAN with non-nephrotic range proteinuria and endocapillary hypercellularity. This patient had given consent for surplus tissue to be used for research. Limited clinical information was available from this case so we used it as proof of the potential for double IF protocols to be applied to cases of IgAN. I then identified ten patients from The Causes and Predictors of Outcome in IgA Nephropathy study (UK Natinoal RES reference 14/LO/0155) with surplus stored FFPE tissue available and applied the same staining techniques to these cases. Clinical and follow-up information was available from the ten cases. These were the only progressive and stable cases with tissue available at the time of analysis. Five of the cases had progressive and five and stable IgAN. Due to tissue availability, I applied four double IF combinations to the cohort of ten IgAN patients. The IF stains applied were IgA with FHR5, C3b/iC3b/C3c with FHR5, C3dg with FHR5 and C3b/iC3b/C3c with properdin. DAPI stains were compared to check IF exposures were uniform across the cohort. I compared IF staining in the stable and progressive cohorts.

Due to difficulties comparing antigen deposition by subjective visual assessment, I used Image J software[273] to quantify the amount of fluorescent deposition. I selected a brightness of 55. This threshold was applied to both red and green light spectrum immunofluorescence staining. To quantify the size of deposits containing co-localised antibodies of red or green immunofluorescence, I determined colour parameters that excluded pure red or green deposits but selected all deposits with a colour between the red and green spectra; I set the hue to between 17 and 60. The co-localised deposits also had to have a brightness above 55. I used the region of interest (ROI) tool to trace around the glomerulus in the section (to eliminate tubular staining from the assessment). After ensuring all images were of identical size, I applied the techniques to one representative and non-sclerosed glomerulus from each case. Deposit sizes were calculated as total number of pixels that met the colour inclusion criteria and brightness threshold.

I also calculated how closely the glomerular locations of two antigens co-localised. This used the COLOC2 plug-in for Image J[273]. This compares whether the equivalent pixel in two images are occupied by the specified colour or not. A step-by-step method is described in the Methods chapter.

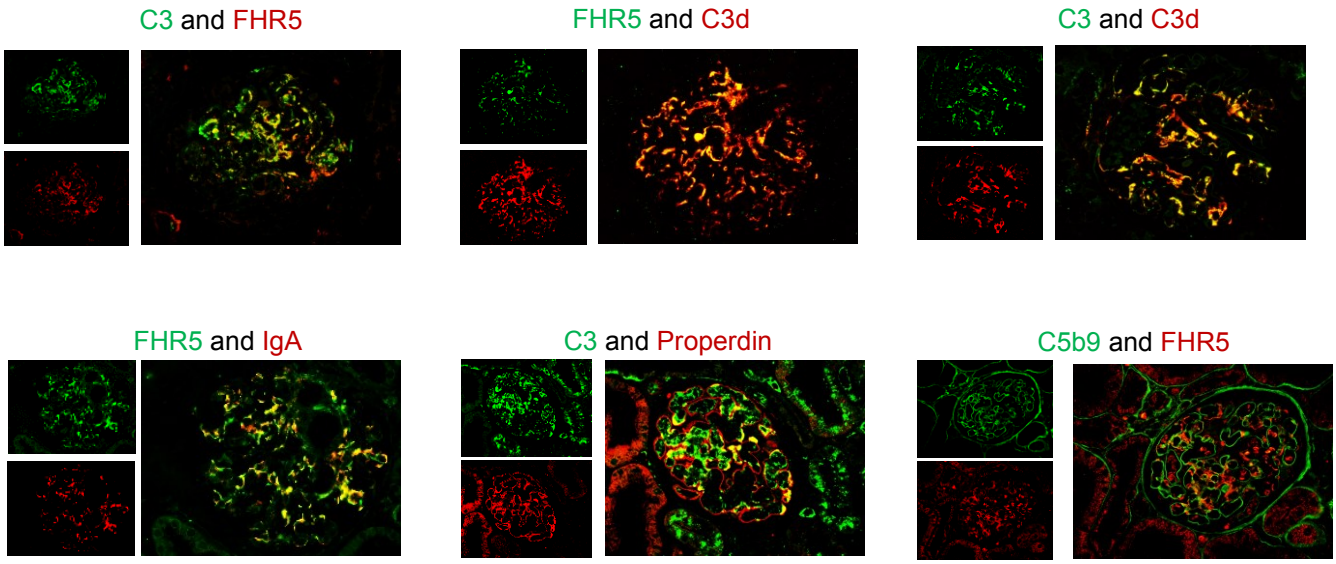
Results

a. IgA and complement co-localisation in IgAN

The double IF protocols optimised for C3G cases produced well defined images of IgA and complement glomerular deposition and co-localisation when applied to a case of IgAN (Figure 41). This case seemed to demonstrate close co-localisation of C3b/iC3b/C3c with FHR5, C3dg with FHR5, C3dg with C3b/iC3b/C3c and FHR5 with IgA. C3b/iC3b/C3c and properdin seemed to co-localise in mesangial deposits. Properdin could be seen along glomerular capillary walls without obvious C3b/iC3b/C3c deposition. Isolated C3b/iC3b/C3c was apparent in mesangial areas. C5b9 and FHR5 showed co-localisation in some mesangial areas. C5b9 was demonstrated along glomerular capillary walls without FHR5 and mesangial FHR5 could be identified without C5b9 (Figure 41).

Figure 41. Factor H related protein 5 co-localises with glomerular C3 fragments and IgA in a case of IgA nephropathy

I applied our double immunofluorescence (IF) protocols to stored surplus formalin fixed and paraffin embedded renal biopsy sections from a patient with IgA nephropathy with endocapillary hypercellularity and proteinuria.
Images at 400x magnification.

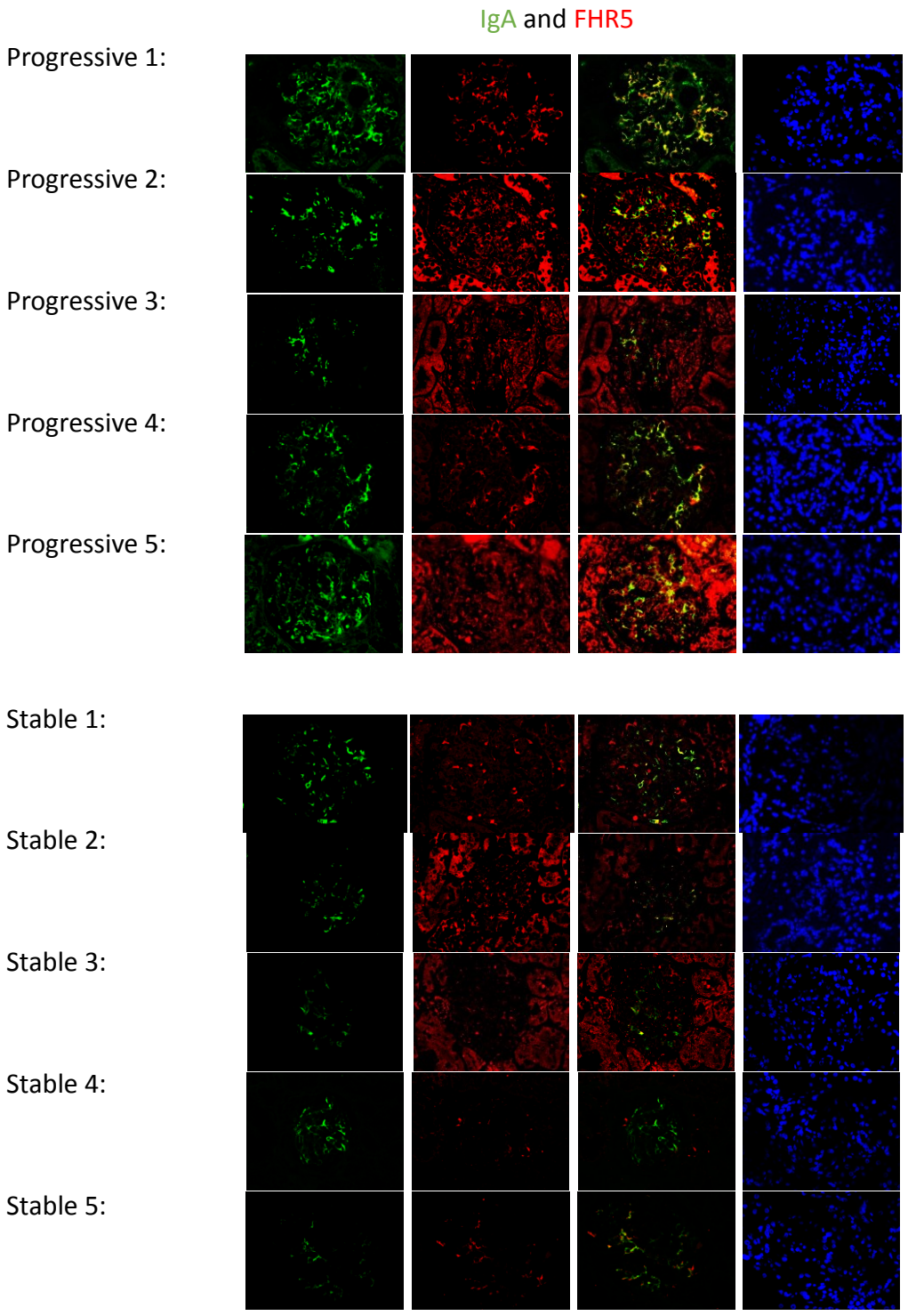


Visual comparison of double IF in five progressive and stable cases of IgAN showed more IgA, C3b/iC3b/C3c and FHR5 deposition in progressive than stable IgAN patients (Figure 42A-D). Properdin deposition did not appear to be significantly different between the stable and progressive IgAN cohorts (Figure 42D).

The pattern of properdin staining was predominantly along capillary walls. C3b/iC3b/C3c deposition seemed to depend on the antibody combination. C3b/iC3b/C3c was seen in both mesangial and some capillary wall areas when co-localised with properdin (Figure 42D). However, the pattern of C3b/iC3b/C3c deposition adopted a capillary wall deposition when co-localised with anti-FHR5 antibodies (Figure 42B). The antigen retrieval used for these two double IF combinations are different. The combination of properdin with C3b/iC3b/C3c follows pressure cooker antigen retrieval in Tris pH9 buffer. C3b/iC3b/C3c with FHR5 follows immersion in sodium citrate tribasic buffer at 95°C for 30 minutes and incubation with HistoReveal (Abcam #103720). These protocol differences may explain the apparent differences in glomerular C3b/iC3b/C3c locations in sections from the same biopsies. Therefore, C3b/iC3b/C3c may be located in both mesangial and capillary wall glomerular compartments but only revealed in one compartment depending on the antigen retrieval technique. It is probable C3b/iC3b/C3c is co-localised with both FHR5 and properdin IgAN glomeruli.

Figure 42. IgA, complement C3b/iC3b/C3c and FHR5 double immunofluorescence in IgA nephropathy . (A) FHR5 and complement C3b/iC3b/C3c

We applied double IF protocols to stored surplus formal fixed and paraffin embedded renal biopsy sections from up to five patients with progressive and stable IgAN. The right-hand column of images shows the DAPI counterstain. Images at 400x magnification.



(B) FHR5 and C3b/iC3b/C3c double immunofluorescece in IgAN

C3b/iC3b/C3c and FHR5

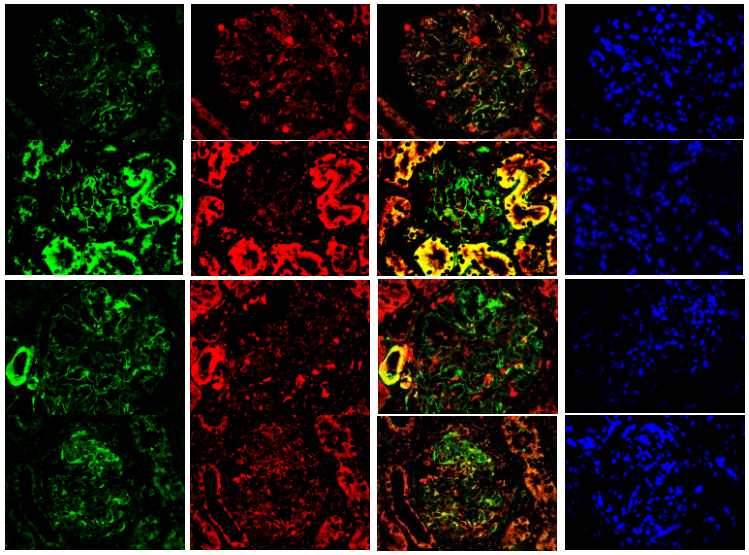
Progressive 1:

Progressive 2:

Progressive 3:

Progressive 4:

Progressive 5:



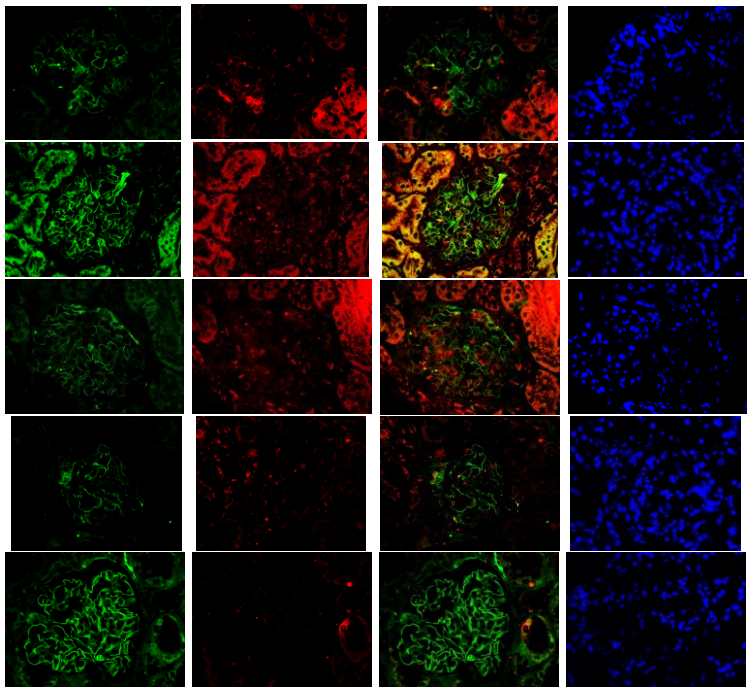
Stable 1:

Stable 2:

Stable 3:

Stable 4:

Stable 5:



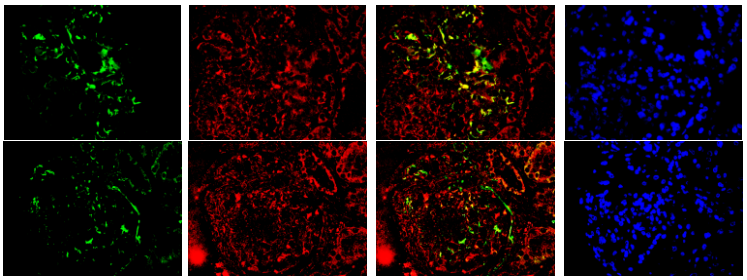
(C) C3dg (C3d) and FHR5 double immunofluorescence in IgAN

C3d and FHR5

Progressive 1:

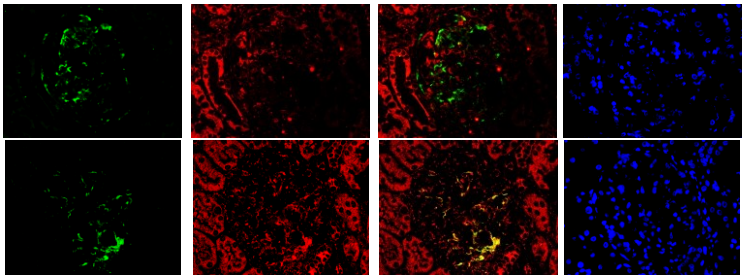
Progressive 2:

Progressive 3:



Progressive 4:

Progressive 5:



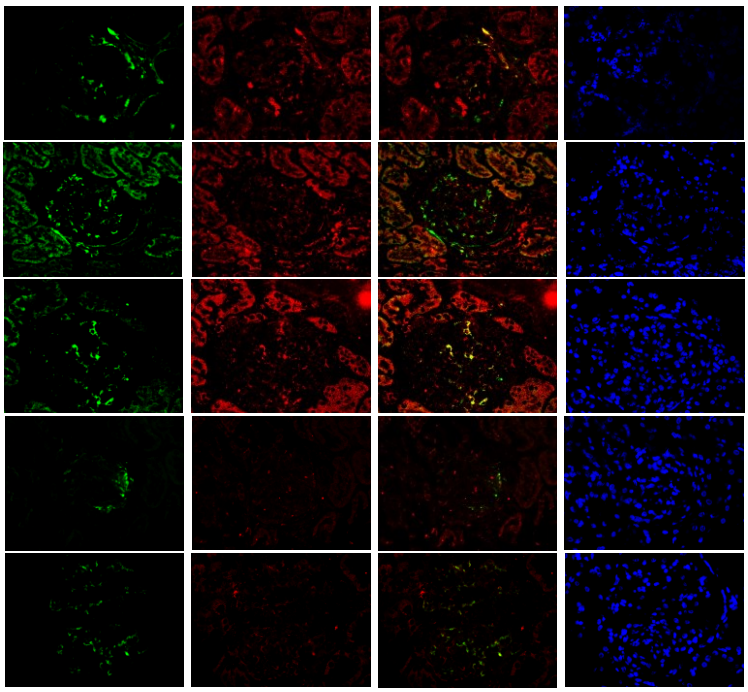
Stable 1:

Stable 2:

Stable 3:

Stable 4:

Stable 5:



(D) C3b/iC3b/C3c and properdin double immunofluorescence in IgAN

C3b/iC3b/C3c and Properdin

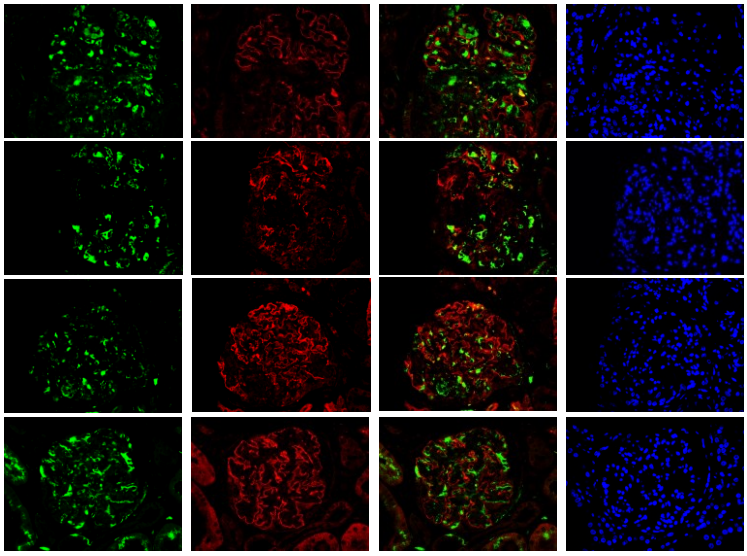
Progressive 1:

Progressive 2:

Progressive 3:

Progressive 4:

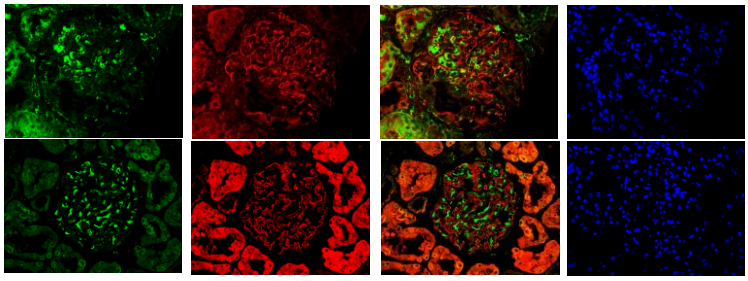
Progressive 5:



Stable 1:

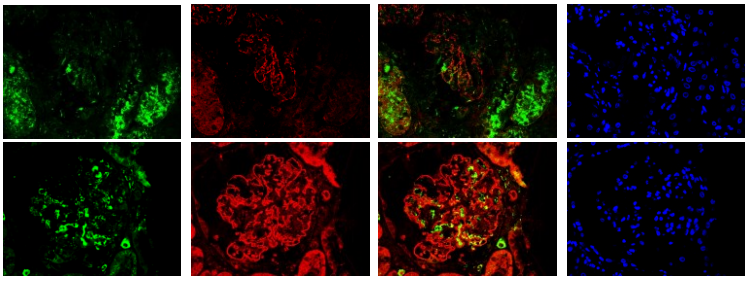
Stable 2:

Stable 3:



Stable 4:

Stable 5:



b. Glomerular FHR5, IgA and C3 deposit sizes in IgAN

I used Image J software to objectively quantify and compare deposit sizes between a representative glomerulus from each IgAN case. FHR5 was detected with three different antibody combinations; FHR5 with IgA, FHR5 with C3b/iC3b/C3c and FHR5 with C3dg. I compared the patient median FHR5 glomerular area (averaged from the three IF combinations). Patients with progressive IgAN had larger areas of FHR5 deposition than patients with stable IgAN (Figure 43A). Immunofluorescence for IgA was performed in one section per patient and I compared one representative glomerulus per section. I found patients with progressive IgAN had larger areas of IgA deposition than patients with stable IgAN (Figure 43B). The size of IgA and FHR5 co-localisation was also significantly greater in patients with progressive than stable IgAN (Figure 43C). There was no difference between progressive and stable patients in the areas of C3b/iC3b/C3c, C3dg or properdin deposition (Figure 44).

Despite no significant difference in the C3b/iC3b/C3c deposition sizes between patients with stable and progressive IgAN, patients with progressive IgAN had larger deposits of co-localised C3b/iC3b/C3c with FHR5 than stable patients (Figure 45). There was no difference between stable and progressive patients in the sizes of deposits co-localised with C3dg and FHR5 or C3b/iC3b/C3c and properdin.

c. Glomerular antigen location correlation in IgAN.

I calculated how closely the glomerular FHR5 locations correlated with the locations of C3b/iC3b/C3c, C3dg and IgA. The glomerular location of C3dg and FHR5 correlated positively (Figure 46). The median Pearson's correlation coefficient (r) for the glomerular locations of C3dg and FHR5 in IgAN was 0.42 (range 0.22 to 0.62, 95% CI of median 0.24-0.60). The glomerular locations of FHR5 with C3b/iC3b/C3c and FHR5 with IgA were closely correlated in some cases, but poorly correlated in others (Figure 46). For C3b/iC3b/C3c and FHR5 glomerular locations, median r was 0.29 (range -0.23 to 0.6, 95% CI 0.14 to 0.53). For IgA and FHR5 glomerular locations, median r was 0.26 (range -0.54 to 0.79, 95% CI -0.23 to 0.57). All the cases with negative correlations of glomerular C3b/iC3b/C3c with FHR5 and IgA with FHR5 locations had stable IgAN.

Figure 43. Progressive IgA nephropathy associates with amount of glomerular FHR5 and IgA deposition and co-localisation

I used Image J software to calculate the size of glomerular deposits showing deposition of (A) FHR5, (B) IgA and (C) co-localisation of IgA and FHR5. Deposit size is measured in number of pixels. I compared glomerular deposit sizes in patients with progressive and stable IgAN. P-values shown have been adjusted for multiple analyses to minimise the false discovery rate.

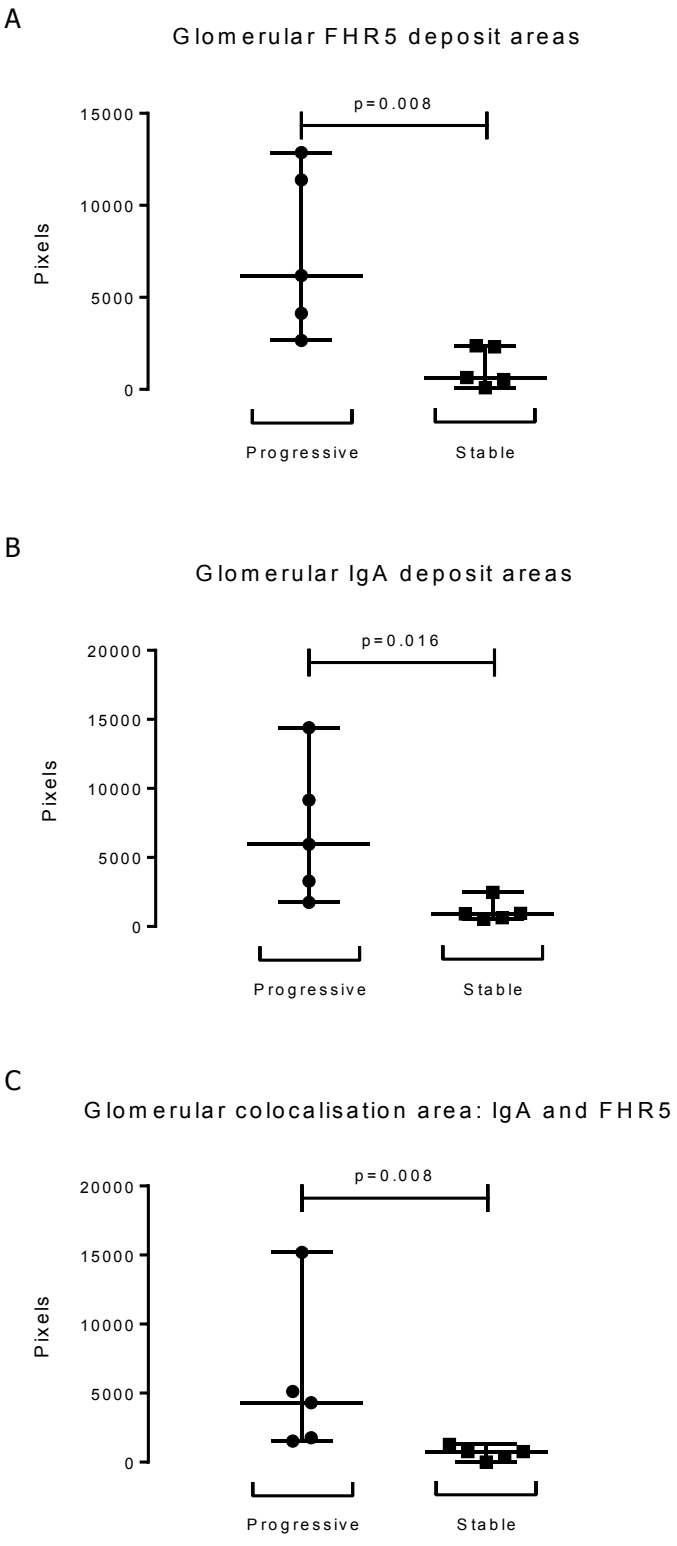


Figure 44. Glomerular deposition of other complement proteins does not associate with progressive IgA nephropathy

I used Image J software to calculate the size of glomerular deposits showing deposition of (A) C3b/iC3b/C3c, (B) C3dg and (C) properdin. Deposit size is measured in number of pixels. I compared glomerular deposit sizes in patients with progressive and stable IgAN.

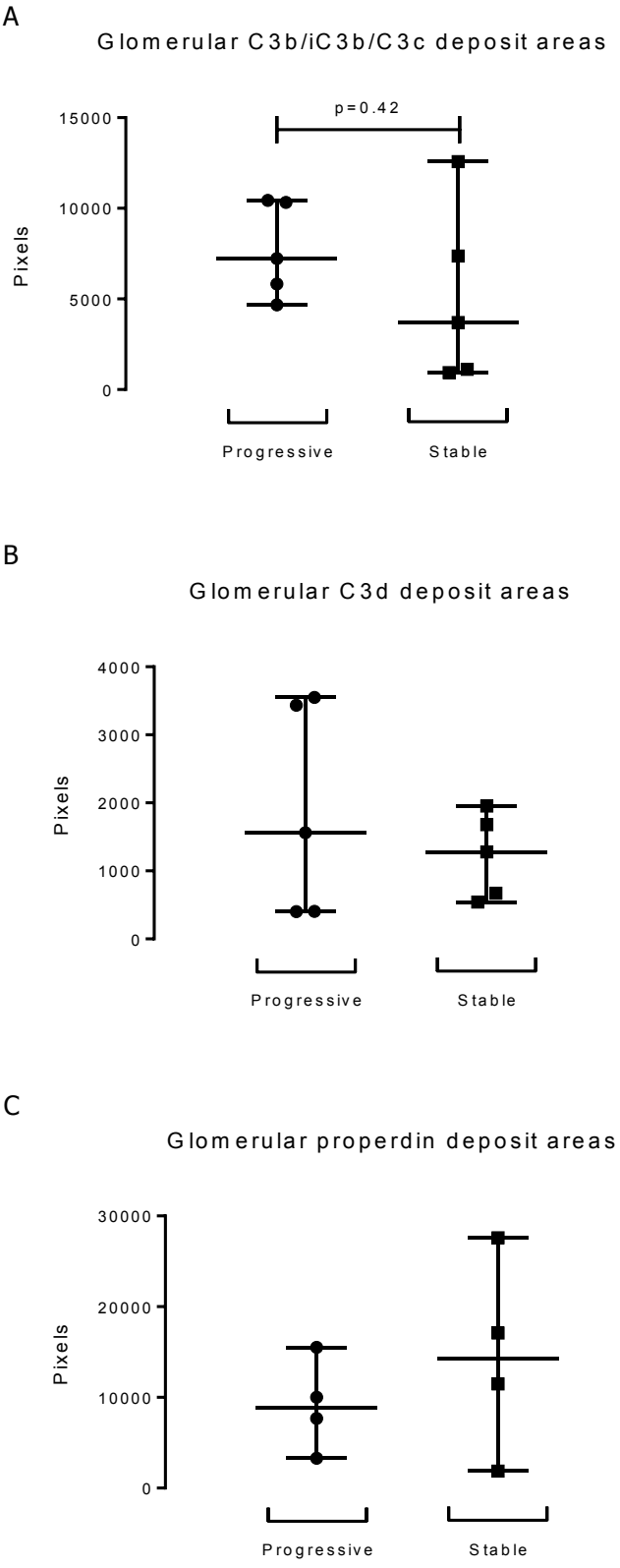


Figure 45. Progressive IgA nephropathy associates with glomerular C3b/iC3b/C3c and FHR5 co-localisation

I used Image J software to calculate the size of glomerular deposits showing co-localisation of C3b/iC3b/C3c (labelled 'C3') and FHR5. Deposit size is measured in number of pixels. I compared glomerular deposit sizes in patients with progressive and stable IgAN.

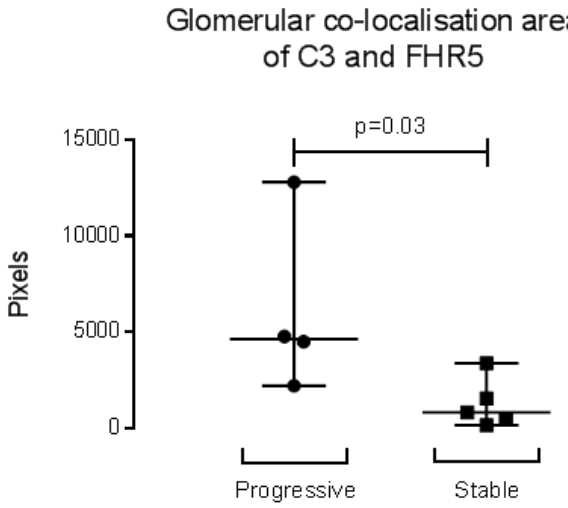
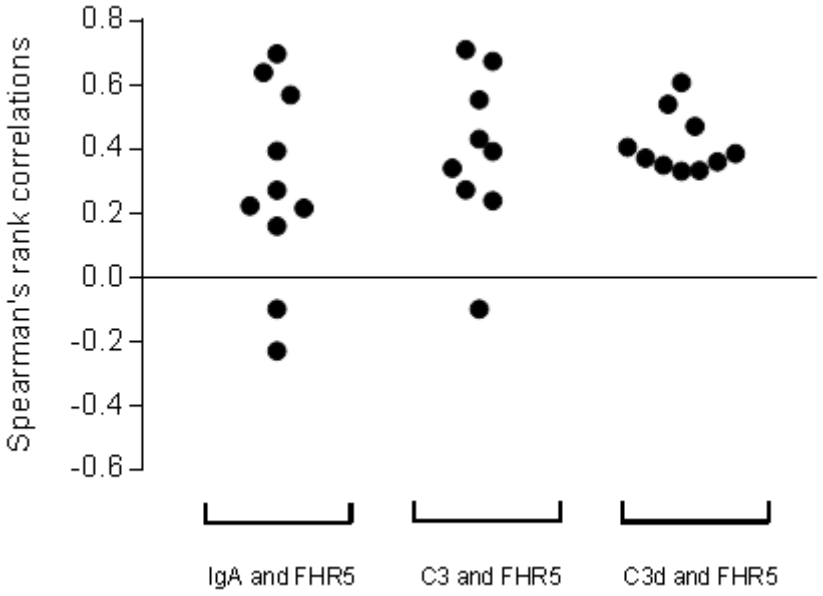


Figure 46. The correlation of glomerular locations for FHR5 and complement antigens in IgA nephropathy

I used Image J software to compare the glomerular locations of FHR5 with three antigens: IgA, C3 (C3b/iC3b/C3c) and C3dg. This provided a correlation coefficient for the locations of each antigen combination. If two antigens are co-localised closely, the correlation coefficient will be high. C3dg and FHR5 showed close correlation of glomerular deposit locations in all cases. The correlation of glomerular locations for FHR5 and complement antigens in IgAN



Discussion

I have demonstrated glomerular co-localisation of FHR5 with both IgA and markers of ongoing (C3b/iC3b/C3c) and previous (C3dg) complement activation in IgAN patient renal tissue. I provided qualitative evidence of antigen co-deposition by IF on diagnostic native renal biopsies. I also attempted to quantify the amount of antigen deposition and co-localisation. Albeit in a small IgAN cohort, I demonstrated associations between the severity of IgAN and quantitative estimates of (1) FHR5 deposition, (2) IgA deposition, (3) FHR5 with IgA co-localisation and FHR5 with C3c/iC3b/C3c co-localisation. This is further evidence of a pathogenic role for FHR5 in IgAN pathogenesis and, specifically, influencing complement activation and glomerular inflammation in response to deposited IgA1 and C3.

The antibody staining quantification I used is a semi-objective surrogate measure of antigen deposition. It is reliant on specifying brightness thresholds and colour hues that define deposits of significant intensity and co-localisation. The outcome calculated is the number of pixels that fulfil these criteria within a region of interest. The results therefore rely on the accurate selection of a representative glomerulus as the region of interest. Finally, all deposits above the brightness threshold are included in the analysis. The analysis does not account for the brightness or intensity of the deposits and therefore may not detect differences in the amount of antigen deposited in a small area. The results therefore do not represent absolute antigen deposition. Other limitations to these techniques include that image analysis of immunofluorescence deposition using Image J software suggested differences in antigen deposition were more obvious than visual interpretation of the images suggest. This may could be interpreted as demonstration of the usefulness and utility of objective software based analysis of immunofluorescence staining. However, the technique still requires semi-subjective brightness thresholds to be set. Furthermore, the correlation of software interpretation with histology and clinical markers needs validation in larger cohorts. How closely immunofluorescence staining quantification correlates with grading of immunohistochemic DAB-based staining intensity needs assessment. Finally, the potential for different antigen retrieval techniques to identify epitopes in different glomerular compartments requires further investigation, for example with super resolution microscopy.

Despite these drawbacks, the uniform application of these techniques to images collected under identical conditions provided results consistent with other modalities. For example, I replicated the association between FHR5 glomerular deposition and IgAN severity identified by DAB dependent IHC. These quantifiable IF

techniques also revealed that patients with progressive IgAN had more C3b/iC3b/C3c and FHR5 co-deposition than stable IgAN cases. However, the amount of glomerular C3b/iC3b/C3c deposition as a single antigen was not significantly different between progressive and stable cases. The anti-C3b/iC3b/C3c antibody will detect C3 fragments produced from both glomerular and extra-glomerular complement cleavage. C3c of systemic origin may deposit in glomeruli and would not participate in ongoing complement activation. FHR5 binds complement activation products which would be relatively more concentrated at points of local complement amplification. The co-deposition of FHR5 with C3b/iC3b/C3c may therefore mark areas of local complement activation. This is consistent with my speculative interpretation of FHR5 and C3 fragment co-localisation in C3G; glomerular FHR5 is identifiable with C3 fragments cleaved from local complement activation, but not C3c from systemic complement cleavage. If glomerular alternative pathway activation determines inflammation and injury and contributes to IgAN severity, the co-localisation of FHR5 with C3b/iC3b/C3c would associate with disease severity. The explanation for me not detecting a difference in IF C3b/iC3b/C3c deposition between stable and progressive IgAN cases may be that a significant proportion of glomerular C3b/iC3b/C3c in IgAN is C3c cleaved in circulation independent of IgAN severity. Perhaps FHR5-C3b/iC3b/C3c co-localisation, in contrast, is greater in progressive IgAN because it marks locally cleaved C3 fragments and glomerular alternative pathway activation. The associations of co-deposited FHR5 and local complement alternative pathway activity with IgAN severity *in vivo* is key evidence supporting a role for FHR5-dependent complement deregulation in IgAN pathogenesis.

The correlations of glomerular antigen locations in IgAN provided interesting observations. The glomerular locations of C3dg and FHR5 correlated positively in both stable and progressive IgAN. This may reflect the binding affinity of FHR5 for C3dg and the production of glomerular C3dg following local complement activation, where it commonly encounters FHR5. This may not drive disease severity. The glomerular locations of FHR5 with IgA and C3c/iC3b/C3c showed variable correlations. In some cases, the antigen glomerular locations correlated closely. However, glomerular locations showed negative correlations in other cases. There were trends towards more positive location correlations in progressive cases. Negative correlations were seen in stable disease only. These data suggest that, in addition to the amount of glomerular deposited FHR5, whether FHR5 co-localises with IgA and C3b/iC3b/C3c is also important in determining IgAN severity. However, this theory needs testing it a larger cohort of progressive and stable IgAN patients.

Conclusions

- IgA, C3b/iC3b/C3c and FHR5 glomerular deposition appeared greater in progressive than stable IgAN.
- Progressive patients have more glomerular FHR5 and IgA and more C3b/iC3b/C3c and FHR5 co-localisation than stable patients.
- The amount of glomerular Ceb/iC3b/C3c and C3dg did not differ between stable and progressive cases.
- The glomerular locations of C3dg and FHR5 correlate closely.
- The glomerular locations of FHR5 with IgA and FHR5 with C3b/iC3b/C3c correlate positively in some cases and negatively in others and this may correlate with disease severity.

F. Factor H related protein 5 is identified with C3 in purified circulating IgA1

Introduction

I have demonstrated associations between circulating FHR5 levels, glomerular FHR5 deposition, and glomerular co-deposition of FHR5, C3b/iC3b/C3c and IgA with IgAN severity. I have also identified correlations between FHR5 glomerular deposition intensity and the severity of C3G, the development of which is dependent on alternative complement pathway deregulation. These data indicate FHR5 might be pathogenic in alternative complement pathway activation and IgAN development. However, FHR5 may be a sensitive marker of alternative pathway activation but not directly pathogenic. In order to determine whether the associations of FHR5 and IgAN are causative, I need to identify mechanisms of FHR5 contributing to complement activation and IgAN development.

Activated C3, C3b, is a labile and promiscuous protein that binds molecules and surfaces in its immediate vicinity via its exposed thioester domain. The co-deposition of FHR5 with C3 activation products implies FHR5 binds products of glomerular C3 activation. This would be predicted to occur as FHR5 flows through kidney capillary networks and interacts with capillary endothelium and adjacent mesangial areas. However, C3 is found bound to IgA1-containing circulating immune complexes in IgAN[304]. FHR5 could also be bound to these IgA1-containing immune complexes and accompany their glomerular and mesangial deposition. Subsequently, the presence of FHR5 and C3 with deposited immune complexes could amplify local complement activation in response to IgA1. To test whether this mechanism of FHR5 glomerular deposition and complement deregulation might be relevant, I aimed to examine the presence of FHR5 and complement proteins in IgA1 containing complexes from IgAN patients and healthy controls.

Humans have two isotypes of circulating IgA: Subclass 1 (IgA1) and 2 (IgA2). Only IgA1 is detected in mesangial deposits of patients with IgAN. IgA1 and IgA2 are differentiated by an extended hinge region with O-linked glycans between the first and second constant domains (Fc) of IgA1. The O-linked glycans consist of GalNAc that can be bound by sialic acid and galactose. Patients with IgAN have increased circulating levels of IgA1 without galactose bound to the O-linked glycans[207, 246]. Furthermore, poorly O-galactosylated IgA1 is higher in IgAN patients with progressive compared to stable disease[246]. In vitro assays show human mesangial cell cultures produce cytokines and proliferate in response to patient-derived gd-IgA1[305, 306]. Their response is increased with polymeric, as opposed to monomeric or dimeric gd-IgA1[306, 307].

The protocol for IgA1 purification was shared with me by Dr Theresa Page. It uses Jacalin bound to agarose (Vector Laboratories, UK)[308]. Jacalin is a lectin that binds serum or secretory human IgA1 but does not bind IgA2 nor other immunoglobulin classes. Jacalin binds only O-glycosidically linked oligosaccharides, preferring the structure galactosyl (β-1,3) GalNac. The Jacalin specificity for IgA1 is due to O-linked oligosaccharides located in the IgA1 hinge region.

Hypothesis and aims

I hypothesised FHR5 interacts directly with patient-derived circulating IgA1, but not circulating IgA1 from healthy individuals. The FHR5 is likely to be complexed with C3. I aimed to purify IgA1 from serum or plasma from IgAN patients and healthy controls using Jacalin-bound agarose and then determine if I could detect FHR5 in the IgA1 preparations.

Methods

See the Methods chapter for a detailed description of the experimental techniques used.

Results

a. Purification of IgA1 from plasma and serum from an IgAN patient and healthy control

I purified IgA1 containing preparations from plasma and serum samples from a patient with IgAN and a healthy control. The patient was consented and enrolled in the Causes and Predictors of IgA Nephropathy trial. The plasma used was plasma exchange fluid removed during a flare of IgAN with rapidly progressive glomerulonephritis (RPGN) and crescentic changes on biopsy. The healthy control was an age, gender and ethnicity matched individual who consented separately to study involvement.

I purified 6ml of IgA1 preparation at a concentration of 1.7mg/ml from 10ml of patient plasma. This equates to about 10.2mg of IgA1 from 10ml of patient plasma. I obtained 4.5ml of IgA1 preparation of concentration 1.5mg/ml from 16ml of serum from the healthy control. This equates to 4.2mg IgA1 from 10ml of serum.

I assessed whether my results were equivalent in efficiency to other groups who use similar IgA1 purification methods. Allen *et al* obtained about 4mg of IgA1 from each 10ml of serum sample[308]. This is comparable to the quantity I purified. I estimated the IgA1 concentration of our plasma and serum samples. At the time of plasma exchange, the patient had a serum IgA level of 3.0g/L. Approximately 85% of serum IgA is subclass IgA1[309]. Plasma exchange removes about 66% of circulating protein. Therefore, we could estimate 10mL of plasma exchange fluid to contain about 16.8mg of IgA1. The 10.2mg I purified is about 61% of the total IgA1 in the sample.

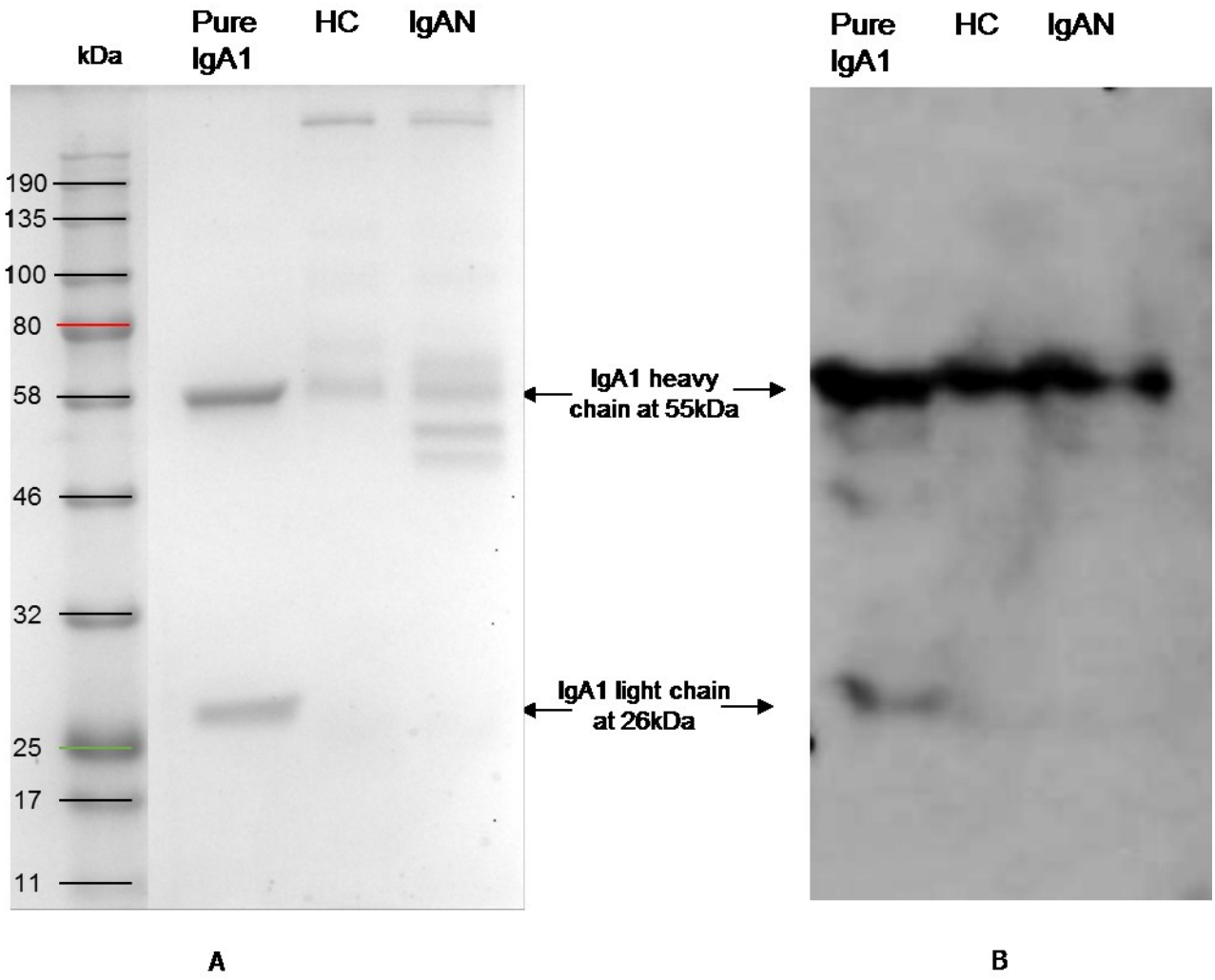
The healthy control serum IgA level was not measured. However, the total circulating pool of IgA1 is 101 +/- 26mg/kg[218] and 55% of this is in the intravascular compartment. The health control individual weighed about 75kg. He therefore had about 4166mg IgA1 in circulation. Assuming his circulating blood volume is about 6 litres, this equates to about 0.69mg/ml of serum, or 6.9mg of IgA1 in 10ml of serum. The 4.2mg I purified is about 61% of the available igA1 in the sample.

b. Demonstration of IgA1 in Jacalin purified serum and plasma preparations

I next demonstrated that the preparations obtained from Jacalin-agarose purification contained IgA1. I used commercially produced full length human IgA1 protein (Abcam ab91020) as a control. According to the datasheet, this is produced from pooled human plasma, is more than 95% pure and shows no reaction to IgA2 antiserum by immunoelectrophoresis. Its concentration is 4.3mg/ml. I demonstrated bands by Coomassie stain under reduced conditions at appropriate size for IgA1 heavy (55kDa) and light (26kDa) chains in the commercial IgA1 preparation (Figure 47). These were seen on western blot of the same samples using a specific anti-IgA1 antibody (Figure 47). The healthy control and IgAN patient preparations showed bands on Coomassie stain and western blot at an appropriate size for the IgA1 heavy chain. No light chain bands were seen. However, additional bands were seen by Coomassie stain and western blot in the healthy control and IgAN patient preparations. These bands may represent the binding of other proteins to IgA1 light chains *in vivo*.

Figure 47. Jacalin-purified preparations contain IgA subtype 1 (IgA1)

(A) Coomassie stain of full length human IgA1 protein purified from plasma (Abcam ab91020) and purified IgA1 from a helathy control (HC) and patient with IgAN under reduced conditions.
(B) Western blot assessment of the same samples under reduced conditions with an anti IgA1 antibody (goat anti-human IgA1, the Binding Site BSAU087).



c. FHR5 is detectable in the IgA1 preparations from an IgAN patient and healthy control.

I demonstrated the presence of FHR5 in IgA1 preparations by western blot (Figure 48). I used a mouse monoclonal anti-FHR5 antibody. For control samples I used serum and IgA1 preparations from an age, gender and ethnicity matched healthy control and a patient known to have mutant FHR5 protein due to the CFHR5 nephropathy associated genetic mutation in heterozygosity. Under non-reduced conditions, the band representing wild-type FHR5 was detectable in serum control samples and the IgA1 preparations from both the IgAN patient and healthy control (at about 50kDa). I also detected a band at higher molecular weight (about 64kDa) representing the mutant FHR5 protein in serum and purified IgA1 preparations from the patient with CFHR5 nephropathy (Figure 48). Therefore, mutant FHR5 associated with CFHR5 nephropathy also binds and is identified in complexes with IgA1. I replicated these findings using a rabbit polyclonal anti-FHR5 antibody. An additional higher molecular band is detected in all samples. Given the antibody is raised against full-length FHR5 protein and the sequence homology between FHR5 and FH, I initially assumed this band is likely to represent FH. However, I was subsequently not able to detect FH using a polyclonal antisera to FH (see below). It is therefore possible the higher bands are FHR5 dimers or multimers detectable under non-reduced conditions, although the absence of FH requires confirmation with additional technique such as ELISA.

d. IgA1 preparations contain detectable C3 but not FH or FHR1.

I next questioned whether other proteins involved in complement deregulation could be detected with the IgA1-purified complexes (Figure 49). For control samples I used serum from an individual with the *CFHR3-1* deletion polymorphism in homozygosity, and therefore no FHR1, and serum from an age, gender and ethnicity matched healthy control. Using an anti-human FH antibody that detects an epitope expressed in both FH, FHR1 and FHR3, I was unable to detect FH or FHR1 in the IgA1 preparations (Figure 49). I was able to detect FH in both the controls serum samples at about 155kDa. I detected FHR1 in the healthy control serum sample at about 30kDa. This was not detected in the control sample with homozygous deletion of *CFHR3-1.* I detected a band with the anti-FH antibody in both the patient and healthy control derived IgA1 at approximately 56 kDa. This band was not visible in serum samples and is therefore assumed to be an IgA1 fragment bound to a FH or FHR protein. Given the molecular weight, this is most likely to be IgA1 light chain bound to FHR2, which has a molecular weight of about 22KDa. It could also represent FHR5 that is present a greater concentration in IgA1

preparations than in serum, which is why it is not seen in the serum samples. Finally, it could represent non-specific binding of the FH antiserum to FH to the IgA1 heavy chain. The precise components of the IgA1 preparations requires further analysis.

Complement C3 consists of two chains, C3α and C3β, which are linked by disulphide bonds and non-covalent interactions and that have molecular weights of about 120kDa and 75kDa respectively[310]. Under reduced conditions, bands for C3α and C3β chains, and C3α chain fragments are seen in the purified IgA1 samples from both the IgAN patient and healthy control (Figure 50). In summary, C3 but neither FH nor FHR1 were detectable in purified IgA1 samples from IgAN patients and healthy controls.

Figure 48. FHR5 is detectable in IgA1 preparations

Western blot under non-reduced conditions for human FHR5 in IgA1 preparations (IgA1) and human serum from a patient with IgAN, a patient with CFHR5 nephropathy (CHFR neph) and a healthy control (HC). A mouse monoclonal anti-FHR5 antibody (Abnova 81494-B01P) was used to detect bands in both serum and the Jacalin-purified IgA1 preps at about 50 kDa representing wild-type FHR5. The CFHR5 neph patient had an additional band at about 64kDa in both serum and IgA1 preps representing the mutant FHR5 protein (CFHR5 nephropathy is associated with a CFHR5 gene mutation in heterozygosity so both the wild-type and mutant proteins are detected). The antibody also detects FHR1 and FHR2 that are likely detected by the band at about 40kDa (FHR1) in the serum samples. The band at about 90kDa in both IgA1 and serum samples could be a FHR protein dimer or could represent non-specific antibody binding.

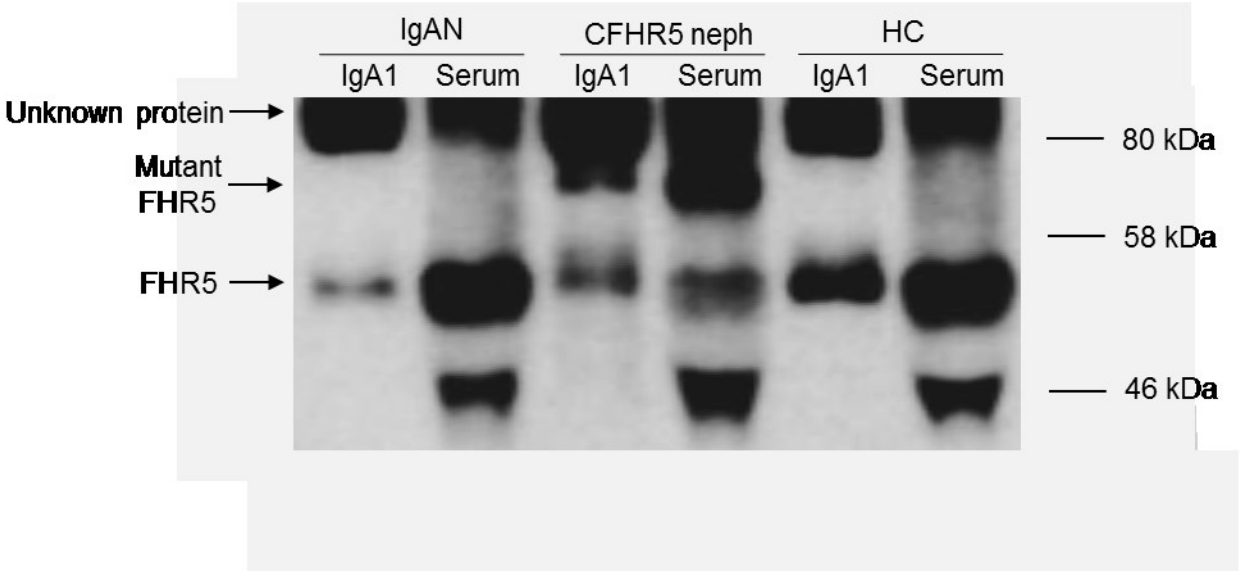
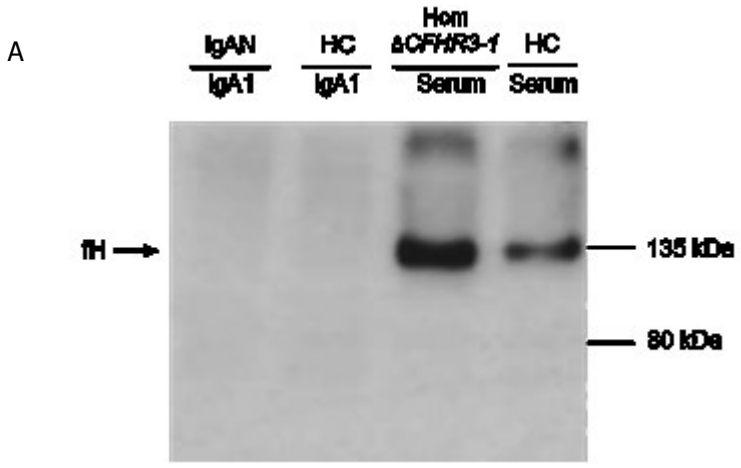


Figure 49. FHR1 and FH are not detectable in IgA1 preparations by western blot

Western blot under non-reduced conditions for (A) human FH and (B) FHR1 in IgA1 preparations (IgA1) and human serum. Samples were from a patient with IgAN, a healthy control (HC), and an individual with the deletion polymorphism of the genes for FHR1 and FHR3 in homozygosity (Hom ΔCFHR3-1). The Hom ΔCFHR3-1 individual therefore has no circulating FHR1. FHR1 was detectable in the serum of the healthy control but in neither IgA1 preparation. I used a goat polyclonal antisera to human FH antibody (Quidel A312). Given the sequence homology between FH and the FHR proteins, the antisera would be predicted to detect other FHR proteins and FH-like protein 1 (FHL1) that has a predicted molecular weight of 45kDa.



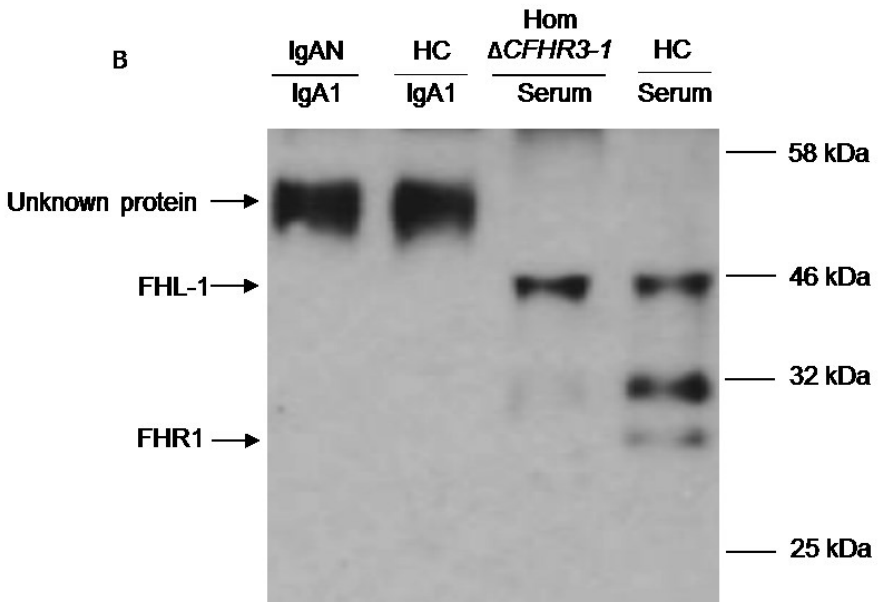
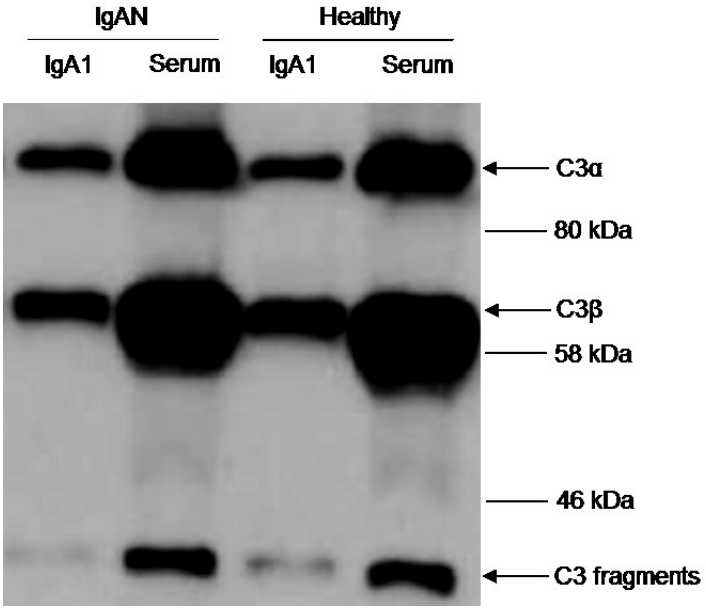


Figure 50. Complement C3 is detectable in IgA1 preparations

Western blot under reduced conditions for human complement (C) 3 in IgA1 preparations (IgA1) and human serum. Samples were from a patient with IgAN and a healthy control (Healthy). I used a HRP-conjugated goat polyclonal anti-human C3 antibody (Cappel 55233). Complement C3 is detectable in IgA1 preparations



e. The presence of FHR5 in IgA1 preparations is dependent on the presence of IgA1 and C3

The demonstration of C3 but no FH in IgA1 preparations led me to question wither the binding of FHR5 to IgA1 is dependent on C3. I also wanted to test whether FHR5 might be binding Jacalin directly. I tested IgA1 preparations and serum from a patient with C3 deficiency and a patient with selective IgA deficiency for FHR5 by western blot. From each case, FHR5 was detectable in serum but not in the IgA1 preps (Figure 51), suggesting the presence of FHR5 in our IgA1 preparations is dependent on the presence of C3 and IgA1.

f. Detection of FHR5 in monomeric, dimeric and polymeric IgA1 preparations from an IgAN patient.

Based on the inflammatory reaction of cultured human mesangial cells to polymeric more than monomeric IgA1[306], I next questioned whether FHR5 would be detectable in polymeric, dimeric and monomeric IgA1 fractions from both IgAN patients and individuals without IgAN. I first separated IgA1 preparations from the IgAN patient with rapidly progressive GN and the matched healthy control into monomeric, dimeric and polymeric fractions by size exclusion chromatography. I concentrated the fractions to 1.33-1.39mg/ml. In reflection of the greater quantity of IgA1 purified from the IgAN patient, I had more IgA1 of each size from the patient than the healthy control. I therefore initially interrogated the complement and FHR5 binding of IgA1 fractions from the IgAN patient only.

By western blot I was able to detect C3α and C3β chains in the monomeric, dimeric and polymeric IgA1 fractions from the IgAN patient (Figure 52). Although I concentrated purified samples to the same concentration and loaded the same volume, the results should not be used to compare relative protein abundance because I can not ensure equal protein loading in each lane. Under non-reduced conditions, FHR5 was detectable in the monomeric but not the dimeric or polymeric IgA1 fractions from the IgAN patient. Under reduced conditions, double bands at about 50 and 54kDa were detected with the anti-FHR5 monoclonal antibody in unfractionated, polymeric and dimeric IgA1 preparations from an IgAN patient (Figure 53). These bands were not seen on a western blot of the IgA1 preparations and secondary antibody alone. The reason the change to reduced conditions causes the band to decrease in molecular weight and split to a double band is not known. FHR5 is highly glycosylated, and variation in N-terminal glyclosylation may explain this finding[311]. However, this finding could be due to non-specific binding or sample degradation.

Figure 51. FHR5 is not detected in IgA1 preparations from individuals with C3 deficiency and selective IgA deficiency

Western blot under non-reduced conditions for human FHR5 in IgA1 preparations (IgA1) and human serum from a patient with IgA nephropathy (IgAN), a patient with selective IgA deficiency (IgA def) and a patient with C3 deficiency (C3 def). A mouse monoclonal anti-FHR5 antibody (Abnova 81494-B01P) was used to detect bands at about 50 kDa representing wild-type FHR5.

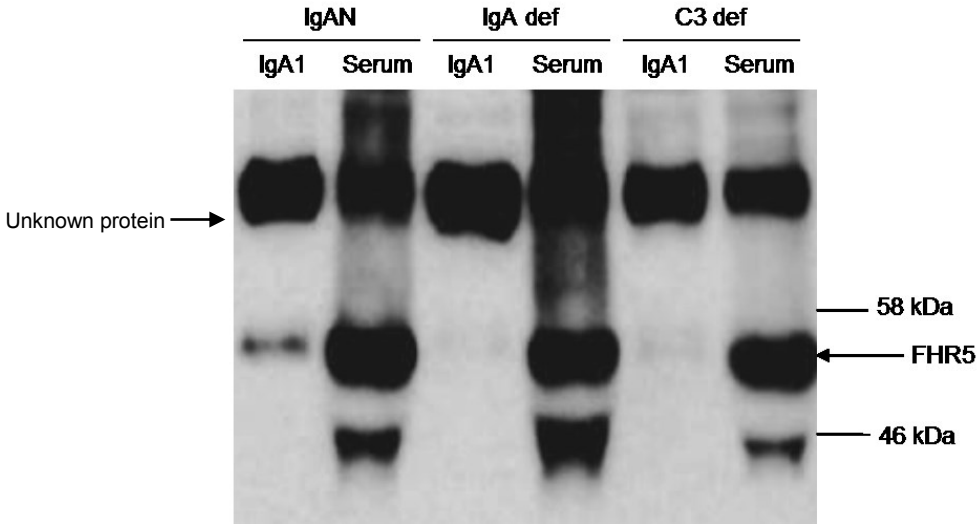
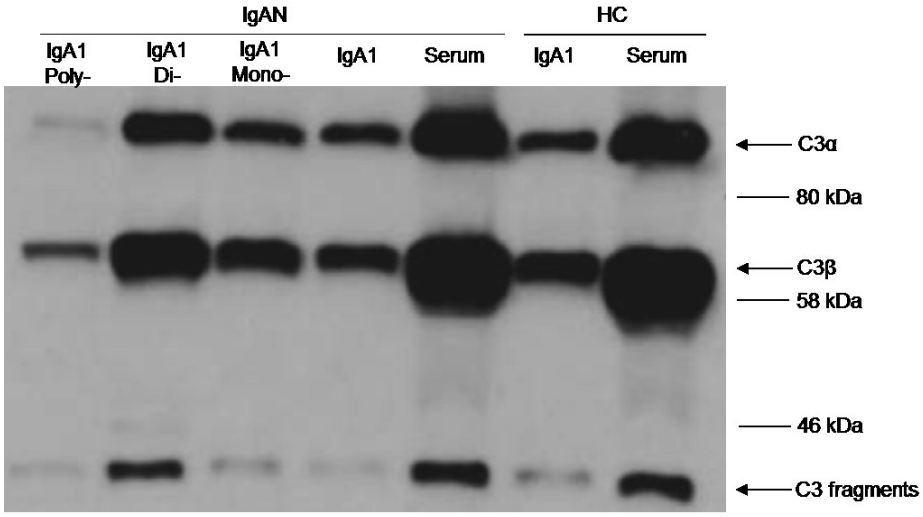
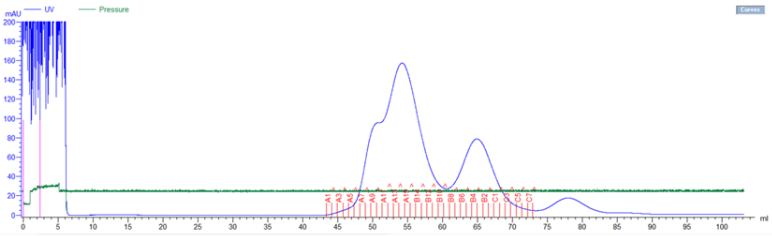


Figure 52. C3 is present in monomeric, dimeric and polymeric IgA1 fractions from an IgA nephropathy patient

Western blot under reduced conditions for human complement (C) 3 in non-separated IgA1 preparations (IgA1), monomeric (IgA1 Mono-), dimeric (IgA1 Di-) and polymeric (IgA1 Poly-) IgA1 fractions and human serum. Samples were from a patient with IgAN and a healthy control (HC). Fractions were separated by size exclusion chromatography. I used a HRP-conjugated goat polyclonal anti-human C3 antibody (Cappel 55233). The size exclusion chromatography trace from the separation of IgA1 samples is shown below. Fraction collection is represented by short red lines and started from 43ml. Fractions A1-A11 were combined as IgA1 Poly-, fractions A12-B9 were combined as IgA1 Di- and fractions B8-C6 were combined as IgA1-mono.





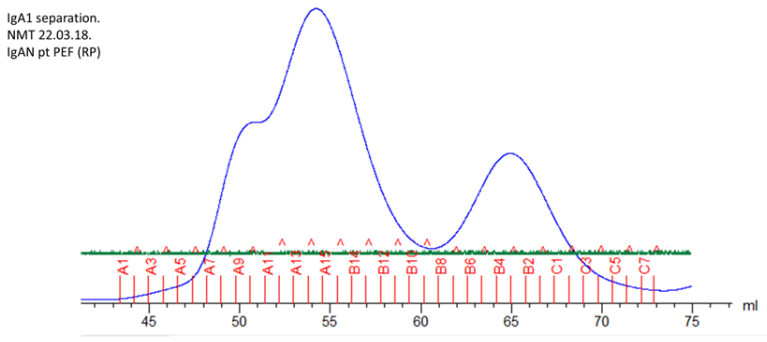
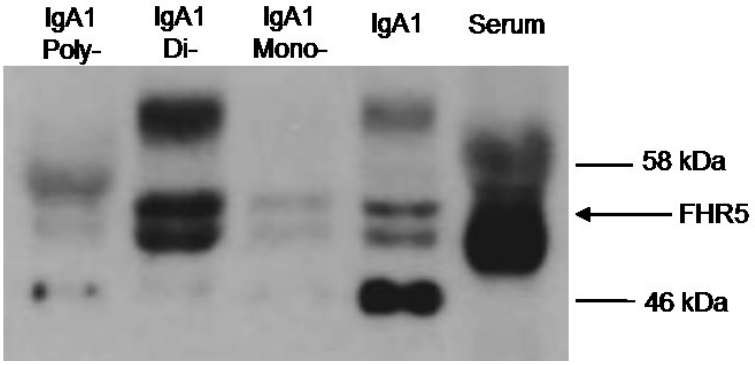


Figure 53. FHR5 is present in monomeric, dimeric and polymeric IgA1 fractions from an IgA nephropathy patient

Western blot under reduced conditions for human FHR5 in non-separated IgA1 preparations (IgA1), monomeric (IgA1 Mono-), dimeric (IgA1 Di-) and polymeric (IgA1 Poly-) IgA1 fractions and human serum. Samples were from a patient with IgA nephropathy. A mouse monoclonal anti-FHR5 antibody (Abnova 81494-B01P) was used to detect double bands at about 50 and 54 kDa representing wild-type FHR5.



g. FHR5 in dimeric and polymeric IgA1 preparations from an IgAN patient and healthy control.

I compared the presence of FHR5 in monomeric, dimeric and polymeric IgA1 fractions from the RPGN IgAN patient and the matched healthy control. I was able to detect FHR5 in the monomeric IgA1 fractions from both the RPGN IgAN patient and the healthy control. However, the dimeric and polymeric IgA1 from the patient only, and not the healthy control, had detectable FHR5 (Figure 54).

I next questioned whether FHR5 would be detected in monomeric and dimeric fractions from other patients with IgAN of different severity, other healthy control individuals, and patients with renal diseases other than IgAN. Dr Theresa Page donated aliquots of monomeric and dimeric IgA1 that had been purified from serum and separated by size exclusion chromatography using identical methods to those I used. FHR5 was detectable in monomeric IgA1 from two other IgAN patients, one of whom had stable and one who had progressive IgAN, and one healthy control (Figure 55). FHR5 was detectable in dimeric IgA1 from one other patient with progressive IgAN but was not clearly seen in dimeric IgA1 preparations from other individuals, including other IgAN patients with progressive disease (Figure 56). I did not detect obvious correlations with FHR5 in IgA1 preparations and renal impairment, proteinuria, serum IgA levels, serum gd-IgA1 levels, or FHR5 levels measured at the sample time. The clinical and assay data available is summarised in table 16.

Figure 54. FHR5 is detectable in dimeric and polymeric IgA1 fractions from an IgA nephropathy patient but not a healthy control

Western blot under either reduced (R) or non-reduced (NR) conditions for human FHR5 in, monomeric (Mono-), dimeric (Di-) and polymeric (Poly-) IgA1 fractions. Samples were from a patient with IgA nephropathy and a helathy control. A mouse monoclonal anti-FHR5 antibody (Abnova 81494-B01P) was used to detect double bands representing wild-type FHR5.

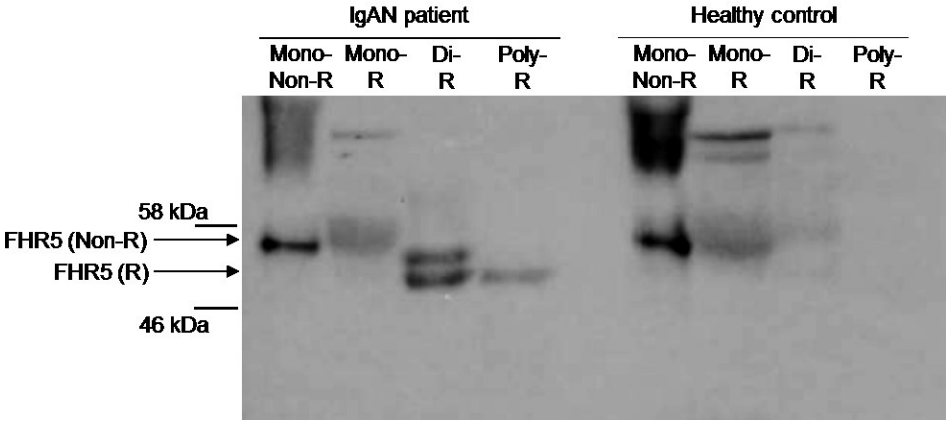


Figure 55. FHR5 in monomeric IgA1 fractions in individuals with and without IgA nephropathy

Western blot non-reduced conditions for human FHR5 in unfractionated (IgA1) and monomeric (IgA1 Mono-) IgA1 fractions and human serum. Samples were from a patient with rapidly progressive IgA nephropathy (RP IgAN), other patients with IgA nephropathy (IgAN 1, IgAN 5, IgAN 10, IgAN 27 and IgAN 28), healthy control individuals (HC 1, HC 2, and HC 3), a patient with diabetic nephropthy (DN), a patient with chronic kidney disease of unknown cause (CKD) and a patient with vasculitis (PR3). A mouse monoclonal anti-FHR5 antibody (Abnova 81494-B01P) was used to bands representing wild-type FHR5.

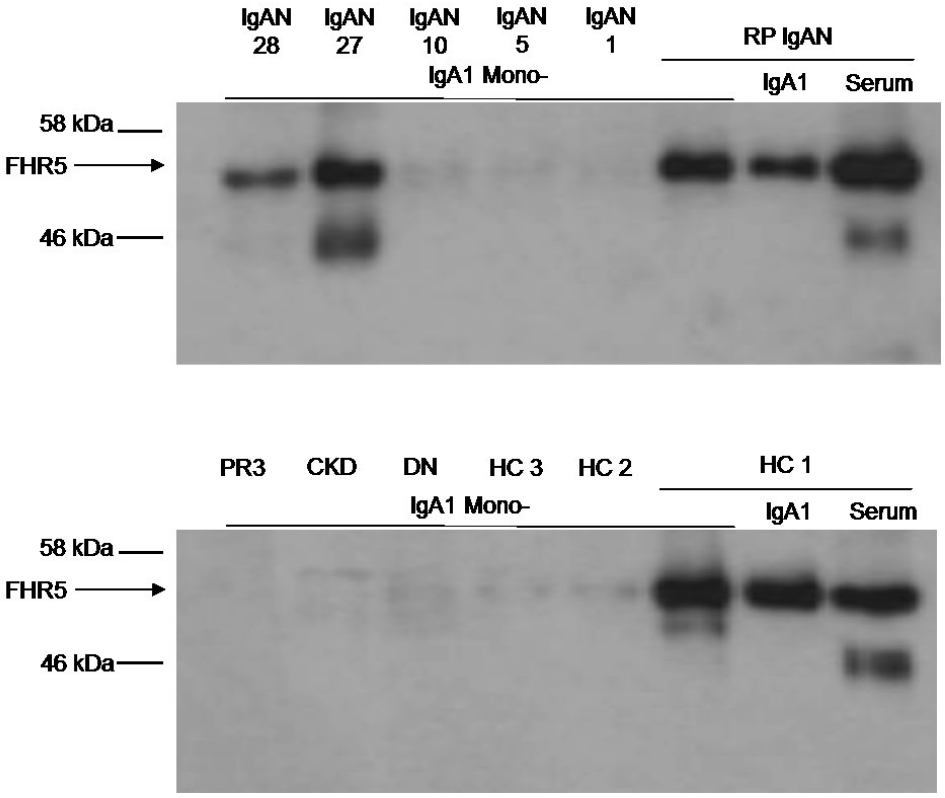
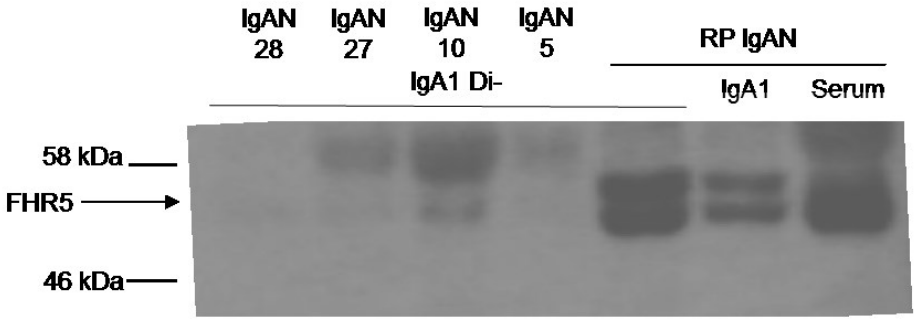
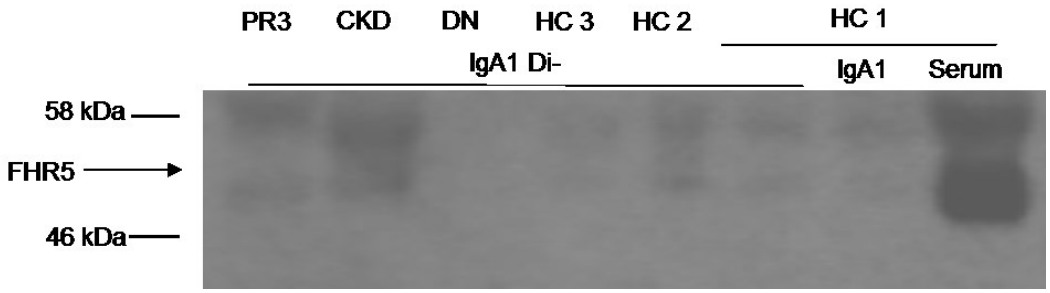


Figure 56. FHR5 in dimeric IgA1 fractions in individuals with and without IgA nephropathy

Western blot reduced conditions for human FHR5 in unfractionated (IgA1) and dimeric (IgA1 Di-) IgA1 fractions and human serum. Samples were from a patient with rapidly progressive IgAN (RP IgAN), other patients with IgA nephropathy (IgAN 1, IgAN 5, IgAN 10, IgAN 27 and IgAN 28), healthy control individuals (HC 1, HC 2, and HC 3), a patient with DN, a patient with chronic kidney disease of unknown cause (CKD) and a patient with vasculitis (PR3). A mouse monoclonal anti-FHR5 antibody (Abnova 81494-B01P) was used to bands representing wild-type FHR5.





Sample	Serum IgA (0.8-4g/L)	gdIgA (AU)	Serum FHR5 (ug/mL)	Urine PCR (mg/mmol)	Serum Cr (umol/L)	Stable or progressive
IgAN1	4.3			24	87	Stable
IgAN5	1.7	0.49	3.2	170	360	Progressive
IgAN10	3.7			685	326	Progressive
IgAN27	1.5	0.32	3.4	54	69	Stable
IgAN28	2.6			350	227	Progressive
RP IgAN	5.4	0.55	3.9	143	377	Progressive

Table 16. Clinical data from IgAN patients with monomeric and dimeric IgA1 fractions available

Discussion

In this chapter, I investigated how FHR5 might interact with circulating IgA1 and C3. This is important in determining how FHR5 contributes to complement activation, glomerular inflammation and renal injury in IgAN. The detection of FHR5 in purified immune complex preparations is novel as is the detection of FHR5 in specific IgA1 preparations. The demonstration of C3 in IgA1-containg complexes is consistent with other analyses of both engineered and patient-derived IgA1 containing circulating immune complexes[248, 304]. Specifically, targeted proteomic analyses of immune complexes engineered from galactose deficient IgA1 myeloma proteins and cord blood-derived anti-glycan IgG revealed C3, iC3b, C3c, and C3dg[248]. Due to the absence of FHR5 in IgA1 preparations from a patient with C3 deficiency, I demonstrated FHR5 is likely complexed with C3 and IgA1 in circulation. These data allow me to speculate on the mechanisms linking IgA1-containing immune complex deposition with complement activation and glomerular inflammation; the presence of FHR5 in immune complexes would deregulate and amplify complement activation on circulating immune complexes and following mesangial deposition. Furthermore, FHR5 could scaffold de novo alternative pathway activation, convertase formation and amplification at the site of deposited IgA1. FHR5 could also interfere with the phagocytosis and excretion of complement-bound immune complexes.

I hypothesised FHR5 would be identifiable bound to IgA1 from IgAN patients but not healthy controls. This was not demonstrated in total purified IgA1 preparations and monomeric IgA1 fractions. However, the identification of FHR5 in dimeric and polymeric IgA1 preparations from only IgAN patients and, potentially, only IgAN patients with progressive disease, would be consistent with FHR5 bound to multimeric IgA1 contributing to disease development and severity.

Novak *et al* reported that IgAN patient derived IgA1 containing immune complexes stimulated the proliferation of mesangial cells and this effect was most pronounced with large molecular weight fractions[306]. Uncomplexed IgA1 did not affect mesangial cells and smaller IgA1-containing circulating immune complexes had inhibitory effects on cultured mesangial cells. It is not clear why circulating immune complexes of different sizes differentially effect mesangial cell proliferation. The identification of FHR5 in IgAN-patient derived multimeric IgA1 fractions may help explain this phenomenon; larger complexes may contain more C3, C3 fragments and FHR5 and therefore activate complement and amplify inflammation. This is supported by the observation that

gd-IgA1 containing immune complexes require a heat-sensitive serum factor to stimulate cultured primary mesangial cells[312]. The serum factor could be complement proteins and FHR5.

These results are preliminary and require further evaluation. Importantly, many of the western blot findings have inconclusive interpretations and the findings require validation with other technique. I plan to complete unbiased proteomic analysis of purified IgA1 by mass spectroscopy. Furthermore, I will perform ELISA on purified samples to confirm the presence of FHR5 and absence of FH and FHR1. This may also allow quantification of complement proteins in samples from different patients with IgAN, and other conditions, of different severities.

Importantly, the size exclusion chromatography separation of purified IgA1 may not separate fractions into monomeric, dimeric and polymeric fractions. The fractions of increasing sizes may infact represent IgA1-containing immune complexes of increasing sizes because of the presence of additional pro-inflammatory molecules and complement proteins. Interestingly, the molecular weight of an IgA1 monomer, C3 and FHR5 complex would be predicted to be similar to an IgA1 dimer, at about 320kDa. This might explain findings from other groups that IgA1 containing immune complexes of larger molecular weight result in significantly more mesangial cell proliferation *in vitro*[306]. The content of the different sized fractions requires further investigation but the fractions should be referred as containing IgA1 complexes of increasing sizes, not monomeric, dimeric and polymeric IgA1.

I have tested fractions contiang IgA1 complexes of different sizes (‘monomeric’ and ‘dimeric’ IgA1 fractions) from five IgAN cases and five non-IgAN individuals only. Furthermore, although FHR5 was detected in dimeric IgA1 preparations from patients with progressive IgAN only, some dimeric IgA1 from progressive IgAN patients had undetectable FHR5. Although we shared methods, eight of the samples were purified and separated by a different group. This involved a different batch of Jacalin-agarose and different sample storage times, potentially with different freeze-thaw cycle exposure, all of which may influence the results. Circulating IgA1-containing complexes are likely to include many other bound proteins and the potential for FHR5 to associate with another pathogenic complex component must also be considered. Furthermore, polymeric IgA1 fractions were available from one IgAN patient and one healthy control only. It is therefore impossible to conclude that the presence of FHR5 with IgA1 fractions associates with IgAN or IgAN severity. This requires replication in

more individuals. Ideally, the finding would also be replicated with a second technique, such as mass spectrometry proteomic analysis of circulating IgA1-containing immune complexes.

FHR5 detected in multimeric IgA1 fractions under reduced conditions had a double band appearance. Similar appearances in other proteins have been attributed to variations in protein N-terminal glycosylation[311]. FHR5 is a glycoprotein that consists of 9 SCRs and includes two potential N-glycosylation sites in SCR2 and SCR7. The double band appearance could represent different complexed FHR5 glycoforms revealed under reduced conditions. This could be tested by eliminating the double band with enzymatic deglycosylation of the IgA1 fractions before western blot detection of FHR5. Monomeric IgA1 preparations did not show a double band for FHR5 under reduced conditions. The presence of multiple FHR5 glycoforms in only multimeric IgA1 complexes indicates FHR5 glycosylation directs FHR5 binding to either monomeric or multimeric IgA1 complexes. FHR5 glycosylation may also influence the affinity of FHR5 for IgA1-bound C3 or galatose-deficient moieties in the hinge region of IgA1. This may explain the associations of galactose-deficient IgA1, polymeric IgA1 immune complexes, FHR5 binding, and complement activation and disease severity. The detection of FHR5 in IgA1-containing complexes and the potential for IgAN disease severity to associate with presence of FHR5 in multimeric IgA1 complexes is novel, exciting and demands further investigation.

Conclusions

- I detected FHR5 in IgA1 preparations purified from plasma and serum from IgAN patients and a healthy control.
- The mutant FHR5 protein associated with CFHR5 nephropathy was identified with purified IgA1 complexes from CFHR5 nephropathy patient serum.
- The presence of FHR5 in circulating IgA1 complexes was dependent on C3 and IgA1.
- FHR5 was detected in monomeric IgA1 fractions from IgAN patients and a healthy control
- FHR5 was detected in IgA1 fractions of larger-sized complexes from IgAN patients but was not detected in large IgA1-containing complexes from healthy controls or patients with non-IgAN renal conditions.
- These results need validation with other techniques and in larger patient cohorts.

G. Circulating complement lectin pathway levels in IgA nephropathy

Introduction

In addition to the alternative pathway, the lectin complement pathway is implicated in IgAN pathogenesis. The lectin pathway is an essential component of innate immunity. The lectin pathway PRMs include MBL, L-ficolin, M-ficolin, H-ficolin, collectin liver 1 and collectin kidney 1. The PRMs bind a number of PAMPs and activate a set of three serine proteases[313-317], called MASP-1, MASP-2, and MASP-3. MASP activation mediates lectin pathway effector function, traditionally thought to occur via formation of the classical and lectin complement pathway C3 convertase, C4bC2b. Additionally, novel links between the lectin and alternative complement pathways have recently been described. MASP-3 is able to cleave Pro-factor D to factor D (fD), a key enzyme in alternative complement pathway convertase formation[318]. The lectin pathway PRMs may also complex with MASP splice variants that lack protease activity called MBL-associated proteins (MAp). Two MAp have been described. With MASP-1 and MASP-3, MAp44 is encoded by the *MASP1* gene. MAp19 is encoded by the *MASP2* gene[319]. Most lectin complement pathway components are expressed in the liver and lung. M-ficolin, however, is predominantly expressed by monocytes[320].

The lectin pathway is a plausible candidate for the link between gd-IgA1 deposition, immunological activation, and glomerular inflammation in IgAN pathogenesis. Due to a deficiency in O-linked glycosylation, IgAN is associated with higher levels of IgA1 with exposed GalNac[247]. GalNac is one of the PAMPs recognised by the lectin pathway PRMs[21]. Also, IgAN is characterised by episodes of macroscopic haematuria and disease flares following respiratory or gastrointestinal tract inflammation[238]; both IgA and the lectin complement pathway are important mediators of innate immunity at these sites. Furthermore, MBL binds polymeric IgA and triggers complement activation *in vitro*[249]

Other studies have looked for evidence of lectin complement pathway activity in IgAN[251-253, 321-323]. However, the detection of M-ficolin, H-ficolin, MASP-3, MAp19 and MAp44 in IgAN patient samples had not been attempted. This is particularly relevant considering the homology between lectin pathway proteins. For example, because MASP-3 differs from MASP-1 only in its 15 C-terminal residues and serine protease domains[324], the antibodies previously used to detect MASP-1 deposition in IgAN patients[251, 252, 322] could have also reacted with MASP-3. This research is of particular relevance considering the recent development of therapeutic agents that target lectin complement proteins, such as the monoclonal antibody targeting MASP-

2, OMS721 (presented at the European Renal Association-European Dialysis and Transplant Association Congress in Madrid, 2017).

Hypothesis and aims

I hypothesised that complement activation by the lectin pathway contributes to glomerular inflammation and disease severity in IgAN. Specifically, I questioned whether (1) I could identify differences in circulating lectin pathway levels in IgAN, (2) lectin plasma levels associate with IgAN disease severity.

Methods

Plasma samples and essential clinical information were available on all 322 IgAN patients. Using the criteria detailed in methods, 190 and 83 patients were categorised into cohorts of progressive and stable IgAN, respectively. Due to a lack of available information, we were unable to confidently classify 49 patients to either cohort. I sent anonymised samples from all patients and some healthy controls to Professor Steffen Thiel. Professor Steffen Thiel and Dr Anne Troldborg (Aarhus University, Denmark) quantified circulating plasma levels of the 11 known lectin components. I analysed the levels and differences between patient and controls groups. I discussed the relevance of these findings and planned additional analyses with Professor Thiel and Dr Troldborg.

Results

a. Plasma M-ficolin, L-ficolin and MAp19 are increased, whilst plasma MASP-3 levels are reduced in IgA nephropathy.

We used a two-stage strategy to measure and compare lectin pathway protein levels. We first screened a subgroup of 125 IgAN patients for quantitative differences in circulating levels of all known lectin pathway components; MBL, M-ficolin, H-ficolin, L-ficolin, CL-L1, CL-K1, MASP-1, MASP-2, MASP-3, Map19, and Map44. At this time, control samples were available from a cohort of 211 healthy volunteers of Caucasian ethnicity from Denmark. We identified lower MBL and MASP-3 levels and higher M-ficolin, L-ficolin, MASP-1 and MAp19

levels in the patient cohort (Table 17). Before confirming our initial findings in the remainder of our 322 patient cohort, we recruited a second healthy control cohort. The second control group cohort showed a more similar ethnicity profile to our patient cohort and, like our patients, was from the UK. We noted significant differences between our two control cohorts in a number of lectin markers, including MBL (1839 ng/ml and 1052 ng/ml in the Danish and London cohorts; difference -787 ng/L, 95% CI -912 to -10 ng/ml; *P* =0.037), the levels of which are influenced by ethnicity[47]. I also found differences between the Danish and London control cohorts in plasma levels of M-ficolin (4201 ng/ml and 3810 ng/ml in the Danish and London cohorts, difference -391 ng/ml, 95% CI -762 to -115 ng/ml; *P* =0.01), and MASP-1 levels (7789 ng/ml and 11247 ng/ml in the Danish and London cohorts, difference 3458 ng/ml, 95% CI 2357 to 4122 ng/ml; *P* <0.0001). It is not known why the two control groups showed differences in circulating levels. The samples were collected and stored similarly and analysed the same laboratory using identical techniques. As mentioned, MBL levels showed marked variation in healthy individuals and are influenced by a complex set of polymorphisms, the frequency of which correlates with ethnicity[49, 50]. This could explain the differences in the control groups. However, other factors, such as diet, may also play a role. Based on more similar ethnicity and locality with our study group, we used the London-based control cohort for the remaining analyses. The lectin components that showed differences in the screening assessment, MBL, MASP-1, MASP-3, M-ficolin, L-ficolin and MAp19, were measured in the remaining 198 IgAN patients and compared with the second control cohort of 51 healthy volunteers from London.

Plasma levels of M-ficolin, L-ficolin and MAp19 were increased in IgAN patients compared to healthy controls (Table 18, figure 57). In contrast, MASP-3 levels were lower in IgAN patients compared to controls (Figure 57, Table 18). Notably as they are splice variants of the same gene, one patient had a very low plasma Map19 level (<65 ng/ml) but plasma MASP-2 level was normal (180ng/ml). The proportion of MBL-deficient individuals, defined as a plasma concentration <100ng/ml, did not differ between patients and control cohorts (Table 18).

	IgAN Patients. Median (range). n=125	Healthy Controls. Median (range). n=211	Difference Between Medians	95% CI
MBL, ng/ml	1086 (<10-7202)	1839 (<10-7202)	-753	-587 to 17
MBL, Caucasian only, ng/ml	1062 (<10-6005). n=85	1839 (<10-7202). n=211	-777*	-714 to -9
Proportion with plasma MBL <100ng/ml	15 (12.0%)	26 (12.3%)		
M-ficolin, ng/ml	4570 (1443-18538)	4201 (1458-10243)	369**	181 to 702
H-ficolin, ng/ml	35466 (9617-82262)	36303 (6928-74806)	-837	-3881 to 1185
L-ficolin, ng/ml	3082 (963-8500)	2751 (982-8267)	341*	49 to 576
CL-L1, ng/ml	535 (295-969)	534 (337-789)	1	-22 to 15
CL-K1, ng/ml	405 (111-1926)	391 (206-555)	14	-1 to 27
MASP-1, ng/ml	10325 (4343-20322)	7789 (3188-15494)	2536***	1949 to 2936
MASP-2, ng/ml	514 (114-1376)	490 (110-1698)	24	-20 to 69
MASP-3, ng/ml	6248 (2924-12101)	7038 (2942-14922)	-790**	-1092 to -218
MAp19, ng/ml	588 (<60-928)	489 (186-1140)	99***	73 to 125
MAp44, ng/ml	2408 (1022-4355)	2351 (1323-4417)	57	-80 to 174

Table 17. Lectin pathway protein levels in a screening cohort of IgA nephropathy patients and Danish healthy controls. P-values shown have been adjusted for multiple analyses to minimise the false discovery rate.

*P <0.05; **P <0.005; ***P <0.0001.

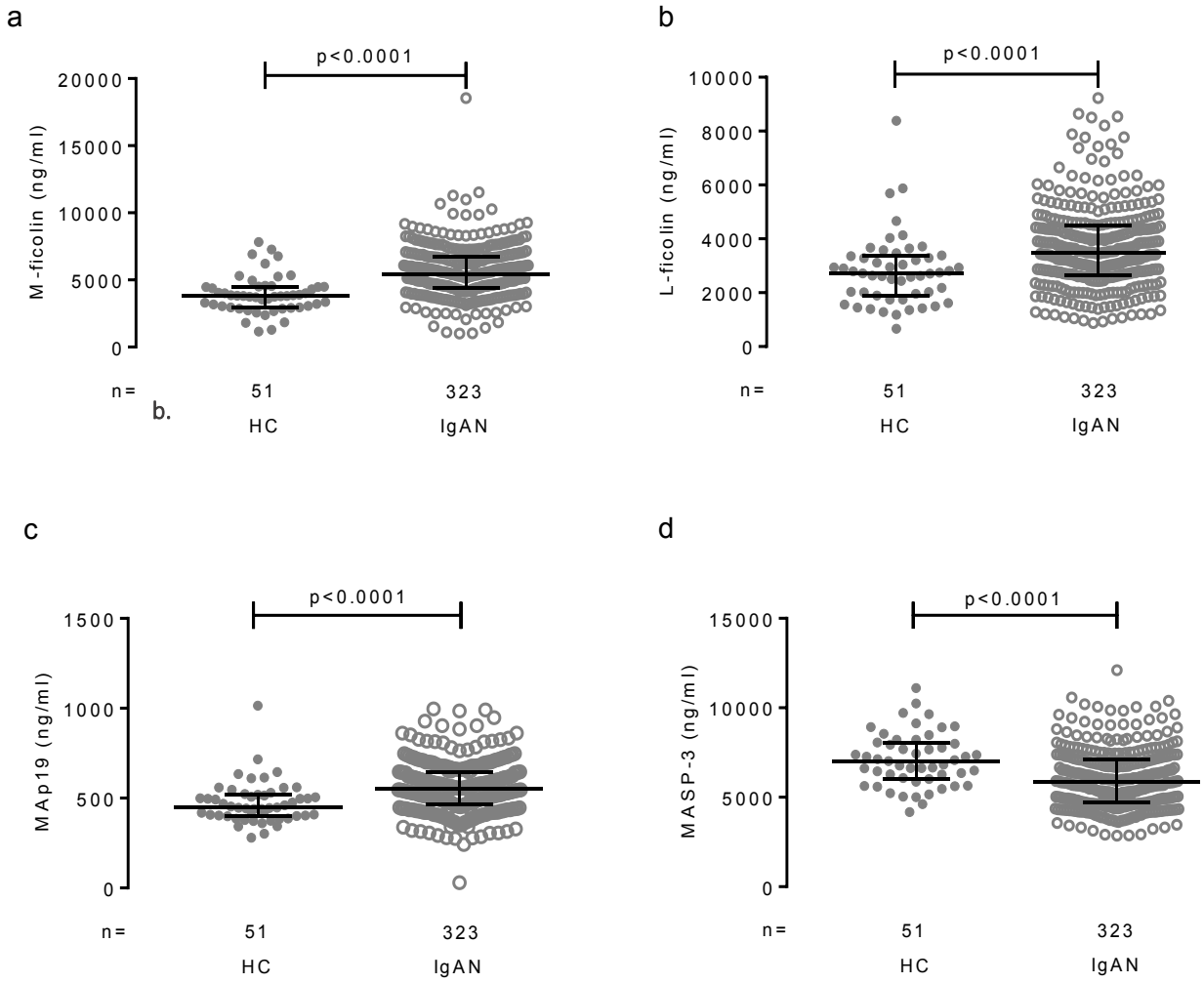
	IgAN Patients Median (range). n=323	Healthy Controls. Median (range). n=262	Difference between medians	95% CI
MBL, ng/ml	1507 (<10-7002)	1557 (<10-7202)	-50	-249 to 145
MBL, Caucasian only, ng/ml	1471 (<10-6556). n=270	1534 (<10-7202). n=258	-63	-235 to 170
Patietns with plasma MBL <100ng/ml	43 (13.3%)	32 (12.2%)		
M-ficolin, ng/ml	5422 (1002-18538)	4124 (1159-10243)	1299***	1066 to 1546
L-ficolin, ng/ml	3463 (872-9230)	2740 (659-8381)	723***	543 to 960
MASP-1, ng/ml	10323 (2349-56002)	8091 (4490-18130)	2233***	1246 to 2327
MASP-3, ng/ml	5836 (2856-12101)	7028 (2942-14922)	-1192***	-1411 to -825
MAp19, ng/ml	552 (<60-996)	485 (186-1140)	67***	51 to 89

Table 18. Lectin pathway protein levels in IgA nephropathy. P-values shown have been adjusted for multiple analyses to minimise the false discovery rate.

*P <0.05; **P <0.005; ***P <0.0001.

Figure 57. Plasma lectin complement pathway components in IgA nephropathy

M-ficolin (a), L-ficolin (b) and MAp19 (c) levels are elevated in IgA nephropathy (IgAN, empty circles) compared to healthy controls (HC, solid circles). Plasma MASP-3 levels are reduced in IgAN (d) The bar represents the median value, box represents the interquartile range, and whiskers represent the range of values. P-values shown have been adjusted for multiple analyses to minimise the false discovery rate.



Lectin complement pathway plasma changes correlate with glomerular filtration.

Changes in glomerular filtration rate can influence plasma complement protein levels. I have previously shown that plasma FHR1 increased with falling eGFR whilst plasma FHR5 levels did not. I identified associations between renal function and lectin pathway plasma levels; M-ficolin and MAp19 showed negative, and MASP-3 showed positive correlations with eGFR sampled at study enrolment (Figure 58). However, the associations were marginal so it is difficult to conclude whether change in eGFR associates with changes in circulating levels or not. There was no association between L-ficolin and eGFR (Figure 58). To delineate whether the observed lectin plasma differences were the result of IgAN disease activity or reduced glomerular filtration, I compared three groups; (1) healthy controls, (2) patients with eGFR preserved at more than 60ml/min per 1.73m^2, and (3) patients with renal function reduced to eGFR less than 30ml/min per 1.73m^2. M-ficolin and MAp19 levels were significantly increased in IgAN patients with preserved renal function compared to healthy controls. Levels were also higher in patients with reduced compared to preserved renal function, suggesting both disease activity and impaired glomerular filtration may contribute to the higher levels in IgAN (Figure 59). Consistent with this, patients with eGFR less than 30ml/min had lower MASP-3 levels than patients with preserved eGFR, who had lower MASP-3 levels than healthy controls (Figure 59).

Due to the marginal nature of the correlations between eGFR and circulating levels of lectin pathway proteins, I aimed to further investigate the effect of eGFR change on lectin plasma levels. I compared M-ficolin, MASP-3 and MAp19 levels within 4 weeks before and 16 weeks after renal transplantation. We measured levels in the same cohorts of IgAN and ADPKD patients described in a previous Results chapter. Reflecting an improvement in eGFR, average M-ficolin and MAp19 levels were significantly reduced after transplantation in both ADPKD and IgAN cohorts (Figure 59). This suggests M-ficolin and MAp19 levels in circulation are infact significantly influenced by changes in eGFR, regardless of the pathogenesis driving eGFR change. MASP-3 levels showed no significant change following transplantation, suggesting the observed differences in MASP-3 levels are independent of eGFR changes and may associate with disease activity.

Proteinuria data from the time of plasma sampling was available from 223 patients but showed no association with reduced plasma MASP-3 levels. Although we identified a positive correlation between M-ficolin and white cell count (WCC), and MASP-3 and alanine aminotransferase (ALT) in our patient cohorts, there was neither a significant difference in WCC nor ALT between our progressive and stable IgAN cohorts.

Figure 58. Associations between lectin complement pathway protein plasma levels and renal function

Correlation between eGFR and (A) M-ficolin, (B) MAp19 , (C) MASP-3 levels, (D) L-ficolin, and (E) MASP-1 levels. P values derived from Spearman's rank correlation.

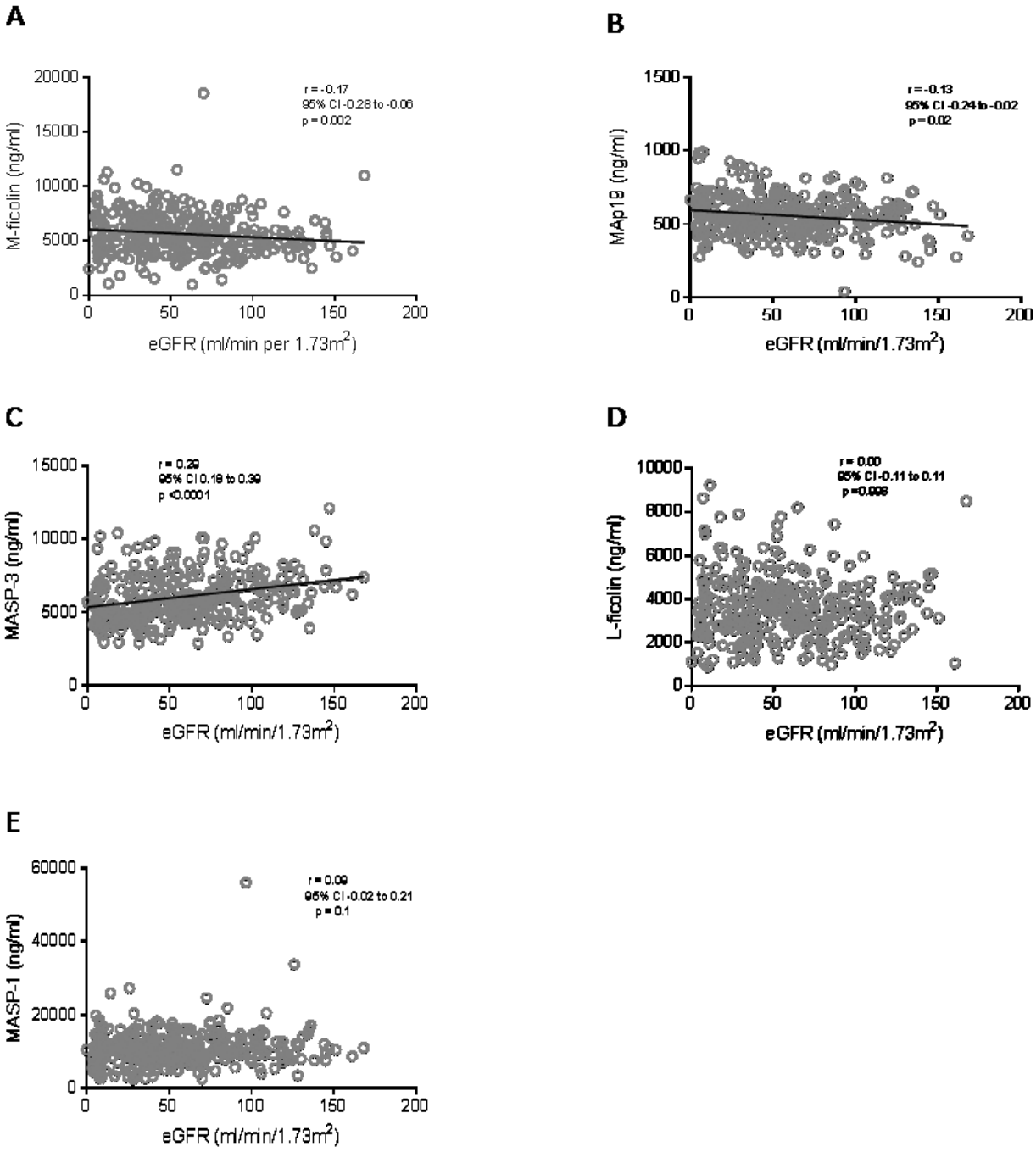
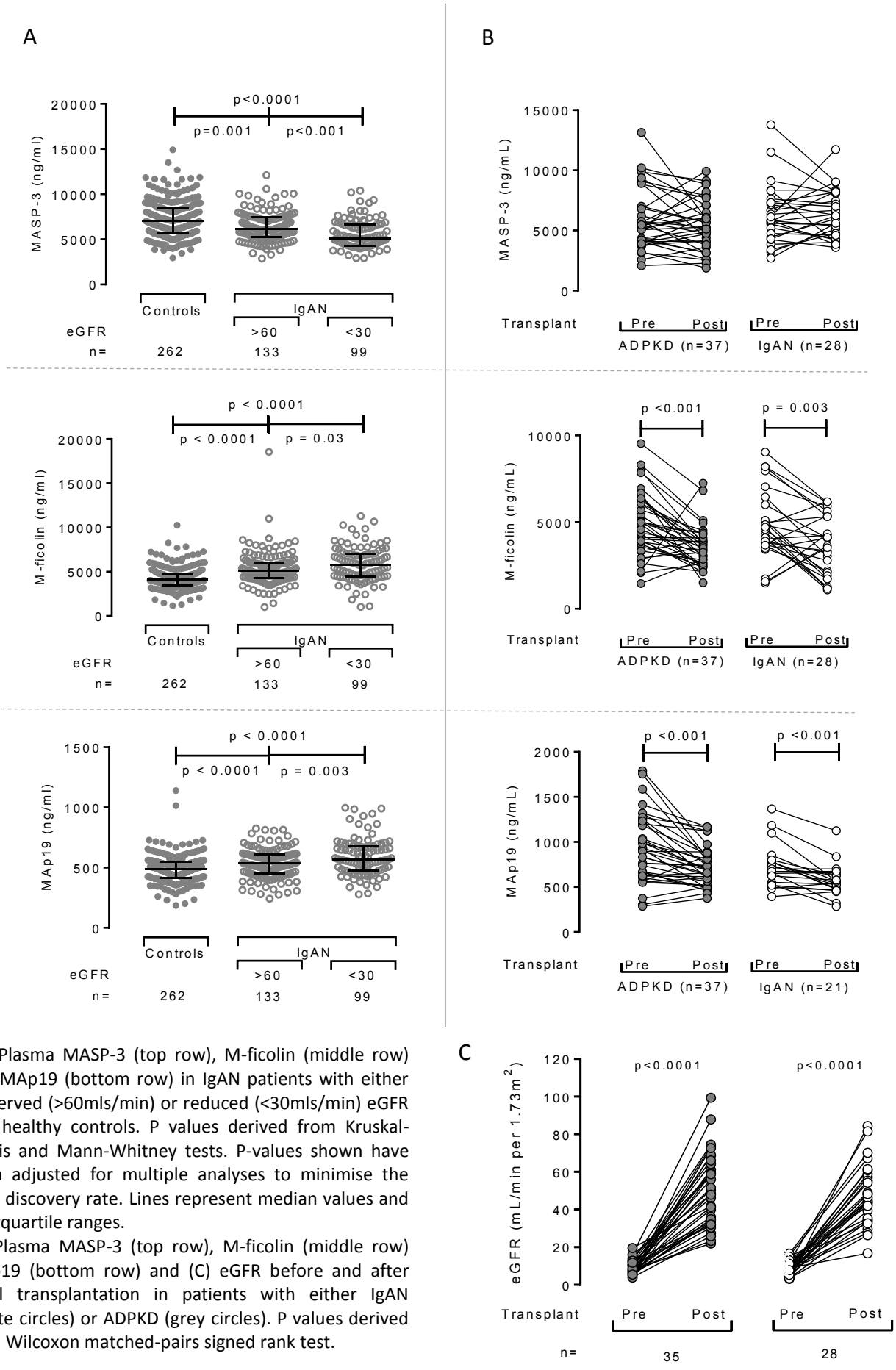


Figure 59. Plasma M-ficolin, MAp19 and MASP-3 levels associate with renal impairment and IgA nephropathy



(A) Plasma MASP-3 (top row), M-ficolin (middle row) and MAp19 (bottom row) in IgAN patients with either preserved (>60mls/min) or reduced (<30mls/min) eGFR and healthy controls. P values derived from Kruskal-Wallis and Mann-Whitney tests. P-values shown have been adjusted for multiple analyses to minimise the false discovery rate. Lines represent median values and interquartile ranges.

(B) Plasma MASP-3 (top row), M-ficolin (middle row) MAp19 (bottom row) and (C) eGFR before and after renal transplantation in patients with either IgAN (white circles) or ADPKD (grey circles). P values derived from Wilcoxon matched-pairs signed rank test.

c. MASP-3 plasma levels are associated with the severity of IgAN

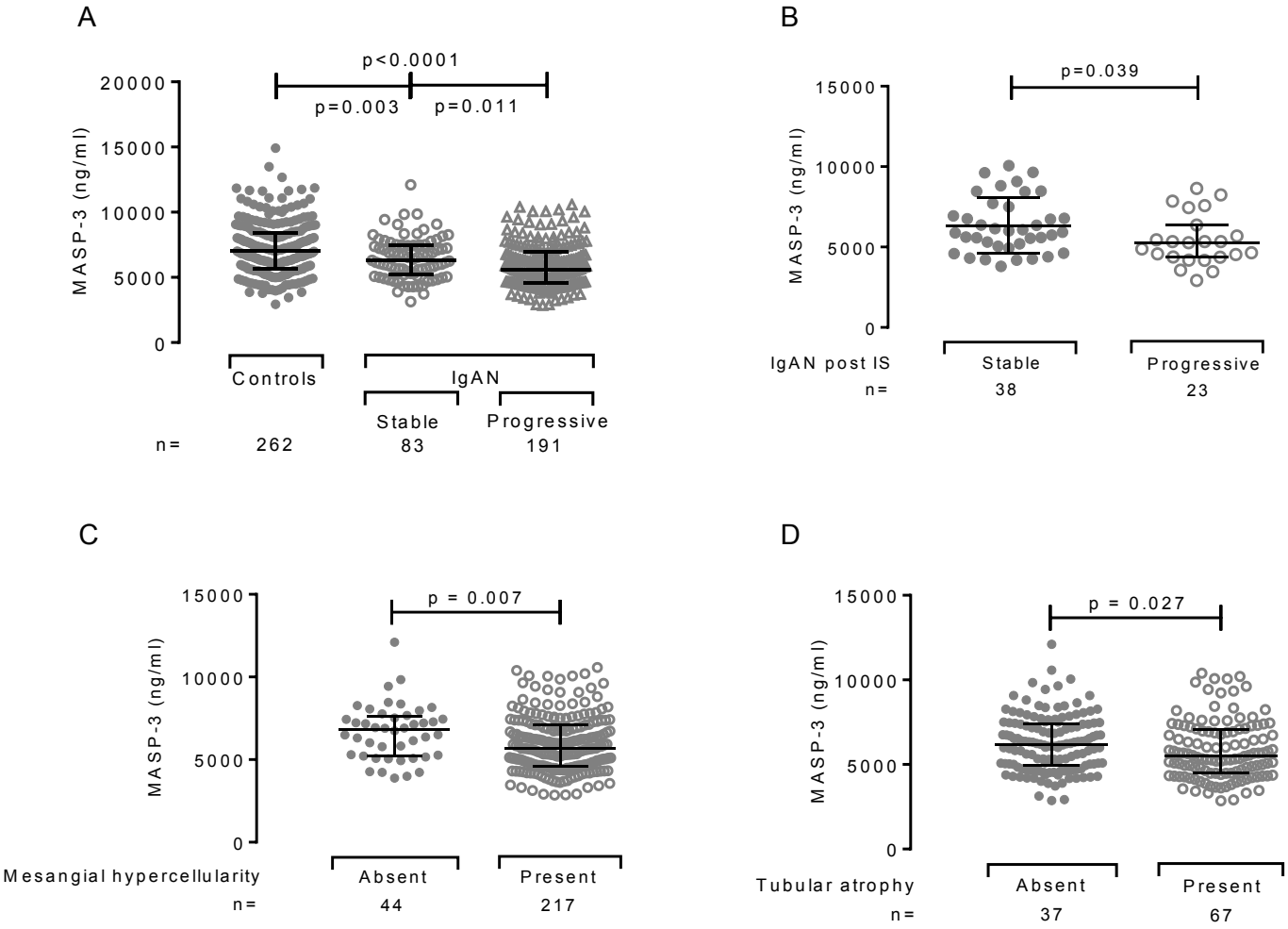
To explore the significance of the altered M-ficolin, L-ficolin, MAp19 AND MASP-3 levels in IgAN, I next assessed whether concentrations differed between stable and progressive IgAN. M-ficolin, L-ficolin and MApP19 did not differ between these groups (Table 18). However, MASP-3 levels were further reduced in patients with progressive disease (mean 5907ng/ml in progressive and 6470ng/ml in stable IgAN; difference -563ng/ml; 95% CI -994 to -133ng/ml; p = 0.011, figure 60). MASP-3 levels were lower in a sub-group who had progressive disease despite immunosuppression compared to the cohort who improved following immunosuppression treatment (5404ng.ml versus 6335ng/ml; difference -931ng/ml; 95% CI -1815 to 47ng/ml; p = 0.039, figure 60). Furthermore, 84.9% (56 of 66 patients) of the quartile with lowest MASP-3 levels had progressive IgAN, compared with 69.3% (190 of 274) of the three quartiles of patients with MASP-3 above this level (OR for progressive disease 2.48, 95% CI 1.25 to 5.01; p = 0.014,).

d. Low plasma MASP-3 and high MAp19 associated with histology features of IgAN severity

I next questioned whether low MASP-3 levels also showed an association with histology features of disease severity. Given the demonstrated stability of MASP-3 serology levels over time[325, 326], we compared levels sampled at recruitment with the Oxford Classification of IgA Nephropathy[194] scores from the native diagnostic renal biopsy. Median plasma MASP-3 levels were lower in the patient cohort with mesangial hypercellularity (median MASP-3 5667 ng/ml with, and 6822 ng/ml without mesangial hypercellularity; difference -1155 ng/ml; 95% CI -1333 ng/ml to -219 ng/ml; p = 0.007, figure 60). A similar relationship was seen for biopsy evidence of tubular atrophy (median MASP-3 5503 ng/ml with, and 6154 ng/ml without tubular atrophy; difference of medians -652 ng/ml; 95% CI -866 to -55 ng/ml; p = 0.027, figure 60). Median plasma MAp19 levels were higher in patients with compared to without biopsy evidence of segmental sclerosis (mean MAp19 576 ng/ml with and 539 ng/ml without segmental sclerosis; difference in means 37 ng/ml; 95% CI 2 ng/ml to 73 ng/ml; p = 0.040).

Figure 60. Plasma MASP-3 and progressive IgA nephropathy

(A) Plasma MASP-3 levels in patients with stable or progressive IgAN compared to healthy controls. P values derived from Kruskal-Wallis test. (B) Plasma MASP-3 levels in patients with stable or progressive IgAN despite immunosuppressive therapy (IS). (C) Plasma MASP-3 plasma levels in IgAN patients without (solid circles) and with (empty cir les) mesangial hypercellularity (M). (D) Plasma MASP-3 plasma levels in IgAN patients without (solid circles) and with (empty circles) tubular atrophy. P-values derived from Mann-Whitney test. P-values shown have been adjusted for multiple analyses to minimise the false discovery rate. Line represent median interquartile range.



e. Low plasma MASP-3 and high MAp19 associated with histology evidence of complement activation in IgA nephropathy

As described in a previous Results chapter, 41 patients from the IgAN cohort had stored surplus renal biopsy tissue available for assessment of immunohistologic evidence of complement activation, 23 of whom had progressive and 18 had stable disease. Progressive IgAN associated with glomerular evidence of complement activation, specifically C3b/iC3b/C3c, C3dg, C4d and C5b9 deposition. I questioned whether the associations between plasma lectin complement pathway levels and IgAN severity would also associate with glomerular complement deposition. Specifically, I asked whether glomerular complement deposition associated with low MASP-3 and high MAp19 plasma levels in progressive and stable IgAN. I first compared the prevalence of glomerular complement staining in IgAN patients with MASP-3 levels below and above the lower cohort quartile and Map19 levels above and below the upper cohort quartile. I categorised antibody staining intensities of 1+, 2+ and 3+ as 'present' and 0 and 0.5+ as 'absent'. I saw non-significant trends towards more prevalent C3b/iC3b/C3c, C4d, and C5b9 in cases with MASP-3 levels less than the lower cohort quartile. I also saw a trend towards more C3b/iC3b/C3c, C3dg, C4d and C5b9 in patients with Map19 levels above the upper cohort quartile (Figure 61). I compared three cohorts of IgAN patients; (1) progressive IgAN and plasma MASP-3 or MAp19 levels associated with IgAN (less than the lower cohort quartile for MASP-3 and higher than the upper cohort quartile for MAp19), (2) progressive IgAN and plasma levels not associated with IgAN (more than the lower cohort quartile for MASP-3 and less than the upper cohort quartile for MAp19), and (3) stable IgAN and MASP-3 or MAp19 levels not associated with IgAN. Example images of the staining compared are shown in figure 62. I was unable to include a cohort of stable IgAN and MASP-3 or MAp19 levels associated with IgAN because of a lack of patients meeting these criteria. For both MASP-3 and MAp19, I demonstrated significant differences in the proportion of patients with positive glomerular C3b/iC3b/C3c, C3dg, C4d and C5b9 immunohistochemistry staining between the three cohorts (Figure 61). Glomerular C3b/iC3b/C3c deposition was significantly more common in progressive IgAN patients with MAp19 plasma levels above, compared to below, the cohort upper quartile (9 of 9 vs 6 of 11 patients, p=0.04, figure 61). I identified non-significant trends towards (1) more frequent C3b/iC3b/C3c and C4d glomerular deposition in progressive IgAN patients with MASP-3 plasma levels below the cohort lowest quartile, and (2) more frequent C3dg and C5b9 glomerular

deposition in progressive IgAN patients with MAp19 above the cohort upper quartile (Figure 61).

Figure 61. Low plasma MASP-3 and high MAp19 associated with glomerular complement deposition in IgA nephropathy

The percentage of patients with (a and e) C3b/iC3b/C3c, (b and f) C3dg, (c and g) C4d and (d and h) C5b9 positive (black) and negative (gray) glomerular IHC from renal biopsy tissue. (a to d) show IgAN patients with plasma MASP-3 less than the lower quartile cohort MASP-3 value (<25%) or more than the lower quartile cohort MASP-3 value (>25%). (e to h) show IgAN patients with plasma MAp19 greater to the upper quartile cohort MAp19 value (>75%) or lower than the upper cohort MAp19 value (<75%).

The percentage of patients with (I and m) C3b/iC3b/C3c, (j and n) C3dg, (k and o) C4d and (l and p) C5b9 positive (black) and negative (gray) glomerular IHC in stable (S) or progressive (P) IgAN. (i to l) show patients with plasma MASP-3 less than the lower quartile cohort MASP-3 value (<25%) or more than the lower quartile cohort MASP-3 value (>25%). (m to p) show patients with plasma MAp19 greater to the upper quartile cohort MAp19 value (>75%) or lower than the upper cohort MAp19 value (<75%).

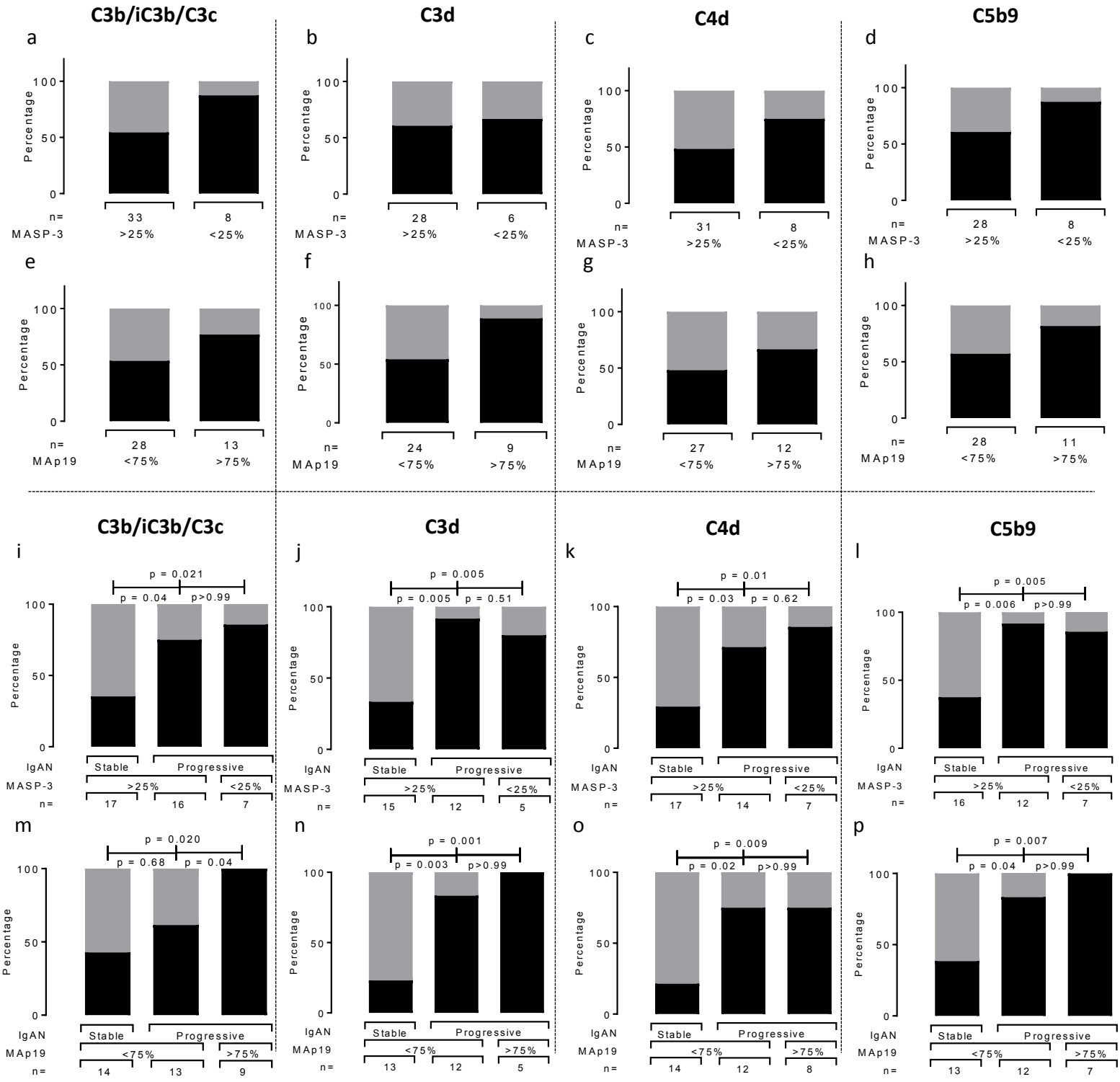
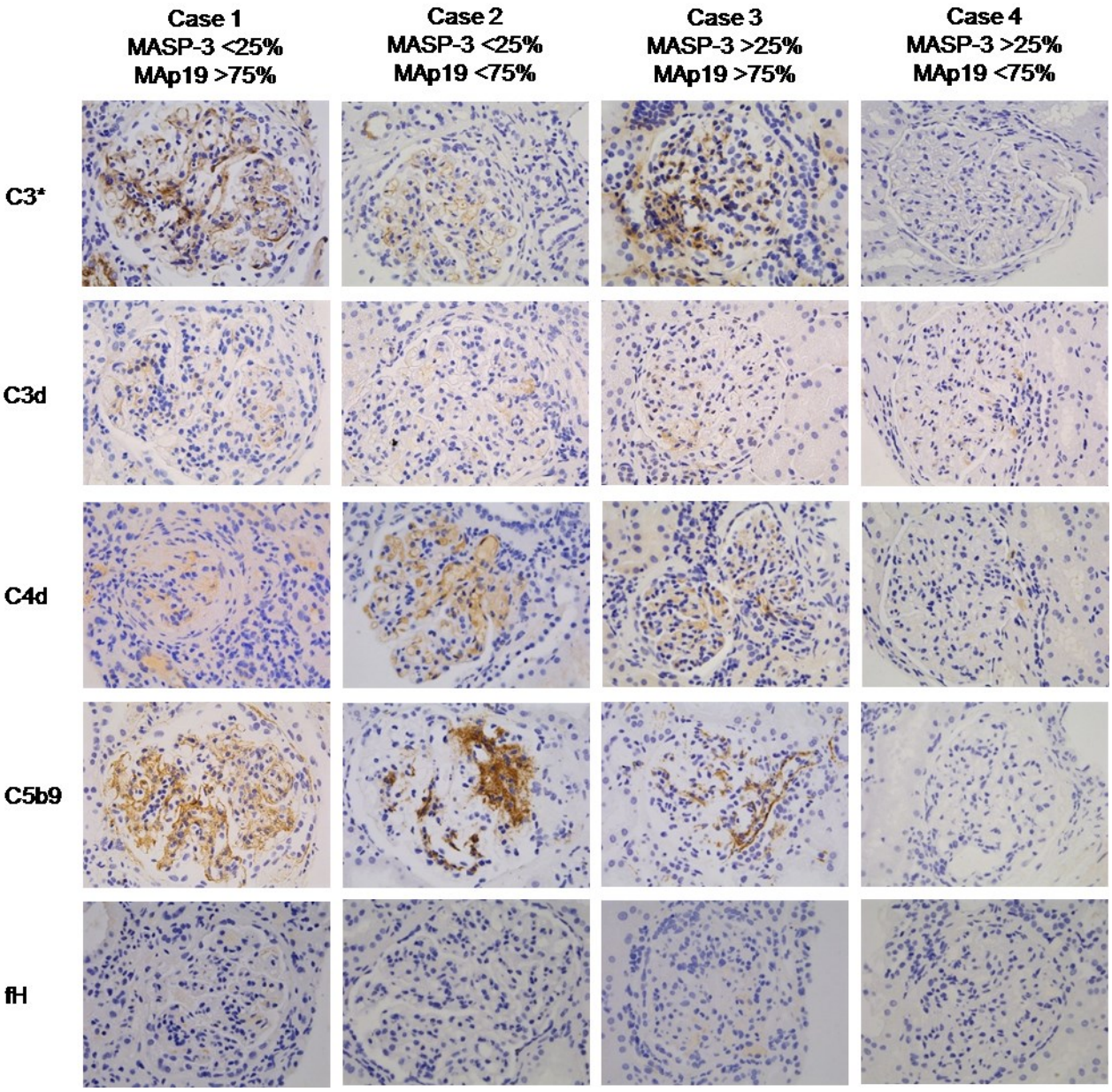


Figure 62. Glomerular complement deposition in renal biopsy tissue from patients with IgAN and low MASP-3 and high MaP19 circulating levels

Representative images of immunostaining for antigens C3*, C3dg, C4d, C5b9 and complement factor H (FH) in sections from the same cases (arranged in columns) from 4 different patients. Each case had a different combination of MASP-3 and MAp19 levels associated with IgAN. FH staining was rarely seen, but the sensitivity of our protocol was confirmed in FFPE healthy liver tissue. All images at 40x magnification.



Discussion

Despite the well established presence of gd-IgA1 containing immune complexes in IgAN[224], the mechanisms linking mesangial complex deposition to glomerular inflammation and injury are unclear. The immune complexes that define IgAN are characterised by an excess of exposed GalNac[327]. Lectin pathway PRMs can recognise GalNac[319, 328], potentially triggering complement activation. Therefore, the lectin complement pathway may link immune complex deposition and glomerular inflammation. However, the mechanism of lectin complement activation in IgAN has not been demonstrated. Consequently, lectin pathway components are currently neither disease biomarkers nor therapeutic targets in IgAN.

In this chapter, I described lectin complement pathway protein levels in a large cohort of IgAN patents. I first comprehensively profiled lectin pathway plasma levels in IgAN patients. To avoid the logistic difficulties of quantifying 11 proteins in all 323 IgAN patients and controls, I achieved this in two stages. I first measured all known lectin proteins in 125 IgAN patients and a healthy control cohort. Due to healthy control availability, the control cohort at this time was exclusively Caucasian individuals from Denmark. I then confirmed the results from this assessment in the remaining IgAN patients. By the time I undertook the second stage of the analysis, I had recruited a new control cohort with more similar demographics to the IgAN study population. However, I noted differences between our two control cohorts in a number of lectin markers. Subsequently, an association between low plasma MBL levels and IgAN identified during the screening assessment was not replicated when using the London control cohort for comparison, highlighting the importance of control cohort selection. Subsequently, I identified increased circulating M-ficolin, L-ficolin, MASP-1 and MAp19 levels in IgAN patients compared to the London healthy control cohort. Conversely MASP-3 levels were reduced in IgAN and associated with progressive disease, both in the whole cohort and following immunosuppression treatment. I also found low MASP-3 and high MAp19 plasma levels correlated with histology features of IgAN severity.

I noted correlations between some lectin component plasma levels and eGFR. Renal impairment partially explained the differences in M-ficolin and MAp19 levels since these levels changed significantly following renal transplantation. This, in addition to the small differences in measured lectin pathway protein levels between cohorts, makes the biological and pathogenic relevance of the results uncertain. However, two of our assessments suggest IgAN disease activity also independently contributes to lectin plasma levels. First, we found that IgAN patients with preserved eGFR show significant differences in plasma lectin levels compared to

healthy controls. Second, we looked at plasma changes in patients with IgAN and APCKD before and after transplantation, after which the eGFR shows dramatic improvement. MASP-3 serum levels did not show significant differences after transplantation, despite a marked change in eGFR.

M-ficolin is mainly synthesised in monocytes and granulocytes[329], but almost all of our patients had a normal white cell count. Although MASP-3 showed a positive correlation with ALT, we identified negative correlations with MASP-3 levels and IgAN severity. I therefore think it is unlikely that the M-ficolin and MASP-3 results are explained by confounding with leucocyte count and liver inflammation respectively. Furthermore, MBL[330], M-ficolin[331], L-ficolin[332], MASP-1[333], and MAp19[334] are not acute phase proteins. Circulating MASP-3 levels did not correlate with proteinuria, excluding this as a cause for the low levels. My research has not included genetic analyses of lectin pathway polymorphisms or mutations. However, the relative importance and effect of MASP-3 and MAp19 polymorphisms[45], and therefore how documenting their prevalence in our cohort would improve our analysis, is unclear.

In order to investigate whether circulating lectin protein variants might be pathogenic in complement dependent glomerular injury, I looked for associations with injurious glomerular complement activation. Because I used the same IgAN cohort as previous analyses, I was able to compare circulating lectin levels and glomerular evidence of complement activation and deposition in a subset of IgAN patients. I demonstrated trends towards more glomerular complement deposition in progressive IgAN patients with low plasma MASP-3 and high plasma MAp19, compared to progressive IgAN patients without these serological characteristics. However, there is a risk that multiple assessments have revealed biologically unimportant correlations. Furthermore, due to the small sample sizes and modest differences between them, the biological significance of these results, and how they relate to IgAN pathogenesis is unclear.

Increased MASP-1 and MAp19 levels have been associated with markers of SLE activity, specifically low serum C3 and high anti-DNA antibody titres[335]. Therefore, these changes are not specific to IgAN. Glomerular L-ficolin and MASP1/3 staining correlated with IgAN severity[252], but circulating L-ficolin levels did not[252]. In fact, reduced circulating L-ficolin levels have been reported in lupus nephritis[336], although this was not replicated in a Japanese cohort[337]. Reduced M-ficolin levels have also been found in lupus nephritis[338]. M-ficolin and L-ficolin are capable of triggering lectin pathway activation, and MASP-1 is essential for MASP-2 activation and lectin complement activity[317, 320, 32]. So higher levels of these three components could be associated with

greater complement activation within the kidney. The significance of the raised MAp19 is unclear. It has been shown to have a regulatory role on lectin pathway activation *in vitro*[339], but the physiological relevance of this is unknown[334].

Interestingly, similar MASP-3 and Map19 plasma changes to those we document in IgAN have been described in association with SLE activity[335]. Specifically, SLE patients with a history of nephritis showed lower MASP-3 plasma levels than patients with no nephritis[335], and SLE patients with low serum C3 levels and positive anti-dsDNA titres showed higher plasma levels of MAp19 than patients without these features[335]. The association of low MASP-3 with lupus nephritis shows this circulating lectin variant is also not specific to IgAN[335]. When MASP-3 binds to PRMs, it can displace MASP-2 and MASP-1[340] and, because it does not lead to convertase formation[341], inhibit activation[324, 340]. Low levels of MASP-3 could be associated with increased complement activation. However, MASP-3 also activates pro-factor D to factor D[342], a requirement for C3-convertase formation[343]. Whether or not glomerular activation of the alternative pathway in either lupus nephritis or IgAN directly influences MASP-3 levels is unknown.

My findings demand further investigation of the mechanistic role of the lectin pathway, and MASP-3 and MAp19 in particular, in IgAN pathogenesis. In particular, it is important to document whether MASP-3, MAp19 and at least one of the lectin PRMs can be identified in IgAN renal tissue and whether they co-localise with each other and other complement pathway components. We have shown reduced MASP-3 and increased MAp19 plasma levels are biomarkers for disease severity and complement glomerular deposition in IgAN. My findings are consistent with a role for lectin pathway activation in IgAN pathogenesis. Modulation of the lectin complement pathway, and particularly MASP-3, may have future therapeutic and diagnostic potential in IgAN.

Vi. Conclusions

- IgAN is associated with higher circulating levels of M-ficolin, L-ficolin, MASP-1 and MAp19 than healthy controls.
- IgAN is associated with lower plasma MASP-3 levels.
- Lower MASP-3 levels also associate with markers of IgAN severity.

- The associations with reduced circulating MASP-3 levels do not seem to be secondary to non-specific renal impairment.
- Low circulating MASP-3 and high circulating MAp19 levels seem to associate with glomerular deposisition of markers of complement activation.

Discussion

I introduced my research with the background data that motivated my work. I described the potency and delicate balance of the complement cascade. I highlighted the potential for inherited and acquired variants to disrupt complement regulation and contribute to a range of pathologies, especially glomerular diseases. This led to my review of the FHR proteins and the literature that implicates them as important in complement deregulation. I focused on *in vitro* data that FHR1[9] and FHR5[8] competed with FH for binding to activated C3 but, unlike FH, interaction of C3b with either FHR1 or FHR5 allowed continued complement activation and prevented the inhibitory actions of FH. I next outlined our understanding of IgAN and C3G. I argued that complement activity associates with the development and severity of IgAN and C3G, and that the pathogenesis of both is incompletely understood. Interestingly, insight into the potential pathogenicity of FHR1 and FHR5 was gained from studies of IgAN and C3G. The del*CFHR3-1* allele associates with protection from IgAN; CFHR5 nephropathy shares phenotypic similarities with IgAN; and abnormal FHR proteins, including the mutant FHR5 that defines CFHR5 nephropathy, associate with familial C3G.[5-7, 122, 138]. I speculated that these observations and associations could be explained by FH deregulation; decreased or absent FHR1 and FHR5 levels could result in reduced or absent FH deregulation and consequently less complement-mediated renal injury. Conversely, increased FHR1 and FHR5 levels, or the presence of gain-of-function variants, could increase FH deregulation and complement-mediated renal injury.

On this background, I hypothesised FHR1 and FHR5 levels and evidence of lectin and alternative pathway activation would associate with IgAN and C3G development and severity. I aimed to identify associations between (1) the amount of circulating and glomerular FHR1 and FHR5 and IgAN, (2) markers of alternative and lectin complement activity and IgAN, and (3) glomerular FHR1 and FHR5 deposition and markers of glomerular complement activation in C3G. I hoped these would improve our understanding of complement dependent glomerular injury in IgAN and C3G and the pathogenicity of the FHR proteins.

I established IgAN and C3G cohorts that were reassuringly representative of other IgAN and C3G cohorts, but that also provided unique disease cohort characteristics. Applying novel serology and immunohistochemistry assessments to patient samples revealed a number of important results. I first demonstrated circulating FHR1 levels and the ratio of FHR1 to FH were increased in patients with IgAN compared to healthy controls and in

patients with progressive compared to stable IgAN. I found IgAN patients had higher circulating FHR5 levels than healthy controls and increased circulating FHR5 levels associated with histology markers of IgAN severity. However, differences in circulating concentrations of FHR1 and, in particular, FHR5, were modest and the biological effect of micromolar and nanomolar differences in circulating concentrations is difficult to conceptualise. Furthermore, circulating FHR1 levels correlated closely with non-specific changes in eGFR and I detected no difference in allelic frequency of the delCFHR3-1 in IgAN patients and healthy controls. Therefore, circulating FHR1 and FHR5 levels may not be pathogenically or biologically important. I therefore looked for local, glomerular evidence of FHR5 and FHR1 dependent FH deregulation through immunostaining analysis of surplus stored diagnostic renal biopsy tissue. Glomerular FHR5 deposition associated with both clinical histologic markers of IgAN severity and IHC markers of complement activation. This demonstrated a trend of similar associations between serological FHR5 and IgAN, and glomerular FHR5 and IgAN. The glomerular absence of FH also associated with IgAN. Unlike circulating FHR1 levels, I did not detect associations between glomerular FHR1 and IgAN.

With the aim of determining whether FHR5 glomerular deposition might be pathogenic in glomerular complement activation, I looked for evidence of glomerular FHR5 in C3G, a rare glomerulopathy caused by inappropriate and deregulated complement activity. Similar to my findings in IgAN, glomerular FHR5 deposition associated with clinical and histology markers of C3G severity and co-localised with glomerular C3 deposits.

I next tried to identify evidence of the mechanisms by which FHR5 might contribute to glomerular complement activation and deregulation. I applied novel double IF techniques to renal biopsy tissue from cases of C3G. I demonstrated glomerular FHR5 deposition co-localised with markers of both ongoing (C3b/iC3b/C3c) and previous (C3dg) alternative complement activation in C3G. This was replicated in IgAN. Additionally, I found glomerular FHR5 deposition co-localised with IgA in IgAN. Importantly, progressive IgAN associated with more co-localisation of IgA with FHR5 and C3b/iC3b/C3c with FHR5. Together, these findings suggest (1) FHR5 is important in the pathogenesis of complement dependent glomerular injury in general and, (2) in IgAN, FHR5 influences the severity of glomerular injury in response to glomerular deposited IgA and C3.

I next investigated how FHR5 might co-localise with glomerular C3b/iC3b/C3c and IgA. Specifically, I questioned whether FHR5, C3b/iC3b/C3c and IgA congregated following glomerular deposition of IgA1-

containing immune complexes, or were C3b/iC3b/C3c and FHR5 detectable with IgA1-containing immune complexes in circulation? In this scenario, the presence of FHR5 and C3b/iC3b/C3c might amplify the complement activity and inflammation that occurs in response to IgA1-IC glomerular deposition, thereby driving disease severity. I detected FHR5 in IgA1-containing immune complexes purified from both IgAN patients and healthy controls and the detection of FHR5 was dependent on C3 in addition to IgA. The presence of FHR5 in dimeric and polymeric IgA1 fractions might associate with IgAN. However, the data from analysis of purified IgA1 was preliminary in nature and requires clarification and further investigation. It will be important to determine the precise composition of and complement proteins detected in IgA1 fractions of different complex sizes. In particular, the fractions of different sizes may not consist of monomeric, dimeric and polymeric IgA1 but may instead consist of many other complexed proteins, including complement proteins. It may be the identity and quantity of non-IgA1 molecules, such as complement proteins, in IgA1-containing complexes determines the pathogenicity of circulating IgA1.

I also investigated how lectin complement pathway activity might contribute to IAN pathogenesis. I replicated the association between C4d glomerular deposition and IgAN severity identified in other IgAN cohorts[253]. I examined variations in circulating lectin pathway protein levels and found that IgAN associated with higher circulating M-ficolin, L-ficolin, MASP-1 and Map19 levels and lower levels of MASP-3. Interestingly, lower circulating MASP-3 levels was the single protein to associate with markers of IgAN severity.

In summary, I identified (1) associations between circulating FHR1, FHR5 and lectin complement protein levels and IgAN development and severity, (2) associations between glomerular complement activation, FHR5 deposition and markers of IgAN and C3G severity, and (3) evidence that FHR5 colocalises with glomerular complement activation and IgA and circulating IgA1-containing immune complexes in progressive IgAN, suggesting mechanisms by which FHR5 may amplify glomerular complement activation and inflammation in response to deposited IgA and complement activation in IgAN and C3G.

I discussed a number of the limitations to my research in the relevant results chapter. In general, my data is limited by differences in timing between diagnostic renal biopsy and study sample collection. I attempted to limit this by analysing plasma antigens shown to be stable over time. Because the long duration of patient follow-up adds certainty to our classification of patients as having stable disease, avoiding this limitation would require a prospective study with significant follow-up or identifying a large cohort with long follow-up and

appropriately stored plasma samples. Multi-centre collaboration through the International IgA Nephropathy Network may facilitate the latter of these.

My immunostaining analyses were limited by the availability of stored surplus biopsy tissue. This was also relevant for control samples; tissue from 'normal' renal transplant biopsy tissue was not readily available. Finally, our definitions of progressive and stable disease inevitably excluded some IgAN patients, especially if adequate information for categorisation was unavailable. My research aim was to understand how complement determines glomerular inflammation in IgAN. I therefore selectively compared IgAN patients with or without features of inflammation. Specifically, I designed criteria to identify patients with immunologically active disease, including those with histological activity but relatively short follow-up and preserved eGFR, while excluding patients with additional pathologies causing renal impairment in the absence of active IgAN.

Many of the associations and differences I detected were modest in magnitude. This may reflect the nature of the samples I studies, that were derived from patients and therefore influenced by infinite unquantifiable factors. The detection of significant associations with circulating and glomerular deposits of FHR5, for example, in such samples could be considered impressive and of probable pathogenic importance. However, the small differences could also be due to FHR5, FHR1 and MASP-3 having only modest biological and pathogenic effects. This uncertainty necessitates further assessment in prospective studies of larger patient cohorts, and elucidating the mechanisms through which FHR5, FHR1 and MASP-3 might drive complement deregulation, glomerular inflammation and disease severity.

My research suggests circulating FHR5, FHR1, and MASP-3 may be useful biomarkers of glomerular inflammation and progressive renal deterioration. I did not aim to assess and develop biomarkers. Consequently, my research cannot make conclusions about biomarker sensitivity and specificity in IgAN. However, the associations I described could lead to novel biomarker development, assessment and translation to clinical practice. This would be important for IgAN because it is relatively common, leads to a poorly understood range of clinical outcomes, and we currently have few tools to routinely, reliably and specifically identify patients who may respond to immunosuppression treatment. Complement biomarkers may be particularly instructive in identifying patients for future therapeutic complement inhibition.

Compared with FHR5, circulating FHR1 seemed to more clearly correlate and separate with IgAN severity. FHR1 also correlated with eGFR; circulating FHR1 levels were higher with reduced eGFR and renal function. My findings were replicated in a large cohort of IgAN patients, polycystic kidney disease patients and healthy controls from Madrid, Spain[254]. I found circulating FHR5 levels were higher in IgAN than healthy controls. The difference between the cohorts was small. However, FHR5 showed no significant correlation with eGFR and associated with histology markers of IgAN activity. Our FHR5 findings were replicated in a large cohort of IgAN patients from Beijing, China. Interestingly, I identified associations between IgAN progression and glomerular deposition of FHR5 but not glomerular FHR1. This may be because FHR5 is important for glomerular complement activation and inflammation, while higher circulating FHR1 levels are exaggerated by non-specific renal impairment.

Although a novel and exciting finding, predicting how and why reduced MASP-3 levels associate with IgAN severity is difficult because the the physiological roles of MASP-3 are unclear. Although MASP-3 binds lectin PRMs, it cannot cleave complement convertases[341], has been shown to displace MASP-2 and MASP-1 from PRM complexes, and inhibits lectin pathway activation when added to MBL-MASP complexes[324, 340]. Low levels of MASP-3 could be associated with a greater proportion of PRM in complexes with MASP-2 and MASP-1, with subsequent increased complement activation. However, MASP-3 is also able to activate pro-factor D to factor D[342], an essential co-factor that cleaves factor B during alternative complement pathway convertase formation[343]. Although the importance of this in disease states is unclear, in this scenario lower plasma MASP-3 would be predicted to reduce alternative complement pathway activation.

My current analysis was based on the assumption that FHR5 and MASP-3 levels were stable over time. And I have not analysed the relationship between FHR1 and histological changes in the renal biopsy because FHR1 levels were influenced by eGFR and the diagnostic renal biopsy was not coincidental with the timing of the study blood sample in our cohort. Testing the hypotheses formed from my serology complement findings will likely necessitate analysis of sequential samples from a range of IgAN patients. This should be completed before assessing the use of FHR5, FHR1 and MASP-3 as clinical biomarkers. I predict it will help me understand how FHR5, FHR1 and MASP-3 are affected by clinical events such as episodes of infection, disease flares, treatment response and transplant recurrence. Analysis of sequential serology samples coincidental with

repeat renal biopsies would also untangle the contribution of circulating levels to histological changes and the influence of non-specific glomerular filtration impairment and CKD on circulating levels.

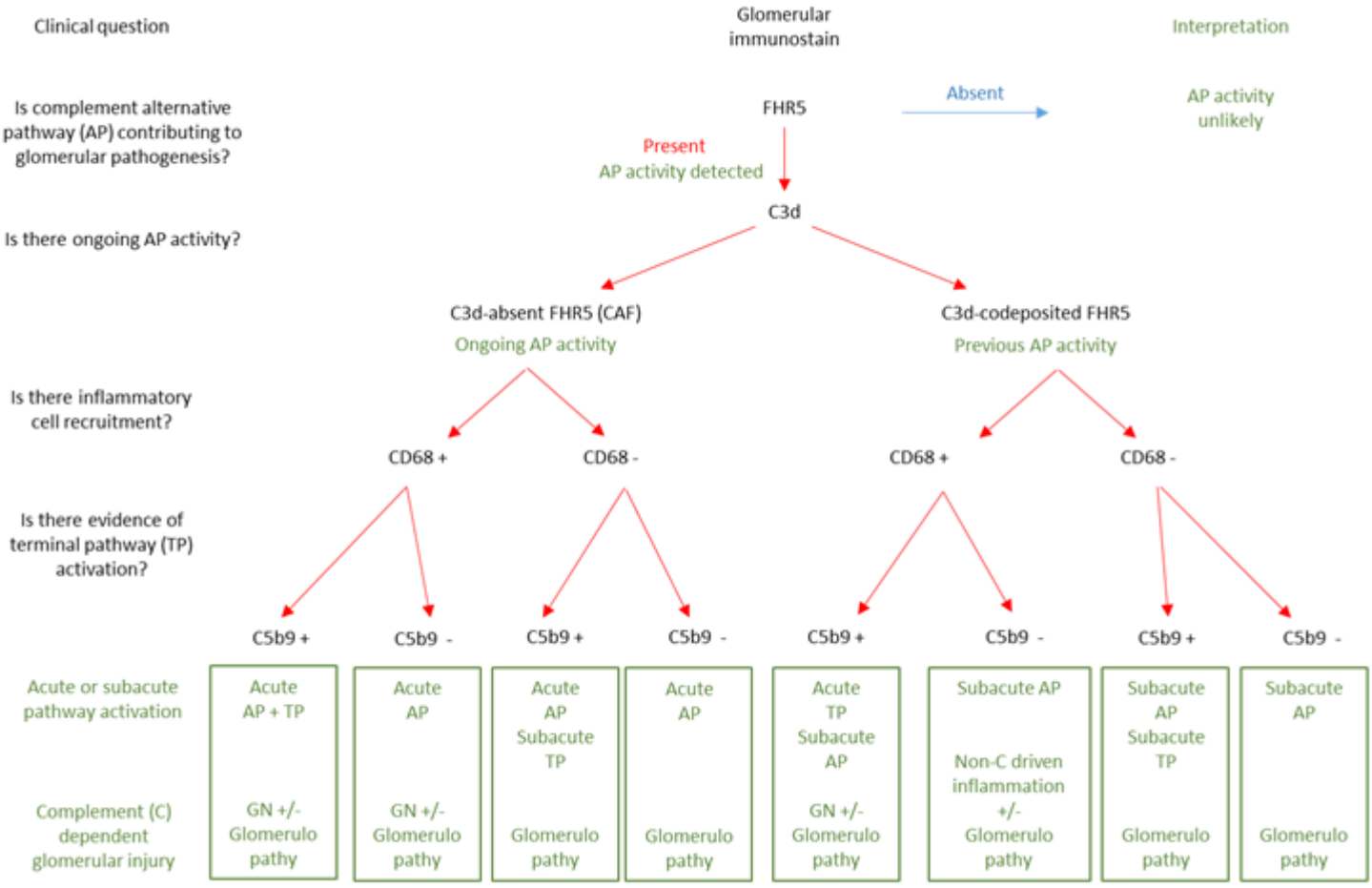
My research indicates glomerular FHR5 deposition, as detected with routine IHC and IF, identifies IgAN and C3G patients with active complement activation and severe disease at risk of clinical progression to ESRD. Reliable immunohistochemistry markers of disease progression are currently not validated for either condition. We are currently unable to reliably and appropriately differentiate patients most likely to improve with immunosuppression. IgAN and C3G patients are therefore at risk of either needless exposure to immunosuppression side-effects or not receiving potentially effective treatments. FHR5 could have dual utilities as an immunostaining marker of disease activity. First, the identification of FHR5 colocalised with C3b/iC3b/C3c could highlight cases of IgAN and C3G with ongoing glomerular inflammation most likely to respond to non-targeted immunosuppression. Second, FHR5 could identify cases likely to benefit from one of the emerging specific complement pathway inhibitors. FHR5 IF could be particularly useful when combined with C3dg to identify C3dg-absent FHR5. I demonstrated FHR5 co-localises with both C3b/iC3b/C3c and C3dg, a marker of previous alternative pathway complement activation. Double IF for C3dg and FHR5 will identify previous (C3dg and FHR5 co-localised) and ongoing (FHR5 with no C3dg, or C3dg-absent FHR5) alternative complement pathway activity. This alternative complement pathway activity could be triggered by classical, lectin or alternative complement pathways, or could result from alternative pathway convertase-dependent complement amplification. Determining the origin of C3 would need the presence of immunoglobulins, C1q, and C4d to be determined. Regardless of the trigger, the presence of C3dg-absent FHR5 would indicate alternative pathway activity is contributing to ongoing pathology. This could be used to identify patients for one of the emerging therapeutic agents that target alternative complement pathway activation, such as factor B or factor D inhibitors. The identification of C3dg with co-localised FHR5 would suggest complement amplification and alternative pathway activity has stopped, which could be a marker of successful therapeutic complement inhibition or defer the use of complement inhibitors.

The biopsy could also be stained for C5b9, the marker of terminal pathway activation (Figure 63). Given the documented persistence of C5b9 in glomeruli following the cessation of lupus nephritis activity[294], I suggest a CD68 biopsy stain should also be performed. This would indicate whether the complement activation is associated with macrophage recruitment and infiltration. If CD68 positive cells are identified, it suggests the

presence of acute complement activation and potential significant benefit from inhibiting complement. The relative abundance of alternative and terminal complement pathway activation could direct which complement inhibitor, which targets either the alternative or terminal complement pathway, is used. This needs prospective assessment and validation. However, it demonstrates my research has the potential to be translated to clinical practice and improve the diagnosis and management of complement-dependent glomerular diseases.

Figure 63. Theoretical application of complement C3dg-negative FHR5 to clinical renal biopsies

AP – alternative pathway; TP – terminal pathway; C3d – detected component of C3dg



Although documented in a small cohort, my results suggest glomerular FHR5 may be identifiable in transplant IgAN recurrence before clinical and immunohistochemic evidence of IgAN recurrence or glomerular injury. This could be important if complement inhibitors are used for IgAN recurrence because they could be introduced at the onset of recurrence before glomerular damage occurs. Demonstrating this utility requires replication of my findings in larger transplant patient cohorts and comparison with transplant recipients with other primary renal conditions. Similar to the assessment of circulating FHR1 and FHR5 levels, I need to better understand how glomerular FHR5 changes with clinical events. This could be achieved with the analysis of serial renal biopsies. Similar analysis of a single C3G patient treated with eculizumab provided *in vivo* evidence of the role of the alternative complement pathway in C3G pathogenesis. However, repeat renal biopsies are indicated less commonly in IgAN. To this end, Professor Terry Cook and I have suggested a project for the complement working group of the International IgAN network to coordinate the analysis of FHR5 deposits in repeat renal biopsies from IgAN patients. It will also be important to investigate and confirm whether tubular FHR5 deposition correlates with disease activity, chronicity, and non-specific glomerular changes. This could be pertinent to the consideration of urinary FHR5 as a biomarker of complement activity in C3G and IgAN.

The number of associations identified between glomerular FHR5, IgAN and C3G suggests FHR5 may directly influence glomerular complement activity, inflammation and damage in these conditions. FHR5 may therefore be a novel therapeutic target in IgAN and C3G. In my opinion, the development of novel therapies directed at FHR5 necessitates an understanding of the mechanisms linking FHR5 and glomerular complement activation in IgAN and C3G. Consequently, my next research goals are to try to describe mechanisms by which FHR5 influences disease development. Based on a host of *in vitro* data, it is likely FHR5 amplifies and deregulates alternative complement pathway activation by one of four mechanisms. (1) FHR5 dimers have been shown to bind with C3 fragments and compete with FH to deregulate complement activation *in vitro*; FHR5 may bind to C3b in the alternative complement pathway convertase and prevent the interaction and regulatory functions of FH. (2) After binding C3 proteolytic fragments such as iC3b and C3dg, which would not participate further in complement activation, FHR5 dimers could bind other C3 and C3b proteins, allowing the formation of C3 convertase and complement amplification. Thereby, FHR5 would convert surface-bound complement fragments that would lead to tolerant immune sequelae to complement fragments that cascade to

complement activation and amplification. (3) There is also evidence that FHR5 could bind to non-complement ligands and scaffold *de novo* C3 activation and convertase formation. A number of the non-complement proteins that FHR5 dimers bind *in vitro,* in particular the laminins, are found in glomerular extracellular matrix. (4) Finally, FHR5 may bind C3 fragments adherent to other structures, such as immune complexes. My demonstration of FHR5 in circulating IgA1-containing immune complexes could be an exciting and important insight that links circulating FHR proteins with glomerular IgA deposition, inflammation and injury. The presence of C3 fragment-bound FHR5 could both increase the inflammatory response to immune complexes and impair the physiologic phagocytosis of deposited and circulating immune complexes, thus leading to a relative abundance of these pro-inflammatory proteins. Investigating the mechanisms of FHR5-dependent complement activation may also help explain why glomeruli seem relatively susceptible to complement-mediated pathology. It is currently not known which mechanisms contribute to complement activation *in vivo*. However, in all scenarios, therapeutic inhibition of FHR5 would be predicted to reduce complement activation and inflammation. My research could therefore be translated to the development of novel therapeutic strategies for IgAN and other complement-dependent glomerulopathies.

Future work

My novel and exciting results implicate FHR5, and potentially FHR1 and MASP-3, in glomerular complement activation and IgAN pathogenesis. However, I have not yet demonstrated mechanisms that drive these associations. This makes the robustness and relevance of my findings uncertain. I next aim to identify these mechanisms. This would facilitate my pursuit of subsequent research fellowships and my overall aim of developing pathogenesis-targeted and disease modifying therapies for IgAN.

There are six parts to my future research plans. First, I hope to investigate how FHR5 deposition correlates with clinical events and morphology changes in IgAN and C3G. The comparison of FHR5 deposition in serial biopsies following disease progression, treatment, remission and recurrence will better indicate the pathogenicity of FHR5 and its potential utility as a biomarker. Furthermore, comparison with C3b/iC3b/C3c deposition and deposit location may be informative. I also aim to re-assess associations in more specific IgAN subtypes, including dialysis patients, and only patients with non-ESRD CKD.

Second, I want to better characterise the associations between circulating IgA1-bound FHR5 and IgAN. It will be essential to characterise purified IgA1 more precisely, especially how the fractions of different complex size differ in IgA1, IgG and complement protein content. Although I identified FHR5 by western blot in circulating IgA1-containing immune complexes purified from IgAN patient serum, I have not established whether FHR5 binding to IgA1 complexes differentiates IgAN patients from controls, or clinically severe from stable IgAN. I therefore plan to extend these experiments to other patients in our IgAN cohort. I also aim to coorborate my findings by detecting FHR5 in IgA1-containing immune complexes by another method, such as mass spectrometry.

Third, I want to investigate human mesangial cell responses to FHR5 and IgA1-containing immune complexes *in vitro*. I have cultured and stored primary human mesangial cells and will measure cytokine production and cell proliferation after incubation with patient and control-derived IgA1-containing immune complexes, complement proteins, FHR5 and FH, the main alternative complement pathway regulator.

In addition to amplifying complement activation and glomerular injury in response to IgA1, FHR5 might interfere with the phagocytosis and elimination of IgA1-immune complexes, particular in glomeruli. The fourth

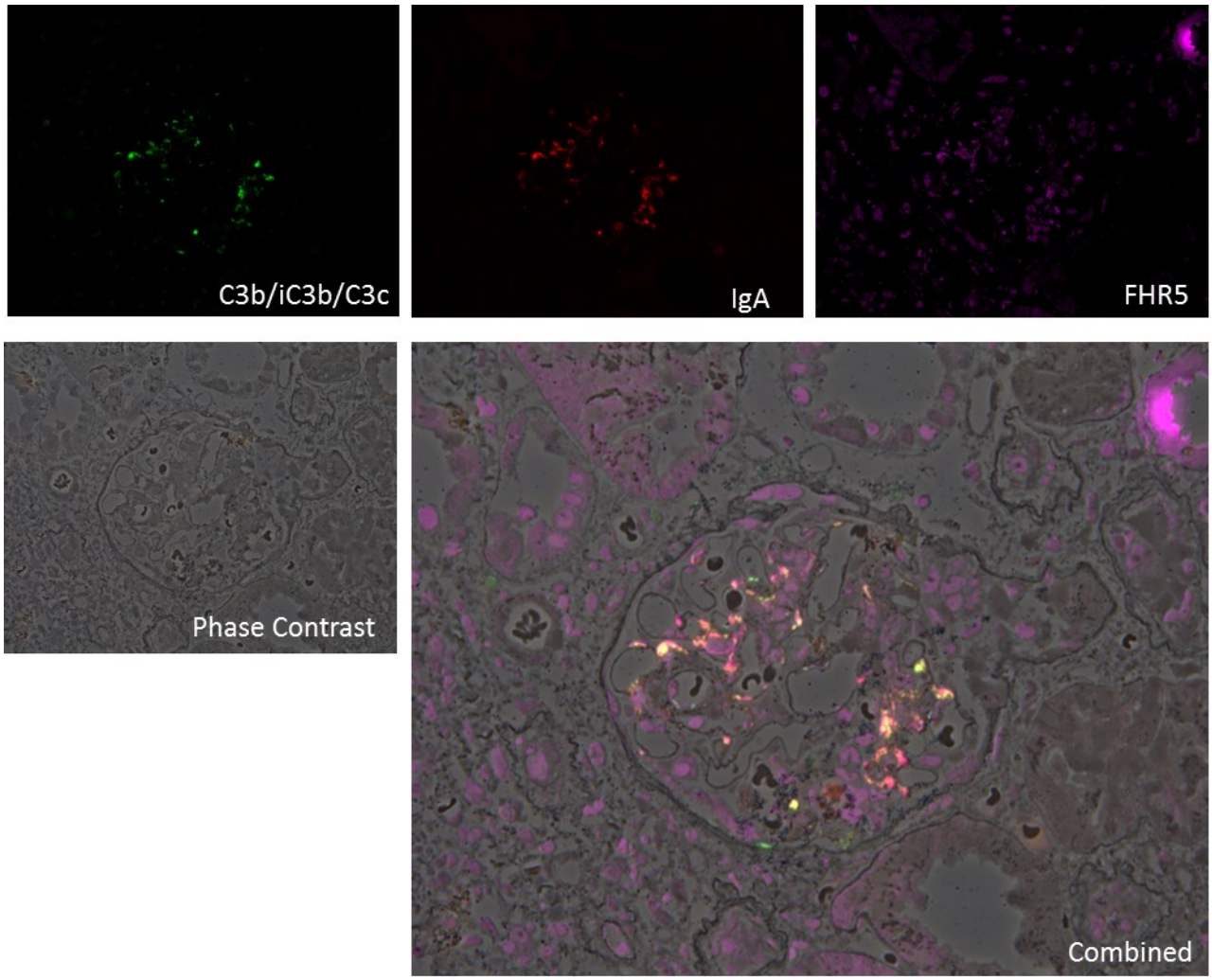
part of my research would use commercially available phagocytosis assays to investigate whether FHR5 influences the elimination of IgA1-containing immune complexes *in vitro*.

Fifth, I want to investigate further the role of the lectin complement pathway in IgAN. In particular, I want to test the relationship between reduced MASP-3 levels and IgAN activity. Following the development of IHC and IF protocols for lectin complement pathway proteins, I aim to identify whether reduced circulating MASP-3 levels are reflected in renal MASP-3 deposition. I also aim to identify the PRM associated with glomerular MASP-3 deposition.

Finally, I want to develop triple IF techniques for FFPE tissue. Hilary (Moffitt) McPhail and I have developed a triple IF protocol for IgA, C3b/iC3b/C3c and FHR5 and have applied it to a case of IgAN as a proof-of principle experiment (Figure 64). These techniques have the potential to identify *in vivo* evidence of proposed pathogenic mechanisms. In particular, it will help demonstrate whether C3b/iC3b/C3c deposition, as a marker of ongoing alternative complement pathway activity, predominantly co-localises with deposited IgA1 and FHR5, or whether complement activity is independent of IgA1 deposition. Furthermore, the use of FFPE tissue with preserved glomerular architecture may demonstrate localisation of complement activity to a particular compartment, such as the mesangium. I am very interested to compare patterns and localities of complement immunostaining in IgAN cases of different severities. I would also like to develop a staining protocol for FHR5, C3b/iC3b/C3c and C3dg and for FHR5, C3b/iC3b/C3c and properdin. These two protocols could indicate how FHR5 co-localises with markers of ongoing and previous alternative complement pathway activity, with relevance to other glomerulopathies characterised by glomerular C3 deposition.

Research I develop could have significant impact on our understanding of IgAN, C3G and other complement dependent kidney diseases and could be translated to provide novel investigations and therapies to improve patient care. My research could establish FHR5, and other complement pathway components, as pathogenesis-specific therapeutic targets for IgAN. Complement pathway inhibitors are undergoing clinical trial in C3 glomerulopathy and ANCA-associated vasculitis. The use of eculizumab, an inhibitor of the terminal complement pathway, has been reported in three cases of IgAN with variable success.[9-11] The identification of mechanisms linking complement activity and IgAN severity in most, some, or no IgAN patients, would inform whether and which IgAN patients should receive specific complement inhibitors in the future.

Figure 64. Example image of triple immnunofluorescence for complement C3b/iC3b/C3c, IgA and FHR5 in IgAN



Conclusions

My research has identified novel associations between FH deregulation by FHR1 and FHR5 and lectin complement pathway activity and IgAN. I have explored these associations to reveal FHR5 as a likely pathogenic protein that links alternative complement pathway deregulation with glomerular complement activation, inflammation and disease severity in IgAN and C3G. My research directly addresses fundamental and significant gaps in our understanding of glomerular complement activation and IgAN pathogenesis. However, my results are confounded by significant limitations common to research on patient-derived samples that make the pathogenic importance of FHR5 in IgAN impossible to conclude. Therefore, it is essential to validate and confirm the findings described in this thesis and determine the mechanisms of FHR5 in complement deregulation and IgAN pathogenesis. If this can be done, my research could be translated to novel and exciting diagnostic tools and therapeutic targets for IgAN, C3G and other complement dependent glomerulopathies.

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Appendix

Publications resulting from work included in the thesis (see below for copies of the manuscripts).

1. Medjeral-Thomas NR, Moffitt H, Lomax-Browne HJ, Constantinou N, Cairns T, Cook HT, Pickering MC. Glomerular Complement Factor H-related Protein 5 (FHR5) Is Highly Prevalent in C3 Glomerulopathy and Associated With Renal Impairment. Kidney Int Rep. 2019:4:1387-1400.

2. Medjeral-Thomas NR, Troldborg A, Constantinou N, Lomax-Browne HJ, Hansen AG, Willicombe M, Pusey CD, Cook HT, Thiel S, Pickering MC. Progressive IgA Nephropathy Is Associated With Low Circulating Mannan-Binding Lectin–Associated Serine Protease-3 (MASP-3) and Increased Glomerular Factor H–Related Protein-5 (FHR5) Deposition. Kidney Int Rep. 2018:3:426-438.

3. Medjeral-Thomas NR, Lomax-Browne HJ, Beckwith H, Willicombe M, McLean AG, Brookes P, Pusey CD, Falchi M, Cook HT, Pickering MC. Circulating complement factor H-related proteins 1 and 5 correlate with disease activity in IgA nephropathy. Kidney Int. 2017:92(4):942-952.

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Abstracts of work included in this thesis presented at international conferences.

1. Medjeral-Thomas NR, Pickering MC, Cook TH. Factor H related protein 5 (FHR5) co-localises with glomerular IgA and C3 and circulating IgA in IgA nephropathy. 15th International Symposium on IgA Nephropathy 2018.

2. Medjeral-Thomas NR, Constantinou N, *et al*. Glomerular FHR5 Associates with Severity in IgA Nephropathy. J Am Soc Nephrol. 2017:28:128. American Society of Nephrology Kidney Week 2017.

3. Medjeral-Thomas N, Troldborg A, Lomax-Browne H, *et al*. Plasma levels of lectin pathway components in IgA nephropathy. Mol Immun. 2017:89:178. European Meeting on Complement in Human Disease 2017.

Prizes for work included in this thesis.

1. Medjeral-Thomas NR, Pickering MC, Cook TH. Factor H related protein 5 (FHR5) co-localises with glomerular IgA and C3 and circulating IgA in IgA nephropathy. Best abstract at the 15th International Symposium on IgA Nephropathy.
2. Medjeral-Thomas NR, Pickering MC, Cook TH. Factor H related protein 5 and IgA nephropathy. Best abstract at the Kidney Research UK Fellows Day 2018.

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CLINICAL RESEARCH

Glomerular Complement Factor H–Related Protein 5 (FHR5) Is Highly Prevalent in C3 Glomerulopathy and Associated With Renal Impairment

Nicholas R. Medjeral-Thomas[1], Hilary Moffitt[1], Hannah J. Lomax-Browne[1], Nicholas Constantinou[1], Tom Cairns[2], H. Terence Cook[1] and Matthew C. Pickering[1]

[1]Centre for Inflammatory Disease, Division of Immunology and Inflammation, Department of Medicine, Imperial College London, UK; and [2]Renal and Transplant Centre, Imperial College Healthcare NHS Trust, UK

Introduction: Therapeutic agents that target complement are increasingly available for glomerular diseases. However, the mechanisms linking glomerular complement deposition with inflammation and damage are incompletely understood. Complement factor H–related protein 5 (FHR5) interacts with complement C3 and is considered to promote activation. Circulating and glomerular FHR5 associates with IgA nephropathy and abnormal FHR5 associates with familial C3 glomerulopathy (C3G). We characterized glomerular FHR5 staining in C3G and assessed its relationships with histological features of glomerular injury and clinical outcome.

Methods: We developed FHR5 staining protocols for formalin-fixed paraffin-embedded (FFPE) renal tissue and applied them to surplus biopsy sections from a C3G cohort.

Results: Glomerular FHR5 was highly prevalent in native and transplant C3G and correlated with glomerular C3 and C5b-9 staining. Glomerular FHR5 staining correlated negatively with estimated glomerular filtration rate (eGFR) ($P = 0.04$, difference of medians 19.7 ml/min per 1.73 m^2; 95% confidence interval [CI] 1.1–43.0) and positively with a membranoproliferative glomerulonephritis pattern at diagnostic biopsy (odds ratio 18; 95% CI 1.6–201; $P = 0.049$). Glomerular FHR5 staining intensity positively correlated with glomerular complement C3b/iC3b/C3c (Pearson's correlation coefficient [R] = 0.59; $P = 0.0008$), C3dg ($R = 0.47$; $P = 0.02$) and C5b9 ($R = 0.44$, $P = 0.02$).

Conclusions: Glomerular FHR5 is highly prevalent in C3G, interacts with glomerular C3, and is associated with markers of disease severity. Glomerular FHR5 likely exacerbates complement-mediated glomerular damage in C3G and its interaction with glomerular complement might be exploited to target complement therapeutic agents.

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See Commentary on Page 1359

Complement dysregulation is associated with renal disease, which includes atypical hemolytic uremic syndrome and C3G.[1,2] Drugs that target specific complement proteins and pathways have emerged as therapeutic options for complement-associated renal diseases.[3] Complement C5 inhibition is an effective therapy for atypical hemolytic uremic syndrome, a condition characterized by complement-mediated glomerular thrombosis.[2] However, the mechanisms through which complement dysregulation contribute to glomerular inflammation are incompletely understood. This limits our ability to select patients for specific complement inhibitors and monitor disease activity. For example, the response of C3G to complement C5 inhibition is heterogeneous.[2]

C3G is associated with significant morbidity. Up to 50% of C3G patients progress to end-stage renal disease within 5 years of diagnosis,[4,5] and recurrent C3G frequently causes transplant dysfunction.[6,7] Abnormal regulation of complement alternative pathway activation is seen in C3G.[8–10] This can be the result of genetic and acquired factors. However, in many cases the cause

Correspondence: Matthew C. Pickering, Centre for Inflammatory Disease, Imperial College London, Hammersmith Campus, London W12 0NN, UK. E-mail: matthew.pickering@imperial.ac.uk
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is unknown. The key negative regulator of C3 activation through the alternative pathway is complement factor H (fH). The fH protein family includes the 5 fH-related proteins (FHR1–5), named because of their structural similarity to fH.[11] Because they lack the complement-regulatory domains of fH but retain C3-binding domains,[12] FHR proteins are currently thought to act as positive regulators that promote complement activity. A heterozygous mutation in FHR5 is associated with a familial form of C3G, known as CFHR5 nephropathy.[13] A number of other FHR protein variants have been identified in association with cases of C3G.[14–18]

C3G includes dense deposit disease (DDD) and C3 glomerulonephritis. The range of histopathology features encompassed by a diagnosis of C3G has uncertain relevance to outcome. In some studies, DDD has been associated with a higher incidence of progression to end-stage renal disease, whereas in a recent cohort no difference between these subsets was evident.[5,6] It is also unclear whether or not glomerular deposition of specific complement proteins influences C3G outcome. Complement activation results in the cleavage of C3 to C3b, which can covalently attach to glomerular tissue. The C3b can contribute to further C3b generation through a positive feed-back loop or it can be cleaved sequentially to iC3b and then to C3dg, releasing C3c. Glomerular C3c is cleared within 24 hours after stopping complement activation in experimental glomerulonephritis models,[19] whereas C3dg persists. Glomerular FHR5 has been documented in a similar staining distribution to glomerular C3 in a range of glomerulopathies, including C3G and IgA nephropathy.[20] FHR5 binds C3b, iC3b, and C3dg and demonstrates stronger binding than fH for iC3b and C3dg.[21] In addition to the associations between FHR5 variants and familial C3G,[13,15,22] evidence that glomerular FHR5 might increase glomerular injury includes *in vitro* studies demonstrating FHR5 can (i) bind extracellular matrix components and support assembly of the alternative pathway C3 convertase formation, thereby promoting further C3b generation, and (ii) antagonize the ability of fH to limit amplification of C3b (a process termed fH deregulation).[21,23,24] Furthermore, both circulating FHR5 levels[25,26] and the intensity of glomerular FHR5 deposition[27] associate with IgA nephropathy severity.

In this study, we characterized the spectrum of glomerular FHR5 deposition in C3G in native and transplant kidneys, its relationships with other complement components, and, where possible, its change over time in the same patient. We show that glomerular FHR5 is highly prevalent in both native and transplant kidney C3G, colocalizes with C3b/iC3b/C3c and C3dg *in vivo*, and associates with disease severity.

METHODS

Patient Cohort

We reviewed the diagnosis of all kidney biopsies performed at Imperial College Healthcare NHS Trust over a 20-year period. This totaled 6592 native and 2577 transplant biopsies. We included all patients with a biopsy diagnosis of C3G. After review of each case, we excluded patients with thrombotic microangiography, immune-complex dependent disease, or membranoproliferative glomerulonephritis (MPGN) type 1. We derived clinical and pathology data from medical records. Ethical approval was through the Natural History Study of C3 Glomerulopathy: Discovery of Histological Predictors of Outcome study (UK National Research Ethics Service reference 16/WM/0497) and the Imperial College Healthcare Tissue Bank. The Imperial College Healthcare Tissue Bank is supported by the National Institute for Health Research Biomedical Research Centre based at Imperial College Healthcare NHS Trust and Imperial College London. The Imperial College Healthcare Tissue Bank is approved by the National Research Ethics Service to release human material for research (12/WA/0196). Outcome parameters were 50% of eGFR from presentation and end-stage renal disease (defined as commencing dialysis or receiving a renal transplant). Patients with less than 12 months of follow-up were excluded from outcome analyses. eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration equation[28] for adults and the Schwartz formula[29] for children and corrected to a maximum of 120 ml/min per 1.73 m^2. Nephrotic syndrome was defined as a combination of serum albumin of less than 32 g/l, edema, and urine protein:creatinine ratio of at least 250 mg/mmol or 24-hour urine proteinuria of at least 3 g. We defined crescentic C3G as the presence of cellular or fibrocellular crescents in at least 50% of glomeruli.

Immunohistochemistry

FFPE renal biopsy sections were examined for the following proteins: FHR5, FHR1, fH, C3b/iC3b/C3c, C3dg, C5b9, properdin, C4d, C1q, and CD68. Primary antibodies were polyclonal rabbit anti-C3c (#A0062; Dako, Glostrup, Denmark); polyclonal rabbit anti-C3d (#136916; Abcam, Cambridge, UK) that recognizes C3dg; polyclonal rabbit anti-properdin (#2097; Biorbyt, Cambridge, UK); monoclonal mouse anti-C5b9 (#M0777; Dako); polyclonal rabbit anti-C4d (#107-01; BD Biotech, Franklin Lakes, NJ); monoclonal rabbit anti-C1q (#A0136; Dako); monoclonal mouse anti-factor H, OX-24 (#118820; Abcam); monoclonal mouse anti-FHR1 (#3078-M01; Abnova, Taipei, Taiwan); polyclonal rabbit anti-FHR5 (#81494-D01P; Abnova); and

Table 1. Clinical and histology features

Characteristic	Number	Percentage	Median (range)
Biopsy at center	27		
Age, yr			30.4 (9.4–73.8)
Male	14	51.9	
White	16	59.3	
eGFR (ml/min per 1.73 m^2)			74 (5–120)[a]
Urine protein:creatinine ratio (mg/mmol)			263 (0–1653)
Serum albumin (g/l)			29 (11–43)
Hematuria (macro- and microscopic)	16	59.3	
Nephrotic syndrome	14	51.9	
Paraproteinemia (n = 19)	3	17.6	
Diagnostic biopsy features	27		
Glomeruli per diagnostic biopsy			17 (5–46)
Percentage sclerosed glomeruli			10% (0%–57%)
Dense deposit disease	6	22.2	
Light microscopy pattern:			
Mesangial proliferative	11	40.7	
Endocapillary proliferative	5	18.5	
MPGN	16	59.3[b]	
Other	2	7.4	
Cellular crescents	7	25.9	
Endocapillary hypercellularity	17	63.0	
Tubular atrophy:			
None	5	18.5	
1%–10%	9	33.3	
11%–25%	8	29.6	
26%–50%	5	18.5	
Detectable immunofluorescence			Mean intensity (range)
C3	27	100.0	2.4 (1–3)
C1q	9	33.3	0.4 (0–1)
IgA	1	3.7	0.04 (0–1)
IgG	4	14.8	0.1 (0–1)
IgM	13	48.1	0.5 (0–2)
Treatment after diagnostic biopsy (n = 28)	25		
ACEi/ARB	20	80.0	
Any immunosuppression (including corticosteroids)	16	64.0	
- Corticosteroids	14	56.0	
- Mycophenolate mofetil	9	36.0	
- Cyclophosphamide	7	28.0	
Follow-up	25		
Duration follow-up (mo)			77.9 (31–155.7)
Loss of 50% eGFR at follow-up	14	56.0	
Time diagnosis to 50% eGFR loss (mo)			12.9 (0.1–80.8)
ESRD	12	48.0	
Time diagnosis to ESRD (mo)			40.0 (1–69.2)
Transplant	9	36.0	
Transplant recurrence of C3G (n = 9)	9	100.0	
Time transplant to recurrence (n = 9) (mo)			4.9 (1–26.8)
Recurrence within 6 mo (n = 9)	4	44.4	
Recurrence within 18 mo (n = 9)	9	100	

ACEi, angiotensin-converting-enzyme inhibitor; ARB, angiotensin II receptor blocker; eGFR, estimated glomerular filtration rate; ESRD, end-stage renal disease; MPGN, membranoproliferative glomerulonephritis.
[a]Associates with 50% loss of eGFR at follow-up. Of the 25 patients with follow-up data available, patients with 50% loss of eGFR during follow-up had median eGFR at diagnostic biopsy of 45.0 versus 117.7 ml/min per 1.73 m^2 for those without (P = 0.048, 95% confidence interval −0.2 to −76.6).
[b]Associates with 50% loss of eGFR at follow-up. Of the 25 patients with follow-up data available, 78.6% of patients with 50% loss of eGFR during follow-up had MPGN on biopsy versus 27.3% of those without 50% eGFR loss (P = 0.02; odds ratio = 9.8; 95% confidence interval 1.5-47.1).
eGFR calculated with the Chronic Kidney Disease Epidemiology Collaboration equation[30] for adults and the Schwartz formula[31] for children and corrected to a maximum of 120 ml/min per 1.73 m^2.

monoclonal mouse anti-CD68 (#M0876; Dako). Variation in glomerular FHR5 staining between non-sclerosed glomeruli from the same biopsy was minimal with bacterial enzyme antigen retrieval. FHR5 staining was eliminated after pre-incubation of the primary antibody with purified FHR5 (#3845-F5; R&D Systems, Minneapolis, MN; Supplementary Figure S1). The anti-FHR5 antibody showed minimal binding to plate-bound purified human C3c (#A116; Comptech, Tyler, TX), iC3b (#A115; Comptech), and C3d (#A117; Comptech) in the absence of purified FHR5 (Supplementary Figure 2A). The anti-C3c antibody (#A0062; Dako) recognized plate-bound purified human C3c and iC3b, so we refer to this antibody as anti-C3b/iC3b/C3c. This anti-C3b/iC3b/C3c antibody and the anti-C3d antibody (#136916; Abcam) recognized plate-bound human C3 fragments irrespective of the presence of FHR5 (Supplementary Figure 2B and C). For pre-incubation experiments, we used recombinant full-length human FHR5 (#3845-F5; R&D Systems) and heparin sodium (#H3393; Sigma-Aldrich, St. Louis, MO) at 30 times the antibody concentration.

Two-micron thickness sections from FFPE renal tissue were fixed on Polysine slides, deparaffinized, and hydrated (xylene for 10 minutes followed by an ethanol gradient), washed in distilled water, and subjected to antigen retrieval. For C3b/iC3b/C3c, C5b9, and FHR5 (Supplementary Figure 1): bacterial proteinase XXIV (#P8038; Sigma-Aldrich) at 37 °C for 30 minutes. For C3dg, C4d, fH, and C1q: sodium citrate tribasic buffer pH 6 at 95 °C for 30 minutes. For properdin, FHR1, and CD68: Tris-EDTA buffer at pH 9 and heating in a pressure cooker for 30 minutes. Sections were blocked for 60 minutes at room temperature with hydrogen peroxidase (EnVision, #K4007 or #K4011; Dako) followed by 30% normal goat serum. Primary antibody was applied overnight at 4 °C followed by application of secondary antibodies for 60 minutes at room temperature: horseradish peroxidase–conjugated goat anti-mouse (EnVision+System-HRP, #K4007; Dako) or anti-rabbit (EnVision+System-HRP, #K4011; Dako) IgG. 3′-Diaminobenzidine substrate (EnVision+System-HRP, #K4007 or #K4011; Dako) was applied for up to 15 minutes and sections counterstained with filtered hematoxylin, washed and

Table 2. Complement analyses

Complement assessment at diagnostic biopsy	Number tested	Percentage of cohort	Number	Percentage	Median (range)
Serum C3, g/l (NR 0.7–1.7)	25	100			0.77 (0.10–1.91)
Low serum C3	25	100	11	44.0	
Serum C4, g/l (NR 0.16-0.54)	25	100			0.27 (0.14-0.4)
Low serum C4	25	100	1	4.0	
CH-50, %	22	88			81 (20–130)
CH-50 <50%	22	88	6	27.3	
AP-50 (%)	22	88			84.0 (20–115)
AP-50 <50%	22	88	9	40.9	
Anti-C1q antibody	10	40	4	40.0	
Serum factor H, mg/l	22	88			597 (286–800)
Serum factor I, mg/l	22	88			49 (26–76)
C3 nephritic factor	24	96	2	8.3	
Anti-fH antibody	22	88	1	4.5	
Complement gene variant[a]	12	48	4	33.3	

fH, complement factor H; NR, normal range.
[a]Heterozygous factor I mutation ($n = 1$, c.577I9G>T, p.(Gly367STOP) variant of uncertain significance); heterozygous C3 mutation ($n = 1$, c.463A>C, p.(Lys155Gln) likely pathogenic C3 gene mutation); FHR5 nephropathy[19] ($n = 2$, duplication of exons 2 to 3 of *CFHR5* gene in heterozygosity).

dehydrated and cleared in xylene for 10 minutes before mounting using xylene-based media (Pertex, #SEA-0104–00A; CellPath, Newtown, UK).

Antigen staining intensities were graded as previously described.[27] Because nonspecific staining was seen in sclerosed glomeruli in previous[20] and our studies, only nonsclerosed glomeruli were included in statistical analyses. We tested for correlations between staining intensities and clinicopathologic markers of disease severity: MPGN light microscopy pattern; urine protein:creatinine ratio at biopsy; eGFR at biopsy; loss of 50% eGFR during follow-up; change in eGFR during follow-up; end-stage renal disease during follow-up.

Immunofluorescence

Antibody combinations for double antigen immunofluorescence (IF) staining were as follows: C3c and C3dg: fluorescein isothiocyanate–conjugated sheep polyclonal anti-human C3c antibody (#PA1–36179; ThermoFisher, Waltham, MA) and rabbit polyclonal anti-C3d (#136916; Abcam) with Alexa Fluor AF555-conjugated goat anti-rabbit IgG (#A-21429; ThermoFisher). C3c and FHR5: fluorescein isothiocyanate–conjugated sheep polyclonal anti-human C3c antibody (#PA1–36179; ThermoFisher) and mouse monoclonal anti-human FHR5 antibody (#81494-B01P; Abnova) with AF555-conjugated goat anti-mouse IgG (#150118; Abcam). C3dg and FHR5: rabbit polyclonal anti-C3d (#136916; Abcam) with AF555-conjugated goat anti-rabbit IgG (#A-21429; ThermoFisher) and mouse monoclonal anti-human FHR5 antibody (#81494-B01P; Abnova) with AF488-conjugated goat anti-mouse IgG (#A-11029; ThermoFisher). The anti-C3c antibody used for IF staining recognized plate-bound C3c and iC3b irrespective of the presence of purified human FHR5 and will also be referred to as anti-C3b/iC3b/C3c (Supplementary Figure 2D). The anti-C3d antibody used for immunohistochemistry and IF showed relatively selective binding to plate-immobilized C3d (Supplementary Figure 2C). The anti-C3b/iC3b/C3c used for IF staining showed relatively selective binding to plate-immobilized C3c and iC3b (Supplementary Figure 2D).

Staining was performed using 2- to 4μm-thick FFPE sections fixed on Polysine slides, deparaffinized (xylene for 10 minutes followed by an ethanol gradient), washed in distilled water, and subjected to antigen retrieval: immersion in sodium citrate tribasic buffer pH 6 at 95 °C for 30 minutes and incubation with HistoReveal (#103720; Abcam) for 5 minutes at room temperature. Slides were blocked with 2% bovine serum albumin in phosphate-buffered saline for 60 minutes at room temperature and the first antibody applied overnight at 4 °C. Sections were blocked again with 2% bovine serum albumin at 4 °C for 8 hours followed by incubation of the second antibody overnight at 4 °C. Sections were sequentially incubated with the appropriate secondary Alexa Fluor antibodies, nuclear stain (4′,6-Diamidino-2-Phenylindole, Dilactate, #D3571; ThermoScientific, Waltham, MA), 0.1% Sudan Black B (#199664; Sigma, St. Louis, MO) in 70% ethanol to reduce autofluorescence and mounted using Mowial 4–88 (#475904; Calbiochem, San Diego, CA).

We used ImageJ software (National Institutes of Health, Bethesda, MD) and the COLOC2 plug-in[30] to calculate antigen location correlations. This analyzed the antigen colocalization of each pixel within the image region of interest. We ensured IF images were captured with identical microscope settings, images were of identical size, and the brightness threshold at which monochromatic (red or green) fluorescence was counted as positive deposition was standardized. The

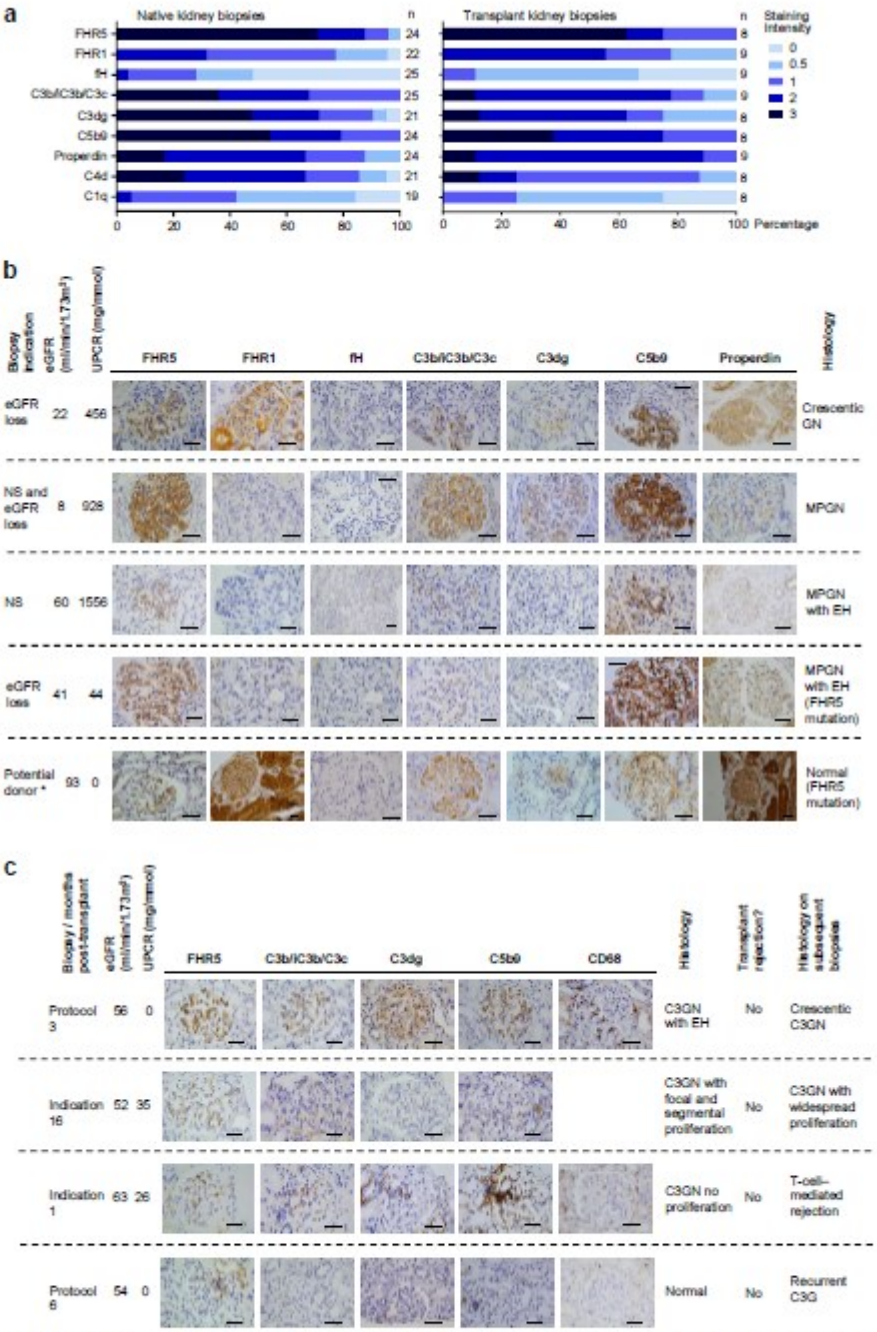

Figure 1. Glomerular FHR5 staining in C3 glomerulopathy. (a) Glomerular staining intensity in native and transplant biopsies. FHR5 was the most frequent protein at 3+ intensity and detected in all of the transplant biopsies. (b) Representative images of complement staining in the native kidney. For each set of images, the biopsy indication, the appearances of the glomeruli by light microscopy, and the estimated (continued)

glomerulus from each section was selected as the region of interest for analysis. Pearson's correlation coefficients were recorded for each antigen combination in all glomeruli. For each antibody combination, this provided a number of antibody location correlation coefficients, which quantified how closely 2 antibodies colocalized on the same tissue section. We wanted to compare how closely each pair of antibodies colocalized. For example, we questioned whether the C3b/iC3b/C3c and C3d antibodies colocalized more or less closely than FHR5 and C3d. To do this, we calculated the median of the correlation coefficients for each antibody pair, and then compared these median values.

Statistical Analysis

We used Pearson's correlation coefficient to calculate IF antigen location correlations with ImageJ software.[30] We used GraphPad (La Jolla, CA) Prism Version 6.00 for Windows for all other statistical analyses. Normally distributed continuous variables were compared using either unpaired *t*-test or (for multiple groups) 1-way analysis of variance. Continuous variables with skewed distribution were compared using either Mann-Whitney *U* test or (for multiple groups) Kruskal-Wallis test. We calculated CIs using the Hodges-Lehmann method and adjusted for multiple analyses with the 2-stage linear step-up procedure of Benjamini *et al.*[31] Boxplots were used to display correlation data. We included repeat biopsies from the same patients because we were interested in the correlation of glomerular FHR5 with other complement proteins and eGFR at each biopsy point. The dependence of these outcomes on repeated measures is not known.

RESULTS

C3G Cohort

We identified 27 individuals with biopsy-proven C3G (Tables 1[32,33] and 2[13]). Two patients were lost to follow-up. A 50% loss of eGFR during follow-up occurred in 14 of 25 patients (56.0%). A MPGN light microscopy pattern at diagnostic biopsy associated with 50% loss of eGFR during follow ($P = 0.02$, $n = 25$, odds ratio for 50% loss of eGFR = 9.8 for MPGN at diagnosis compared with non-MPGN light microscopy patterns; 95% CI 1.5–47.1). Patients with 50% loss of eGFR during follow-up had significantly lower median eGFR at diagnostic biopsy (45.0 vs. 117.7 ml/min per 1.73 m^2; $P = 0.049$, $n = 25$, difference of median eGFR −72.7 ml/min per 1.73 m^2; 95% CI −0.2 to −76.6) but no significant difference in the amount of proteinuria at biopsy (Table 1). Surplus tissue was available from 34 renal biopsies from 19 patients. Nine of these were transplant biopsies from 5 patients. Four of the 19 patients (21.1%) with available biopsy tissue had a diagnosis of DDD.

Glomerular FHR5 is Highly Prevalent in C3G

Using immunohistochemistry staining on FFPE renal biopsy sections,[27] glomerular FHR5 was detected in all the transplant biopsies ($n = 8$ from 4 patients) and all but 1 of the native biopsies (23 of 24 biopsies from 15 patients). The sample sizes varied depending on the availability of surplus stored renal tissue. FHR5 was the most prevalent glomerular complement protein detected at 3+ intensity (Figure 1a–c). We did not detect significant differences in complement protein immunostaining when comparing cases of DDD with C3GN. We have previously reported the prevalence of glomerular FHR5 deposition in IgA nephropathy.[27] We did not detect glomerular FHR5 in 3 thin basement membrane cases (Supplementary Figure S2).

We detected glomerular FHR5, C3, and C5b-9 in 2 cases of CFHR5 nephropathy, 1 with and 1 without glomerular inflammation and clinical signs of glomerulonephritis (Figure 1b). Although C5b-9 and FHR5 staining intensities were greater in the biopsy with glomerular inflammation, the detection of C5b-9 and FHR5 in a potential kidney donor with normal renal function but subclinical CFHR5 nephropathy shows that glomerular complement deposition can be detected in the absence of glomerular inflammation in CFHR5 nephropathy.

Two of the renal transplant biopsies were part of rejection surveillance programs ("protocol biopsy"), thereby providing an opportunity to characterize subclinical C3G (Figure 1c). None of the C3G patients with renal transplant had a diagnosis of antibody-mediated rejection. In 1 protocol biopsy performed 6 months posttransplant, the glomeruli were normal by light microscopy and glomerular C3, C5b9, and CD68 staining were negative. However, glomerular FHR5 staining was detectable. Nine months later, the patient

Figure 1. (continued) glomerular filtration rate (eGFR) and proteinuria at the time of biopsy are indicated. *The cases represented by the fourth and fifth rows of images are from the same family. (c) Representative images of complement staining in transplant kidneys. For each set of images, the biopsy indication (either protocol or due to clinical changes; i.e., indication biopsy), time posttransplant together with the eGFR, and proteinuria at the time of biopsy are indicated. The histology including the presence or absence of rejection and the histology of subsequent biopsies is also shown. C3G, C3 glomerulopathy; C3GN, C3 glomerulonephritis; EH, endocapillary hypercellularity; fH, factor H; FHR1, factor H–related protein 1; FHR5, factor H–related protein 5; FHR5 mutation, the mutation described in CFHR5 nephropathy[13]; MPGN, membranoproliferative glomerulonephritis; NS, nephrotic syndrome; UPCR, urinary protein:creatinine ratio. Original magnification ×400. Bars = 100 μm.

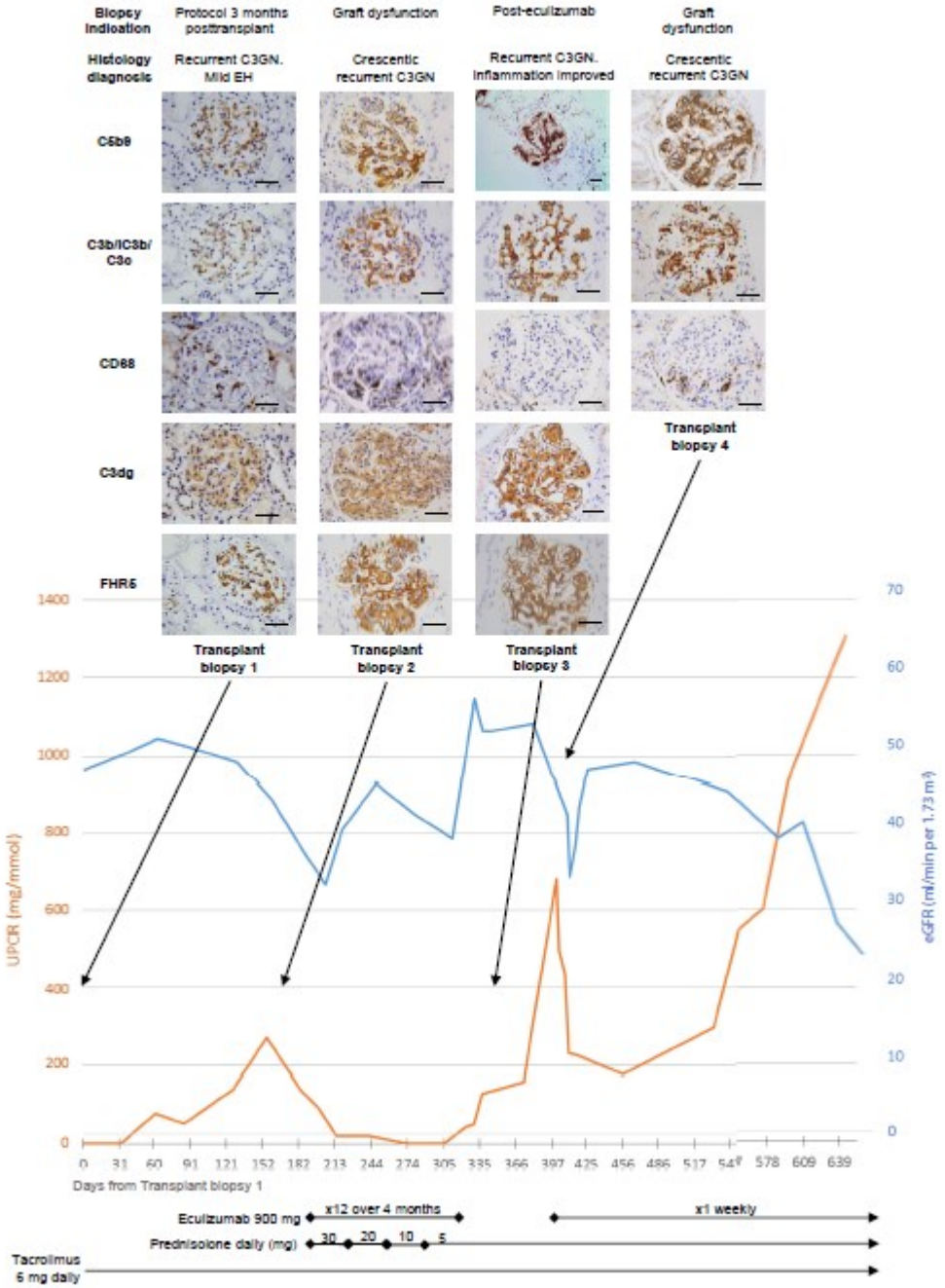

Figure 2. Sequential glomerular FHR5 staining in a single case of C3 glomerulopathy transplant recurrence. Images and information from the same biopsy are organized in columns. A protocol surveillance biopsy performed 3 months after transplantation (Transplant biopsy 1) showed recurrent C3 glomerulonephritis (C3GN) with mild endocapillary hypercellularity (EH). Glomerular factor H–related protein 5 (FHR5), (continued)

developed biopsy-proven C3G recurrence (surplus biopsy tissue unavailable to study). The other protocol biopsy was from an individual who had a 3 further transplant biopsies and treatment with eculizumab. The initial protocol biopsy revealed subclinical C3G recurrence with glomerular endocapillary hypercellularity and glomerular staining for C3b/iC3b/C3c, C3dg, C5b-9, FHR5, and CD68 (Figure 2). A biopsy performed due to proteinuria and fall in eGFR 6 months later showed crescentic C3G and increased glomerular C3b/iC3b/C3c, C3dg, C5b-9, FHR5, and CD68 staining (Figure 2, transplant biopsy 2). A biopsy 4 months after starting eculizumab treatment showed reduced endocapillary hypercellularity and reduced glomerular CD68 infiltration (Figure 2, transplant biopsy 3). Proteinuria and eGFR had improved but glomerular FHR5, C3b/iC3b/C3c, C3dg, and C5b-9 staining were unchanged. Three months after stopping eculizumab, graft function deteriorated again, and a fourth transplant biopsy showed recurrent crescentic C3GN. Rapid clinical improvement was seen after reintroducing eculizumab treatment, but subsequently proteinuria increased despite ongoing eculizumab therapy.

Glomerular FHR5 Colocalizes With Glomerular C3 in C3G

We next assessed the relationship between glomerular C3 and FHR5. Glomerular FHR5 staining intensity positively correlated with the glomerular staining intensities of C3b/iC3b/C3c, C3dg, and C5b9 (Figure 3a). Glomerular C3b/iC3b/C3c and C3dg positively correlated with glomerular C5b9. Glomerular C3b/iC3b/C3c and C3dg intensities also correlated significantly.

In 3 cases of C3GN, we performed double immunofluorescence staining for (i) FHR5 and either C3b/iC3b/C3c or C3dg, and (ii) C3b/iC3b/C3c and C3dg (Figure 3b). At the time of biopsy, serum C3 was normal in case 1 and low in cases 2 and 3. We noted areas of C3b/iC3b/C3c and C3dg codeposition (Figure 3b, right-hand column, arrows), and areas staining for either C3b/iC3b/C3c (Figure 3b, right-hand column, stars) or C3dg (Figure 3b, right-hand column, triangles) alone. Capillary wall (case 3) and mesangial FHR5 (cases 1 and 2) colocalized with C3b/iC3b/C3c (Figure 3b, left-hand column, arrows); however, there were also areas of glomerular C3b/iC3b/C3c staining without FHR5 (Figure 3b, left-hand column, cases 1 and 2, stars) and FHR5 without C3b/iC3b/C3c (Figure 3b, left-hand column, cases 2 and 3, triangles). Glomerular C3dg deposition was detected predominantly along capillary walls in cases 2 and 3, but was less marked and mesangial in location in case 1 (Figure 3b). Capillary wall FHR5 colocalized with C3dg (Figure 3b, middle column, cases 2 and 3, arrows). We also observed areas of FHR5 without C3dg deposition in cases 1 and 2 (Figure 3b, middle column, cases 1 and 2, triangles).

Antigen glomerular locations correlated positively in all glomeruli (Figure 3c). The median values of the antigen location correlations were 0.72 for C3b/iC3b/C3c with FHR5 (8 glomeruli from 3 cases; range 0.61 to 0.79), 0.75 for FHR5 with C3gd (5 glomeruli from 3 cases; range 0.5 to 0.91), and 0.74 for C3b/iC3b/C3c with C3dg (6 glomeruli from 3 cases; range 0.57 to 0.82).

Glomerular FHR5 and C5b9 Staining Intensity Associates With Loss of eGFR in C3G

We next examined whether intense glomerular staining (grade 3+) associated with renal impairment (eGFR) at the time of biopsy (Figure 4). Both glomerular FHR5 and C5b9 negatively correlated with eGFR, whereas glomerular FHR1, C3b/iC3b/C3c, C3dg, and C4d did not. Our correlation excluded sclerosed glomeruli, so any relationships, such as that between glomerular FHR5 and eGFR at time of biopsy, were unlikely to be influenced by the prevalence of sclerosed glomeruli. Furthermore, we did not identify correlations between glomerular FHR5 intensity and the percentage of sclerosed glomeruli per biopsy (Pearson's correlation coefficient $r = 0.1993$, $P = 0.27$). Glomerular FHR5 also correlated with an MPGN pattern at diagnostic biopsy (90% patients with vs. 33.3% without MPGN pattern, $P = 0.049$; odds ratio 18; 95% CI 1.6–201). Glomerular FHR5 deposition intensity did not correlate with age ($P = 0.5$), gender ($P > 0.99$), urine protein:creatinine ratio ($P = 0.27$), the presence of cellular crescents ($P > 0.99$), or endocapillary hypercellularity ($P = 0.27$) at the time of biopsy.

Figure 2. (continued) C5b9, C3b/iC3b/C3c, and C3dg were detected. The estimated glomerular filtration rate (eGFR) was stable and there was no significant proteinuria. Approximately 6 months posttransplant, the patient developed proteinuria and a fall in eGFR. Biopsy showed crescentic C3GN and increased glomerular CD68-positive cells (Transplant biopsy 2). Glomerular FHR5, C5b9, C3b/iC3b/C3c, and C3dg were detected but at increased staining intensity compared with the first transplant biopsy. The eGFR and proteinuria improved with eculizumab and prednisolone treatment. After 4 months of eculizumab treatment, renal biopsy (Transplant biopsy 3) showed resolution of glomerular CD68 staining, but glomerular staining for FHR5, C5b9, C3b/iC3b/C3c, and C3dg remained unchanged. After a further 3 months, proteinuria increased and biopsy showed crescentic C3GN and the recurrence of glomerular CD68-positive cells (Transplant biopsy 4). Proteinuria improved with re-introduction of eculizumab. UPCR, urine protein:creatinine ratio. Original magnification ×200 or ×400. Bars = 100 μm.

a

Correlation coefficient R values	FHR5	C3b/iC3b/C3c	C5b-9
C3b/iC3b/C3c	0.59 (p=0.0003)		
C5b9	0.47 (p=0.008)	0.66 (p<0.0001)	
C3dg	0.44 (p=0.02)	0.42 (p=0.02)	0.42 (p=0.02)

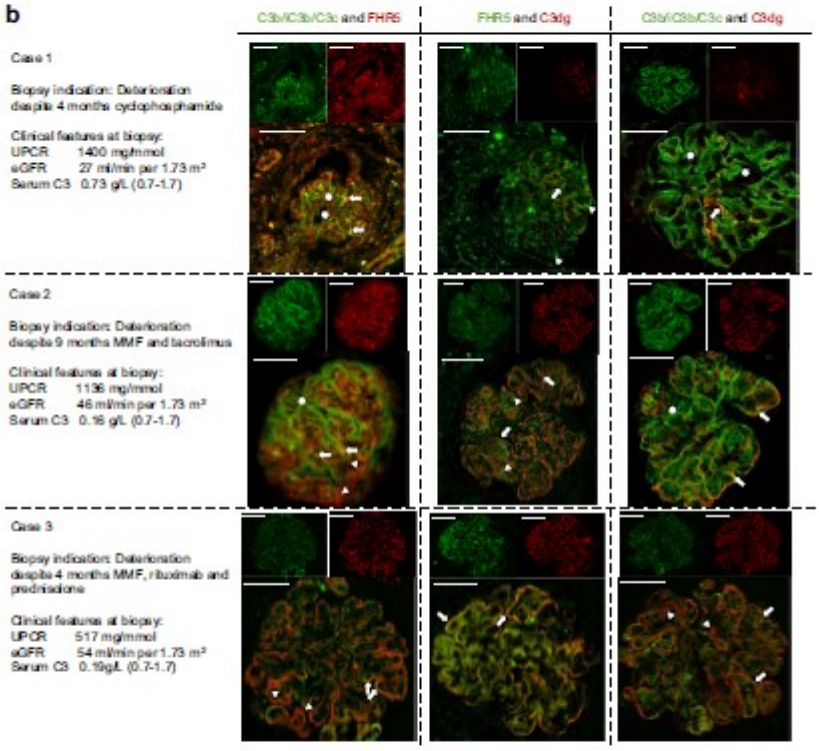

Figure 3. Glomerular factor H–related protein 5 (FHR5) colocalizes with C3 in C3 glomerulopathy. (a) Glomerular FHR5 staining intensity positively correlated with the staining intensity of C3b/iC3b/C3c, C3dg, and C5b9. Both glomerular C3b/iC3b/C3c and C3dg positively correlated with glomerular C5b9. *R* values are calculated from Pearson's correlation coefficient and *P* values are adjusted for multiple (continued)

Tubulo-interstitial FHR1 But Not FHR5 Was Detectable in C3G

We did not detect significant FHR5 staining in the tubulointerstitium of either native or transplant C3G (Figure 5a); however, there was tubular cell staining for both FHR1 and properdin (Figure 5a). This staining did not correlate with proteinuria, eGFR, tubular atrophy, loss of 50% eGFR (data not shown), or tubular deposition of other complement proteins (Figure 5a). Tubular FHR1 staining was detected in non-C3G cases and was absent in a section from a patient with IgA nephropathy and FHR1 deficiency (Figure 5b). Notably, when we used pressure cooker heating for antigen retrieval, we were able to detect tubulointerstitial FHR5 staining in different glomerulopathies, including C3G (Supplementary Figure 1). However, unlike the glomerular FHR5 staining evident using enzyme antigen retrieval, tubulo-interstitial staining could not be inhibited by pre-incubation of the primary antibody with purified FHR5, suggesting it was nonspecific.

DISCUSSION

Glomerular FHR5 was the most prevalent protein in native and transplant renal biopsies in our C3G cohort. FHR5 staining intensity associated with eGFR at the time of biopsy and correlated with the intensities of glomerular C3b/iC3b/C3c, C3dg, and C5b9 deposition. These findings support a role for FHR5 in complement-dependent glomerular injury, and FHR5 staining may improve C3G diagnosis. Murphy *et al.*[20] demonstrated the presence of FHR5 in glomerular diseases, and we have demonstrated an association between IgA nephropathy severity and glomerular FHR5 deposition.[27] It will be interesting to determine the prevalence of glomerular FHR5 deposition in a large population of patients with complement-dependent and complement-independent renal diseases. We identified an association between MPGN light microscopy pattern at diagnostic biopsy and a 50% loss of eGFR. Intense glomerular FHR5 deposition also associated with an MPGN pattern in our cohort. The interaction between glomerular FHR5 deposition and MPGN morphology requires further investigation. FHR5 and complement glomerular deposition would ideally be assessed in serial sections of the same glomeruli; however, the limited amount of surplus biopsy tissue precluded assessment of serial sections in most cases.

Glomerular FHR5 staining intensity correlated positively with the staining intensity of both C3b/iC3b/C3c and C3dg, which identify ongoing and previous complement activity, respectively. Our double-staining immunofluorescence protocols identified areas of FHR5 codeposition with C3b/iC3b/C3c and C3d. Therefore, FHR5 may bind both areas of ongoing (C3b/iC3b/C3c) and previous (C3dg) complement activity.

FHR5 and C5b9 were the only glomerular complement antigens to associate with renal impairment. The interplay of C3G activity, impaired glomerular filtration, and glomerular FHR5 and C5b9 deposition requires further investigation. Notably, glomerular C5b9 has been shown to persist in lupus nephritis following complement activity cessation,[34] so may not be a reliable marker of ongoing glomerular C5 activation in C3G as well.

We made a number of interesting observations from transplant cases. Glomerular FHR5 deposition was identified in the absence of and predated detectable C3b/iC3b/C3c deposition and glomerular inflammation in one transplant patient. Whether this implicates FHR5 in early C3G pathogenicity or represents the sensitivity and affinity of FHR5 for otherwise undetectable C3 fragments requires further investigation. Specifically, a comparison of FHR5 deposition in protocol biopsies from patients with and without clinical signs of C3G recurrence is needed. Serial transplant biopsies during recurrent crescentic C3G showed FHR5 deposition correlated with initial disease activity, but

Figure 3. (continued) comparisons. (b) Representative images of combined immunofluorescence staining in three C3G cases for glomerular FHR5 with either C3b/iC3b/C3c or C3dg, and C3b/iC3b/C3c with C3dg. Renal biopsies in all 3 cases showed C3-dominant membranoproliferative glomerulonephritis, and the biopsy indications together with the urine protein:creatinine ratio (UPCR), estimated glomerular filtration rate (eGFR), and serum C3 levels at the time of biopsy are listed. The staining patterns for C3b/iC3b/C3c and C3dg (right-hand column of images) showed areas of colocalization (arrows), areas of C3b/iC3b/C3c alone (stars), and areas of C3dg alone (triangles). The staining pattern for C3b/iC3b/C3c and FHR5 (left-hand column of images) showed areas of colocalization (arrows), areas of C3b/iC3b/C3c alone (stars), and areas of FHR5 alone (triangles). The staining pattern for C3dg and FHR5 (middle column of images) showed areas of colocalization, particularly along capillary walls in cases 2 and 3 (arrows) and areas of FHR5 alone in cases 1 and 2 (triangles). Notably, we did not detect areas of C3dg without FHR5 staining. (c) FHR5, C3b/iC3b/C3c, and C3dg glomerular locations correlate in C3G. We calculated the correlation of glomerular antigen locations in all available glomeruli from the three C3G cases (c). The Pearson's correlation coefficient for all glomeruli from cases 1 (circles), 2 (squares), and 3 (triangles), and the median values of the correlations for each case (horizontal lines) are shown. The median values of the correlation coefficients (r) for each case were as follows: C3b/iC3b/C3c with FHR5: 0.73 (case 1), 0.76 (case 2), and 0.68 (case 3); FHR5 with C3dg: 0.71 (case 1), 0.90 (case 2), and 0.5 (case 3); and C3b/iC3b/C3c with FHR5: 0.68 (case 1), 0.81 (case 2), and 0.58 (case 3). MMF, mycophenolate mofetil. Original magnification ×400. Bars = 100 μm.

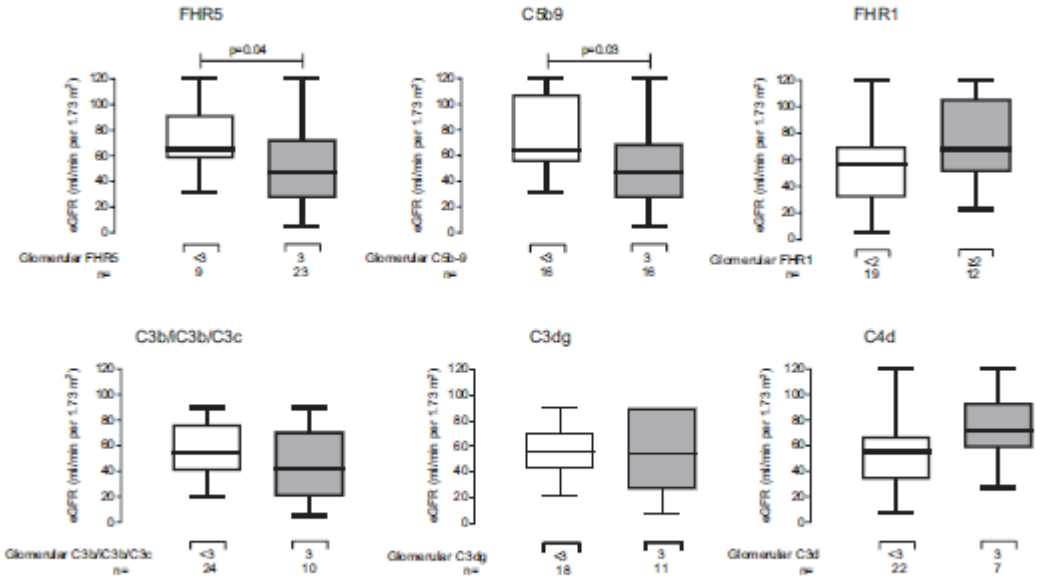

Figure 4. Glomerular factor H–related protein 1 (FHR5) and C5b9 staining intensity associated with lower estimated glomerular filtration rate (eGFR) at biopsy in C3 glomerulopathy. The eGFR at the time of biopsy was significantly lower in biopsies that had maximal staining intensities for FHR5 ($P = 0.04$, difference of medians 19.7 ml/min per 1.73 m^2; 95% confidence interval [CI] 1.1–43.0) and C5b-9 ($P = 0.03$, difference of medians 14.86 ml/min per 1.73 m^2; 95% CI 3.8–46.6). This was not seen for glomerular factor H–related protein 1 (FHR1), C3b/iC3b/C3c, C3dg, and C4d. *P* values are derived from Mann-Whitney *U* tests.

was unaltered by C5 inhibition. This is consistent with a role for FHR5 in alternative but not terminal complement pathway activity. As evidenced by improved CD68-positive cell infiltration, clinical improvement following eculizumab treatment is likely secondary to reduced C5a production and inflammatory cell recruitment. Whether continued terminal pathway blockade would cause long-term disease improvement is unclear. Evidence from C3G animal models suggests alternative pathway activity in the absence of C5 reduces glomerular inflammation, but C3 glomerulopathy persists.[35] The role of C5 inhibition in treating acute exacerbations of disease and its ability to modify long-term outcomes requires further study. Glomerular C4d was prevalent in both native and transplant C3G biopsies, suggesting the absence of C4d as a tool for diagnosing C3G is unreliable in our cohort, and the role of C4d in C3G requires further investigation.

In our protocols, glomerular FHR5 staining could identify complement activation in the absence of anti-C3b/iC3b/C3c staining. We speculate that, by comparing FHR5 and C3dg immunostaining patterns, it might be possible to identify areas within glomeruli where there is either ongoing (glomerular FHR5 without C3dg) or previous (glomerular FHR5 with C3dg) complement activation. Moreover, the combination of glomerular C5b9 and CD68 staining patterns could differentiate ongoing from previous glomerular C5 activation. This approach would rely on CD68 staining as a surrogate marker of complement C5a production. Consequently, ongoing C5 activation would be inferred if both C5b-9 and glomerular CD68 were positive; whereas previous C5 activation would be inferred if glomerular C5b-9 was positive in the absence of glomerular CD68.

Because FHR5 binds all immobilized C3 fragments *in vitro* (Supplementary Figure 2), the identification of glomerular C3b/iC3b/C3c deposits without FHR5 (Supplementary Figure 3) was unexpected. The structural conformation of C3c *in vivo* may hide the epitope that FHR5 binds *in vitro*. Alternatively, we speculate glomerular C3b/iC3b/C3c staining without FHR5 is C3c released from extrarenal complement activation that deposits in glomeruli but does not participate in complement activation[32] and therefore does not interact with FHR5. In this scenario, comparing C3b/iC3b/C3c and FHR5 deposition could differentiate local from extra-glomerular complement activation.

Intratubular complement activation is associated with proteinuria and is properdin-dependent.[33,36,37] We identified tubular FHR1 and properdin staining in our cases and these included those without

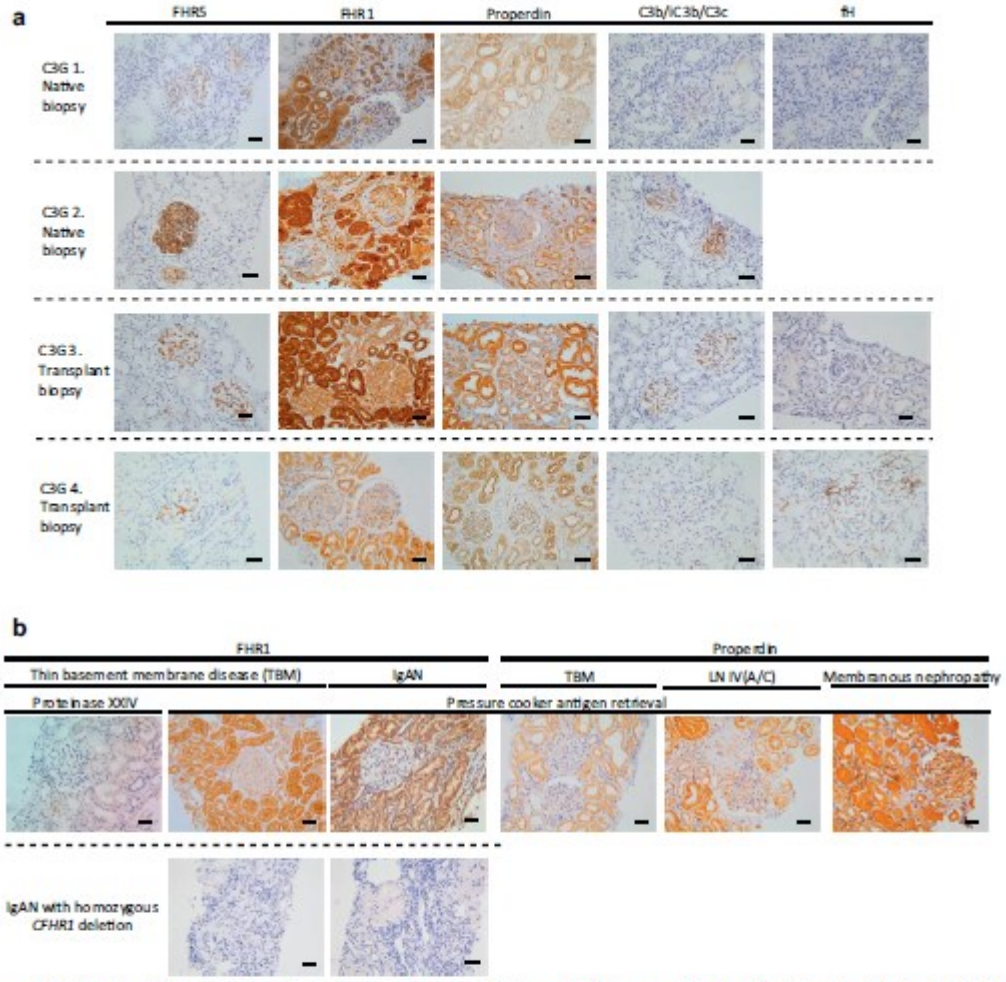

Figure 5. Tubulo-interstitial staining for FHR5 in C3 glomerulopathy. (a) Representative images of staining for factor H–related protein 5 (FHR5), factor H–related protein 1 (FHR1), factor H (fH), properdin, and C3b/iC3b/C3c. No tubulo-interstitial staining for FHR5 or fH was evident but there was strong tubulo-interstitial staining for both properdin and FHR1. (b) Tubulo-interstitial staining for properdin was also seen in other renal diseases (thin basement membrane disease [TBM], lupus nephritis [LN], and membranous nephropathy). Tubulo-interstitial staining for FHR1 was demonstrable in biopsies from patients with TBM and IgA nephropathy. Tubulo-interstitial FHR1 staining in TBM was still detectable but less intense when the proteinase XXIV enzyme was used instead of pressure cooker antigen retrieval. Glomerular and tubulo-interstitial FHR1 was absent in renal tissue from a patient with IgA nephropathy and FHR1 deficiency. C3G, C3 glomerulopathy; LN IV(A/C), lupus nephritis class 4, active and chronic. Original magnification ×200. Bars = 100 μm.

proteinuria. The significance of FHR1 in this context is unclear. Tubulo-interstitial staining for FHR5 was absent when we used enzymatic antigen retrieval but became detectable when we used pressure cooker antigen retrieval. We considered this to be nonspecific staining, as we were unable to inhibit the staining with pre-incubation of the anti-FHR5 antibody with purified FHR5. Nevertheless, the role of the FHR proteins in the tubulointerstitium requires further investigation.

Mass spectrometry analysis of laser-captured glomeruli has identified alternative and terminal complement pathway components and FHR proteins in C3G.[58] Using mass spectrometry spectra as semi-quantitative evidence of glomerular protein abundance, Sethi *et al.*[58] found C9, C3, and, specifically, C3dg were abundant in C3G, and DDD and C3GN cases had similar complement proteomic profiles. These observations seem consistent with our findings. By mass

spectrometry, FHR1 was more prevalent and detected with higher mass spectrometry spectral counts than FHR5.[38] In contrast, using immunohistochemistry, we found glomerular FHR5 to be more prevalent than FHR1; FHR5 was detected (at least 1+ intensity) in 96% (23 of 24) native and 100% (8 of 8) transplant biopsies; FHR1 was detected in 77% (17 of 22) native and 78% (7 of 9) transplant biopsies. Glomeruli FHR5 also appeared to be more abundant than FHR1; FHR5 was detected at 3+ staining in 71% (19 of 24) native and 63% (5 of 8) transplant biopsies, compared with FHR1 that we detected at 2+ intensity in 32% (7 of 22) native and 56% (5 of 9) transplant biopsies. Given the different research techniques and the experimental nature of protein quantification by spectra abundance, it is difficult to conclude why we did not replicate this finding in our cohort.

Our observational data cannot unravel the mechanisms that result in glomerular FHR5 deposition or its relationship with glomerular damage; however, we speculate that glomerular FHR5 interacting with C3 impairs the ability of fH to downregulate glomerular complement C3 activation. In addition, C3dg-bound FHR5 could facilitate C3 convertase formation and local C3 activation.[24] FHR5 could have similar effects after binding directly to glomerular components, such as laminin-521 or -211.[39] In addition, C3-bound FHR5 could impair complement receptor–dependent phagocytosis of deposited C3-coated immune complexes by mesangial cells and macrophages. In these scenarios, inhibition of glomerular FHR5 would be predicted to improve glomerular complement regulation and ameliorate complement-mediated glomerular inflammation and injury.

In summary, glomerular FHR5 deposition associates with C3G severity and colocalizes with C3 fragments *in vivo*. The prevalence of glomerular FHR5 binding to C3 fragments could be exploited to target therapeutic complement inhibitors to areas of complement activation in C3G.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Figure S1. Complement factor H–related protein 5 (FHR5) immunohistochemistry (IHC) protocol optimization.

Figure S2. Antibody specificity to purified human proteins by enzyme-linked immunosorbent assay (ELISA).

Figure S3. The potential value of combination staining with antibodies to complement (C)3b/iC3b/C3c, C3d, and factor H–related protein 5 (FHR5).

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CLINICAL RESEARCH

Progressive IgA Nephropathy Is Associated With Low Circulating Mannan-Binding Lectin–Associated Serine Protease-3 (MASP-3) and Increased Glomerular Factor H–Related Protein-5 (FHR5) Deposition

Nicholas R. Medjeral-Thomas[1], Anne Troldborg[2,3], Nicholas Constantinou[1], Hannah J. Lomax-Browne[1], Annette G. Hansen[2], Michelle Willicombe[4], Charles D. Pusey[5], H. Terence Cook[1], Steffen Thiel[2] and Matthew C. Pickering[1]

[1]Centre for Complement and Inflammation Research, Imperial College London, London, UK; [2]Department of Biomedicine, Aarhus University, Aarhus, Denmark; [3]Department of Rheumatology, Aarhus University Hospital, Aarhus, Denmark; [4]Renal and Transplant Centre, Imperial College Healthcare NHS Trust, London, UK; and [5]Renal and Vascular Inflammation Section, Imperial College London, London, UK

Introduction: IgA nephropathy (IgAN) is characterized by glomerular deposition of galactose-deficient IgA1 and complement proteins and leads to renal impairment. Complement deposition through the alternative and lectin activation pathways is associated with renal injury.

Methods: To elucidate the contribution of the lectin pathway to IgAN, we measured the 11 plasma lectin pathway components in a well-characterized cohort of patients with IgAN.

Results: M-ficolin, L-ficolin, mannan-binding lectin (MBL)–associated serine protease (MASP)-1 and MBL-associated protein (MAp) 19 were increased, whereas plasma MASP-3 levels were decreased in patients with IgAN compared with healthy controls. Progressive disease was associated with low plasma MASP-3 levels and increased glomerular staining for C3b/iC3b/C3c, C3d, C4d, C5b-9, and factor H–related protein 5 (FHR5). Glomerular FHR5 deposition positively correlated with glomerular C3b/iC3b/C3c, C3d, and C5b-9 deposition, but not with glomerular C4d. These observations, together with the finding that glomerular factor H (fH) deposition was reduced in progressive disease, are consistent with a role for fH deregulation by FHR5 in renal injury in IgAN.

Conclusion: Our data indicate that circulating MASP-3 levels could be used as a biomarker of disease severity in IgAN and that glomerular staining for FHR5 could both indicate alternative complement pathway activation and be a tissue marker of disease severity.

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KEYWORDS: complement; IgA nephropathy; lectin; MBL

IgA nephropathy (IgAN) is a common glomerular pathology that frequently causes renal failure, especially in young people.[1,2] IgAN is characterized by glomerular deposits of galactose-deficient IgA1.[3,4] Although a 4-hit theory is proposed for mesangial IgA deposition,[5] the mechanisms leading to glomerular injury remain poorly understood. The clinical course of IgAN is heterogeneous: after 20 years of follow-up from renal biopsy, up to 40% of patients will reach end-stage renal disease, but 20% of patients will have preserved renal function.[6] Our incomplete understanding of IgAN pathogenesis limits the development of biomarkers allowing the identification of patients who may benefit from immunosuppression and disease-specific therapies.[2,7]

The complement system is an important inflammation-generating arm of the immune system. Complement activation occurs in IgAN.[8] Colocalization of glomerular complement C3c with IgA is present in 90% of cases.[3] Serum levels of activated C3[9] and mesangial C3 deposition[10] correlate with loss of renal function. The degree of complement regulation is also important. Imbalances in plasma factor H (fH), an essential negative regulator of C3 activation, and factor H–related (FHR) proteins 1 and 5, that deregulate fH,

Correspondence: Matthew C. Pickering, Centre for Complement and Inflammation Research, Imperial College London, Hammersmith Campus, Du Cane Road, London W12 0NN, UK. E-mail: matthew.pickering@imperial.ac.uk
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associate with IgAN.[11,12] Complement activation leads to the accumulation of C3 proteolytic fragments, such as C3dg, within glomeruli.[8]

The lectin pathway pattern-recognition molecules include MBL (mannan-binding lectin), L-ficolin (also called ficolin-2), M-ficolin (ficolin-1), H-ficolin (ficolin-3), collectin liver 1 (CL-L1, also called CL-10), and collectin kidney 1 (CL-K1 or CL-11). Following interaction with ligands that include pathogen and/or damage-associated molecular patterns, the pattern-recognition molecules trigger complement activation through complexed serine proteases: MBL-associated serine protease (MASP)-1, MASP-2, and MASP-3. Pattern-recognition molecules also can bind nonenzymatic subunits: MBL-associated protein (MAp) 19 and MAp44. The pathway generates a C3-convertase, termed C4bC2b.[13] The C4b is further processed to C4d. The finding of glomerular C4d in the absence of C1q, the activator of the classic pathway of complement, in IgAN is consistent with lectin pathway activation.[14]

IgAN is characterized by disease flares following respiratory or gastrointestinal tract inflammation[15]; both IgA and the lectin complement pathway are important mediators of innate immunity at these sites. IgAN is associated with higher levels of IgA1 with exposed N-acetyl-galactosamine.[5,16] N-acetyl-galactosamine is a structure that may trigger lectin pathway activation due to interaction of ficolins with patterns of acetyl-groups.[17] Furthermore, MBL binds polymeric IgA and triggers complement activation *in vitro*.[18] Both high and very low MBL levels were associated with poor renal outcomes in a Chinese IgAN population.[19] Roos *et al.*[20] demonstrated glomerular MBL, L-ficolin, MASP1/3, and C4d deposition in 25% of patients with IgAN, which associated with disease severity. This finding is supported by the association of glomerular C4d deposition with poor prognosis in IgAN.[12,21]

We hypothesized that the lectin pathway contributes to glomerular inflammation and disease severity in IgAN. We examined (i) levels of circulating lectin pathway components; (ii) glomerular complement deposition; and (iii) glomerular fH, FHR1, and FHR5 deposition in IgAN. Using a cohort of patients with IgAN stratified into those with either stable or progressive disease, we identified circulating lectin pathway components, glomerular complement protein deposition, and immunohistologic evidence of fH deregulation that correlated with disease severity.

METHODS

Study Cohort and Clinical Measurements

We expanded our previously characterized[11] Causes and Predictors of Outcome in IgA Nephropathy study cohort of patients with biopsy-proven IgAN to 323 patients (Supplementary Figure S1, UK National Research Ethics Service Committee number 14/LO/0155). Progressive disease was defined by at least 1 of the following criteria: (i) end-stage renal disease without histology evidence of a second pathology causing renal impairment; (ii) biopsy evidence of endocapillary hypercellularity, or (iii) cellular and/or fibrocellular crescents; (iv) treatment with immunosuppression for native IgAN; (v) clinical Henoch-Schonlein purpura, unless spontaneous resolution and >20 years of follow-up with "stable" criteria; or (vi) 50% loss of estimated glomerular filtration rate (eGFR) or average annual loss of eGFR of more than 5 ml/min without evidence of a second pathology causing renal impairment. Stable disease was defined as meeting all of the following: (i) urine protein-creatinine ratio less than 100 units or daily proteinuria of less than 1 g/24 hours; (ii) combined Oxford classification[22] MEST (mesangial hypercellularity [M], endocapillary hypercellularity [E], segmental glomerulosclerosis [S], interstitial fibrosis/tubular atrophy [T]) score of less than 3; and (iii) average annual loss of eGFR of less than 3 ml/min per 1.73 m^2. The transplantation cohorts have also been characterized.[11] Control samples were obtained from healthy volunteers. The eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration Creatinine Equation.[23]

Protein Measurements

Levels of MBL,[24] M-ficolin,[25] H-ficolin,[26] CL-L1,[27] CL-K1,[28] MASP-1,[29] MASP-2,[30] MASP-3,[31] MAp19,[32] and MAp44[31] were measured using time-resolved immunofluorometric sandwich-type immunoassays as previously described using "in-house" antibodies. Plasma L-ficolin was measured by enzyme-linked immunosorbent assay (Hycult Biotech, Uden, The Netherlands). Serum IgA and galactose-deficient IgA1 levels were measured by enzyme-linked immunosorbent assay.[33]

Histology

Immunohistochemistry protocols were optimized (Supplementary Figures S2–S4) for formalin-fixed paraffin-embedded renal biopsy tissue with the following antibodies: rabbit polyclonal anti-human C3c (Dako, Glostrup, Denmark), rabbit polyclonal anti-human C4d (DB Biotech, Kosice, Slovakia), mouse monoclonal anti-human factor H (OX-24; Abcam, Cambridge, UK), rabbit polyclonal anti-human C3d (Abcam), mouse monoclonal anti-human C5b9 (Dako), mouse monoclonal anti-human FHR1 (Abnova, Taipei, Taiwan), and rabbit polyclonal anti-human FHR5 (Abnova). The anti-C3c antibody cannot distinguish among C3c, C3b, and iC3b, so we refer to this staining as anti-C3b/iC3b/C3c. We graded antigen-staining

intensities from anonymized sections as 0 (absent), 0.5 (minimal), 1+, 2+, and 3+. Staining described as "positive" includes 1+, 2+, and 3+. Staining described as "negative" includes 0 and 0.5. For tubular cell FHR1 staining, we used the area of most intense staining to grade tubular cell FHR1 staining intensity from 0 to 3. We identified 41 IgAN biopsies with median of 6 glomeruli per section (range 2–16). All biopsies had absent or nonsignificant C1q staining documented in clinical reports. We excluded sections that contained <2 nonsclerosed glomeruli.

Statistical Analysis

Analyses were performed using GraphPad Version 6.00 for Windows (La Jolla, CA). Normally distributed continuous variables were compared using unpaired or paired *t*-test and 1-way analysis of variance for multiple groups. Continuous variables with skewed distribution were tested using Mann-Whitney *U* tests, Kruskal-Wallis tests for multiple groups, and Wilcoxon matched-pairs signed rank test for matched transplant samples. Confidence intervals (CIs) were calculated using the Hodges-Lehmann method; categorical data compared using the Fisher exact test; and correlation assessed using Pearson or Spearman rank tests. We adjusted for multiple analyses with the 2-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli.[34]

RESULTS

Plasma M-Ficolin, L-Ficolin, MASP-1, and MAp19 Are Increased, Whereas Plasma MASP-3 Levels Are Reduced in IgAN

Due to the large number of cases and lectin proteins, we quantified lectin pathway plasma concentrations in 2 stages. In the assessment cohort of 125 patients with IgAN and 211 controls, we measured the plasma concentrations of MBL, MASP-1, MASP-2, MASP-3, MAp19, MAp44, CL-K1, CL-L1, M-ficolin, H-ficolin, and L-ficolin (Table 1). We found plasma levels of M-ficolin, L-ficolin, MASP-1, and MAp19 were increased, whereas MASP-3 levels were reduced in patients. This was confirmed in our entire patient cohort ($n = 323$, Table 1). The difference in MBL level seen in the assessment cohort between white patients with IgAN and healthy controls was not replicated. The proportion of MBL-deficient individuals (plasma concentration <100 ng/ml) did not differ between patients and controls (Table 1). Notably, as the 2 proteins are alternative splice products from the same gene (*MASP-2*), 1 patient had a very low plasma MAp19 level (<65 ng/ml) but normal plasma MASP-2 level (180 ng/ml). M-ficolin is expressed in peripheral blood leucocytes.[35] There was a positive correlation between plasma

Table 1. Circulating lectin pathway protein levels in IgA nephropathy

	Assessment cohort				Complete cohort			
Results	Patients with IgAN, median (range), $n = 125$	Healthy controls, median (range), $n = 211$	Difference between medians	95% CI	Patients with IgAN, median (range), $n = 323$	Healthy controls, median (range), $n = 262$	Difference between medians	95% CI
MBL, ng/ml	1066 (<10–7202)	1839 (<10–7202)	−753	−567 to 17	1507 (<10–7002)	1857 (<10–7202)	−50	−249 to 145
MBL, white only, ng/ml	1062 (<10–6000), $n = 86$	1839 (<10–7202), $n = 211$	−777[c]	−714 to −9	1471 (<10–6556), $n = 270$	1534 (<10–7202), $n = 256$	−63	−235 to 170
MBL <100, ng/ml	15 (12.0%)	26 (12.3%)			43 (13.3%)	32 (12.2%)		
M-ficolin, ng/ml	4570 (1443–16536)	4201 (1458–10243)	369[b]	181 to 702	5422 (1002–16536)	4124 (1159–10243)	1298[c]	1066 to 1546
H-ficolin, ng/ml	35466 (9617–62262)	36903 (6928–74906)	−837	−3881 to 1185				
L-ficolin, ng/ml	3062 (963–6400)	2701 (982–6267)	341[a]	49 to 576	3463 (672–9230)	2740 (659–6381)	725[c]	543 to 960
CL-L1, ng/ml	839 (295–999)	834 (337–769)	1	−22 to 15				
CL-K1, ng/ml	405 (111–926)	391 (206–665)	14	−1 to 27				
MASP-1, ng/ml	10326 (4343–20322)	7789 (3188–15494)	2536[c]	1949 to 2936	10323 (2349–66002)	8091 (4490–18130)	2233[c]	1246 to 2327
MASP-2, ng/ml	514 (114–1376)	490 (110–1686)	24	−20 to 69				
MASP-3, ng/ml	6246 (2924–12101)	7036 (2942–14922)	−790[b]	−1092 to −218	6836 (2856–12101)	7026 (2942–14922)	−1192[c]	−1411 to −826
MAp19, ng/ml	588 (<60–926)	489 (186–1140)	99[c]	73 to 125	552 (<60–996)	485 (186–1140)	67[c]	51 to 89
MAp44, ng/ml	2406 (1022–4366)	2361 (1323–4417)	57	−60 to 174				

CI, confidence interval; CL-K1, collectin kidney-1; CL-L1, collectin liver-1; IgAN, IgA nephropathy; MAp, MBL-associated protein; MASP, MBL-associated serine protease; MBL, mannan-binding lectin.
[a] $P < 0.05$.
[b] $P < 0.005$.
[c] $P < 0.0001$.

M-ficolin and white cell count ($r = 0.38$, $P < 0.0001$; Supplementary Figure S5). However, 93% of our cohort had a white cell count in the normal range. L-ficolin, MASP-1, MAp19, and MASP-3 are expressed by hepatocytes.[13] We identified a positive correlation between plasma MASP-3 and alanine aminotransferase ($r = 0.31$, $P = 0.0015$; Supplementary Figure S5), a marker of liver inflammation. No associations were identified between alanine aminotransferase and plasma L-ficolin, MASP-1, or MAp19 concentrations (data not shown). Plasma MASP-3 levels did not correlate with proteinuria (Supplementary Figure S5).

Plasma M-Ficolin and MAp19 Levels Are Influenced by Glomerular Filtration Rate

There was no relationship between eGFR and plasma levels of either L-ficolin or MASP-1 (Supplementary Figure S6). Plasma MASP-3 levels positively correlated ($r = 0.29$, $P < 0.0001$), whereas M-ficolin and MAp19 negatively correlated with eGFR ($r = -0.17$, $P = 0.002$ and $r = -0.13$, $P = 0.02$, respectively; Supplementary Figure S6). Increased plasma M-ficolin and MAp19, and reduced MASP-3 levels, were still evident when we compared patients with preserved eGFR (>60 ml/min) with healthy controls (Figure 1b). These differences increased in magnitude when we compared patients with reduced eGFR (<30 ml/min) with healthy controls (Figure 1b). To determine if these changes were solely due to their association with progressive disease, we compared plasma levels before and after renal transplantation.[11] Although plasma M-ficolin and MAp19 levels fell significantly posttransplantation in both IgAN and a cohort of adult polycystic kidney disease, plasma MASP-3 levels did not change (Figure 1c). We concluded

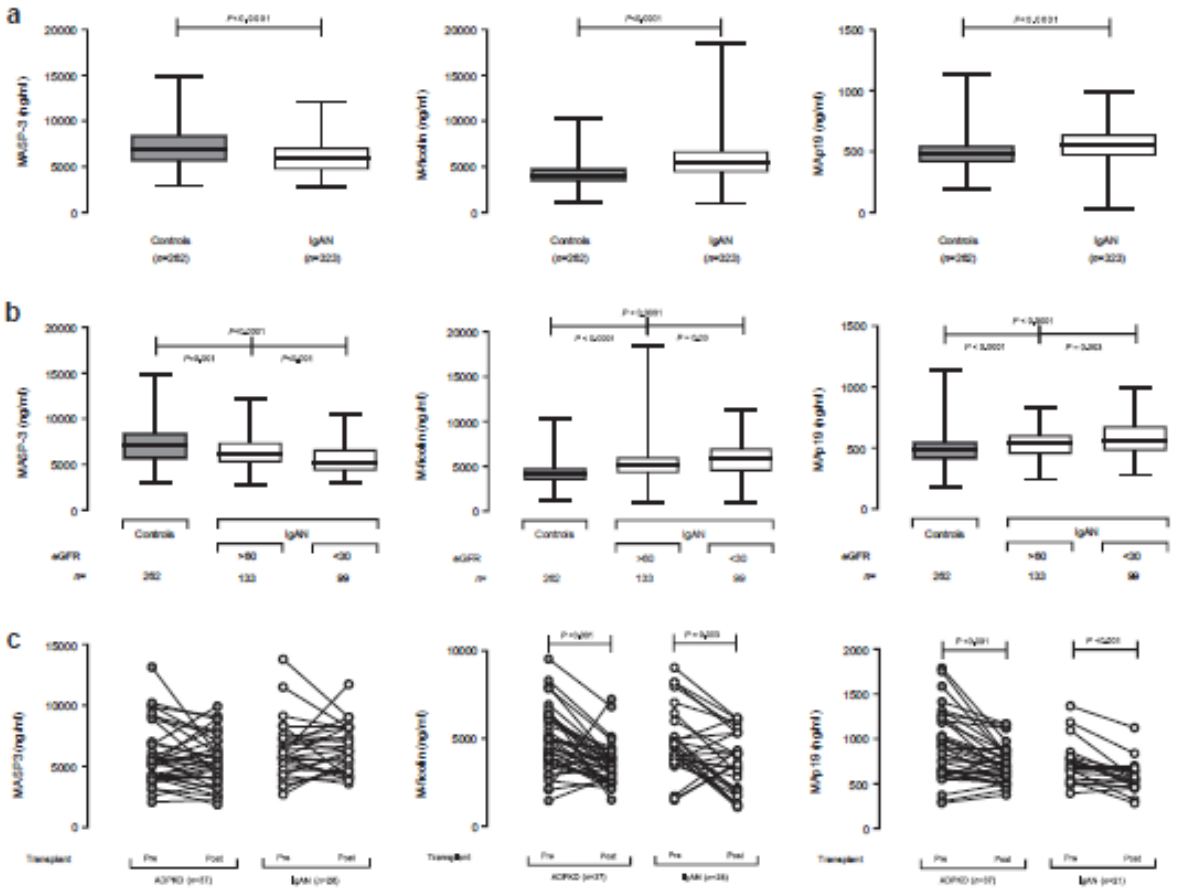

Figure 1. Plasma lectin pathway components in IgA nephropathy. (a) Plasma mannan-binding lectin (MBL)–associated serine protease (MASP)-3 (left), M-ficolin (middle), and MBL-associated protein (MAp) 19 (right) in patients with IgAN and healthy controls. *P* values derived from the Mann-Whitney test. (b) Plasma MASP-3 (left), M-ficolin (middle), and MAp19 (right) in patients with IgAN with either preserved (>60 ml/min) or reduced (<30 ml/min) estimated glomerular filtration rate (eGFR) and healthy controls. *P* values derived from the Kruskal-Wallis test. (c) Plasma MASP-3 (left), M-ficolin (middle), and MAp19 (right) before and after renal transplantation in patients with either IgAN (white circles) or autosomal dominant polycystic kidney disease (ADPKD, gray circles). *P* values derived from Wilcoxon matched-pairs signed rank test.

that M-ficolin and MAp19 levels were influenced by both IgAN and eGFR, whereas MASP-3 levels were influenced only by IgAN.

MASP-3 Plasma Levels Are Associated With IgAN Severity

To explore the significance of the altered lectin pathway levels in IgAN, we compared patients with either stable or progressive disease. M-ficolin, L-ficolin, MASP-1, and MAp19 did not differ between the groups (Supplementary Figure S7); however, MASP-3 levels were reduced in patients with progressive disease (Figure 2a), including those with progressive disease despite immunosuppressive therapy (Figure 2b). Given the demonstrated stability of MASP-3 serology levels over time,[31,36] we compared plasma levels (sampled at recruitment) with the Oxford Classification of IgA Nephropathy[22] scores from diagnostic renal biopsies. Plasma MASP-3 levels were lower in the patient cohort with biopsy evidence of mesangial hypercellularity (Figure 2c) and tubular atrophy (Figure 2d). Plasma MAp19 levels were higher in patients with segmental sclerosis (mean 576 vs. 539 ng/ml; difference 37; 95% CI: 2–73 ng/ml; $P = 0.040$). We did not identify associations between histology parameters and M-ficolin, L-ficolin, or MASP-1 levels.

Glomerular Complement Deposition Is Associated With Progressive IgAN

To understand the significance of the association between MASP-3 levels and progressive IgAN, we assessed complement deposition in our stable and progressive cohorts (Figure 3). In progressive compared with stable disease, there was proportionately greater glomerular staining for C3b/iC3b/C3c (odds ratio [OR]: 5.66; 95% CI: 1.49–23.39; $P = 0.02$), C3d (OR: 17.6; 95% CI: 3.01–89.97; $P = 0.001$), C4d (OR: 8.32; 95% CI: 2.00–30.33; $P = 0.004$), and C5b9 (OR: 12.14; 95%

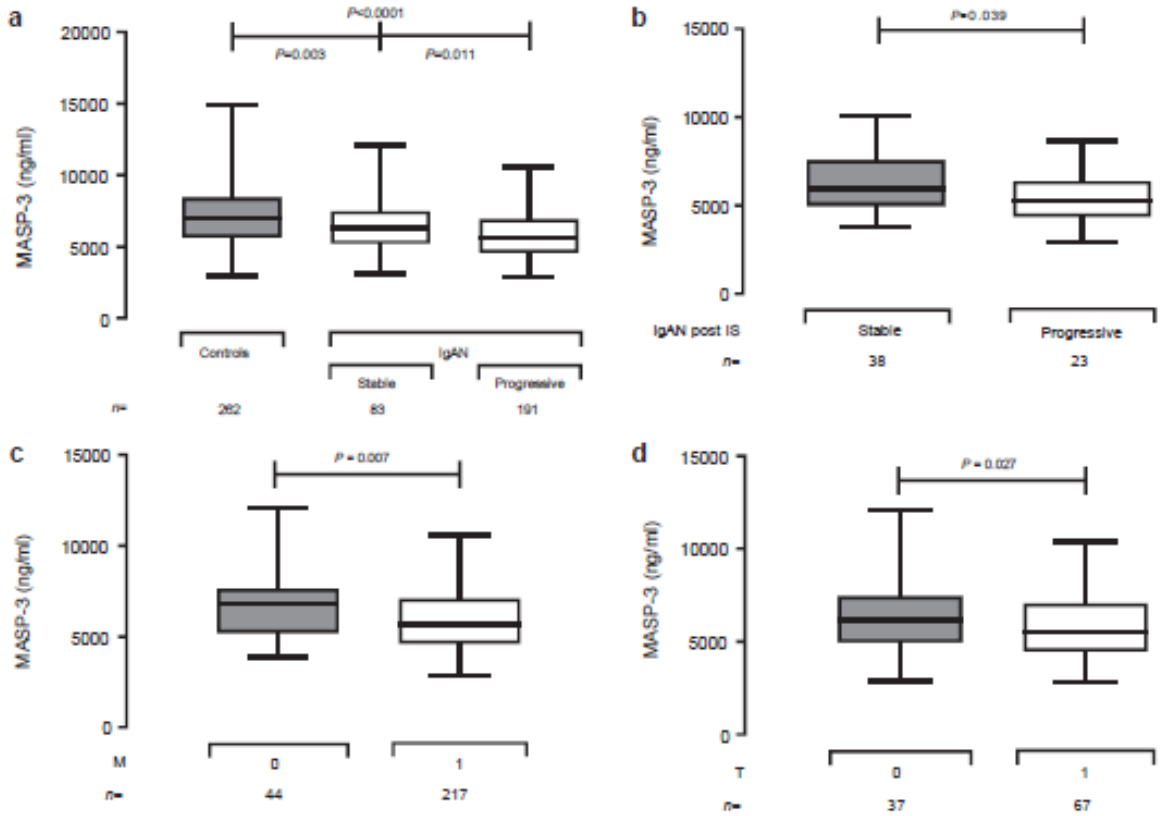

Figure 2. Plasma MASP-3 and progressive IgA nephropathy (IgAN). (a) Plasma mannan-binding lectin (MBL)–associated serine protease (MASP)-3 levels in patients with stable or progressive IgAN compared with healthy controls. *P* values derived from the Kruskal-Wallis test. (b) Plasma MASP-3 levels in patients with stable or progressive IgAN despite immunosuppressive therapy (IS). *P* values derived from the Mann-Whitney test. (c) Plasma MASP-3 plasma levels in patients with IgAN without (0, gray box) and with (1, white box) mesangial hypercellularity (M). (d) Plasma MASP-3 plasma levels in patients with IgAN without (0, gray box) and with (1, white box) tubular atrophy. *P* values derived from the Mann-Whitney test.

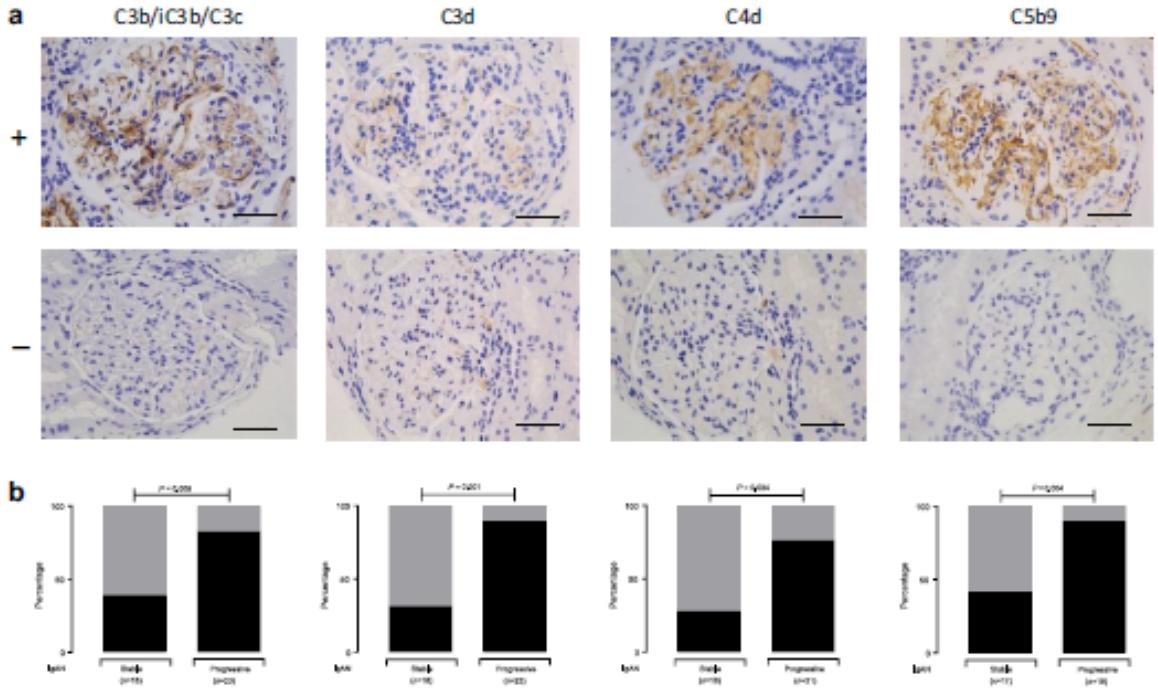

Figure 3. Glomerular complement staining in IgA nephropathy (IgAN). (a) Representative images for complement C3b/iC3b/C3c, C3d, C4d, or C5b9. The top row represents present (+) and the bottom row represents negative (–) staining. Original magnification ×400. Bar = 100 μm. (b) Proportion of cases with present (black) versus absent/uncertain (gray) glomerular staining in stable and progressive IgAN. *P* values derived from the Fisher exact test.

CI: 1.95–61.35; $P = 0.004$). Glomerular C5b-9 staining, a marker of complement terminal pathway activation, significantly correlated with both glomerular C3b/iC3b/C3c and C3d but not C4d staining (Table 2). There was no correlation between glomerular C4d and either C3d or C3b/iC3b/C3c.

Glomerular FHR5 Deposition Is Associated With Progressive IgAN

We have previously shown levels of negative (fH) and positive regulators (FHR1, FHR5) of the complement alternative pathway associated with progressive IgAN.[11] In progressive disease, there was more glomerular staining for FHR5 (OR: 13.4; 95% CI: 2.2–66.9; $P = 0.002$) and a trend for greater FHR1 staining (Figure 4). In contrast, glomerular staining for fH was significantly reduced in progressive compared with stable disease (OR: 0.10; 95% CI: 0.008–0.87; $P = 0.04$). Glomerular FHR5 staining correlated with glomerular C3b/iC3b/C3c, C3d, and C5b-9 but not C4d (Table 2). In aggregate, a heat map of the glomerular staining data showed that the renal biopsies from patients with progressive disease had more staining for C3b/iC3b/C3c, C3d, C4d, C5b9, and FHR5 than those with stable disease (Figure 5). Interestingly, the combination of FHR5 staining with negative fH staining was significantly more common in patients with progressive (15/18, 88.2%) versus stable (4/16, 25%)

Table 2. Correlation of mesangial complement antigen intensity in IgA nephropathy native renal biopsies

Correlation coefficient *R* values	C3b/iC3b/C3c	C3d	C4d	C5b9	fH	FHR1	FHR5
C3b/iC3b/C3c	–	0.45 ($P = 0.01$)	0.31 ($P = 0.09$)	0.57 ($P < 0.001$)	0.06 ($P = 0.52$)	–0.28 ($P = 0.8$)	0.64 ($P < 0.001$)
C3d		–	0.22 ($P = 0.20$)	0.53 ($P = 0.003$)	–0.29 ($P = 0.11$)	–0.11 ($P = 0.42$)	0.68 ($P < 0.001$)
C4d			–	0.34 ($P = 0.08$)	–0.22 ($P = 0.19$)	0.08 ($P = 0.45$)	0.27 ($P = 0.12$)
C5b9				–	–0.26 ($P = 0.14$)	–0.31 ($P = 0.09$)	0.75 ($P < 0.001$)
fH					–	–0.33 ($P = 0.09$)	–0.16 ($P = 0.30$)
FHR1						–	–0.31 ($P = 0.09$)
FHR5							–

A correlation of complement antigen-staining intensities in sections from the same biopsy, using the 0, 0.5, 1+, 2+, and 3+ scale. *R* values are calculated from Spearman's rank correlation. *P* values shown have been adjusted for multiple analyses to minimize the false discovery rate (using the 2-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli). fH, factor H; FHR1, factor H-related protein 1; FHR5, factor H-related protein 5.

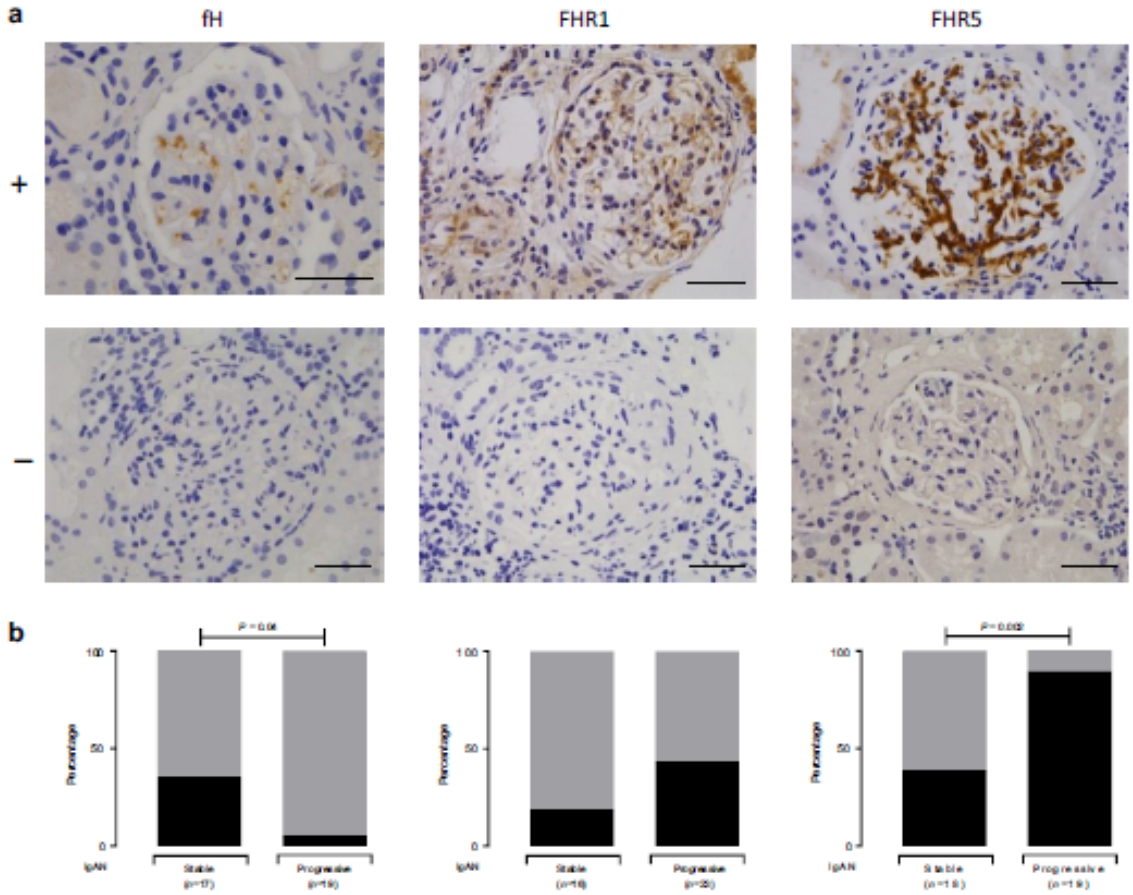

Figure 4. Glomerular staining for alternative pathway regulators in IgA nephropathy (IgAN). (a) Representative images for complement factor H (fH), and factor H–related proteins 1 (FHR1) and 5 (FHR5). The top row represents positive staining (+) and the bottom row represents negative staining (−). Original magnification ×400. Bar = 100 μm. (b) The proportion of cases with positive (black) versus absent/minimal (gray) glomerular staining in stable and progressive IgAN. *P* values derived from the Fisher exact test.

disease (OR: 15; 95% CI: 2.5–62.6; $P = 0.001$). Only 1 of 14 (6.7%) stable patients had glomerular FHR1 staining and negative fH staining compared with 9 of 19 (47.4%) in the progressive cohort (OR: 12.6; 95% CI: 1.7–146.4; $P = 0.02$). Surplus renal biopsy tissue was available from 1 patient with stable and 1 with progressive IgAN who had homozygous deletion of the CFHR3 and 1 genes. Glomerular FHR1 staining was negative in both cases, but glomerular C3b/iC3b/C3c, C3d, C4d, C5b9, and FHR5 was detectable (Figure 5). Surplus renal biopsy tissue was available from 3 patients with MBL deficiency: 1 with stable and 2 with progressive disease (Figure 5). Glomerular C4d deposition was present in the 2 patients with progressive disease but negative in the patient with stable disease. Glomerular C3b/iC3b/C3c was detectable in all 3.

The IgAN cohort demonstrated a range of tubular FHR1 staining prevalence and intensity (Supplementary Figure S8). FHR1 staining was often present and absent in adjacent tubule segments (Supplementary Figure S8). Eleven patients showed no tubular staining for FHR1, 7 had 1+, 19 had 2+, and 4 had tubular cell FHR1 staining of 3+ intensity. The intensity of tubular cell FHR1 staining did not correlate with proteinuria, glomerular filtration, or the presence of tubular atrophy at the time of biopsy. And there was no correlation with disease severity. We did not have access to stored plasma samples to quantify the circulating FHR1 concentration at the time of biopsy.

DISCUSSION

We observed increased circulating M-ficolin, L-ficolin, MASP-1, and MAp19 levels in patients with IgAN. Conversely MASP-3 levels were reduced and associated with progressive disease. Renal impairment partially

IgAN severity	*CFHR3-1* copies	MBL deficient	C3b/iC3b/C3c	C3d	C4d	C5b9	fH	FHR1	FHR5
Stable	2	No	0	0	0	0.5	0	0	0
Stable	2	No	0	0.5	0.5	1	0.5	0.5	0
Stable	2	No	0.5	1	0	0	1		0.5
Stable	1	No	0.5	1	0	0	0	0.5	0
Stable	2	No	0.5	0.5	0	0.5	0.5	0	0.5
Stable	2	No	0.5		0.5	0		1	0
Stable	1	No	0.5	0.5	0.5	1	0	0.5	0.5
Stable	2	No	0.5	1	0.5	2	0	0.5	1
Stable	1	No	0.5	0.5	1	0.5	0.5	0.5	0.5
Stable	1	No	0.5	1	1	0.5	0	1	1
Stable	2	No	0.5	0		1		0.5	1
Stable	1	No	1	0	0	3	0.5	0	2
Stable	2	Yes	1		0.5		1	1	2
Stable	2	No	1	0.5	0.5	0.5	1		0.5
Stable	2	No	1	0.5	1	1	0	0.5	1
Stable	0	No	1	1	1	2	1	0	2
Stable	2	No	1	0	2	0	1	0.5	0
Stable	2	No	2	0.5	0.5	0	1	0	0.5
Stable	2	No			0		0.5		
Progressive	2	No	0.5					1	1
Progressive	2	No	0.5	1	1	1	0	2	1
Progressive	2	No	0.5	2	0.5	1	0	1	2
Progressive	2	No	0.5	1	1	2	0	0.5	2
Progressive	1	No	1		0.5			0	
Progressive	1	No	1	1				0	
Progressive	1	Yes	1	0.5	1	0.5	0	2	0.5
Progressive	2	No	1	0.5	1	0.5	0	2	
Progressive	2	Yes	1	1	1	1	0.5	1	1
Progressive	1	No	1		1	1		0.5	
Progressive	2	No	1	1	1	2	0	2	1
Progressive	2	No	1	1	1	2	0	0	1
Progressive	1	No	1	1	1	2	0.5	0	2
Progressive	0	No	1	1	2	2	0.5	0	2
Progressive	2	No	1	1	2	3	0	1	0
Progressive	1	No	2		0.5		0	1	2
Progressive	2	No	2		0.5	2	0	1	2
Progressive	1	No	2	2	1	2	0	0	3
Progressive	2	No	2	2	1	2	0	0.5	3
Progressive	2	No	2	2	1	3	0	0	3
Progressive	2	No	3	2	2	3	0	0	3
Progressive	2	No	3	1	1	3	0	0.5	3
Progressive	1	No	3	2	0.5	3	1	0	3

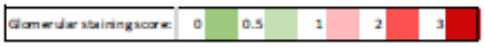

Figure 5. Complement glomerular deposition in IgA nephropathy (IgAN). Glomerular staining intensity scores from surplus native renal biopsy tissue from patients with either stable or progressive IgAN. Each row represents information from a single patient. Staining intensity was scored: 0, absent; 0.5, minimal; 1, mild; 2, moderate; 3, strong. Filled cells indicate insufficient renal tissue to perform staining. Mannan-binding lectin (MBL) deficiency was defined as a plasma level of less than 100 ng/ml. *CFHR3-1*, complement factor H–related 3 and 1 genes; fH, complement factor H; FHR1, factor H–related protein 1; FHR5, factor H–related protein 5.

explained the differences in M-ficolin and MAp19 levels, because these levels changed significantly following renal transplantation. M-ficolin is mainly synthesized in monocytes and granulocytes,[37] but almost all of our patients had a normal white cell count. Although MASP-3 showed a positive correlation with alanine aminotransferase, we identified negative correlations with MASP-3 levels and IgAN severity. We therefore think it is unlikely that the M-ficolin and MASP-3 results are explained by confounding with

leucocyte count and liver inflammation, respectively. Furthermore, MBL,[38] M-ficolin,[25] L-ficolin,[39] MASP-1,[40] and MAp19[32] are not acute-phase proteins. Circulating MASP-3 levels did not correlate with proteinuria, excluding this as a cause for the low levels, and there is no clear genotype-phenotype correlation to explain the variation in MASP-3 levels.[41]

How these changes relate to IgAN pathogenesis is unclear. Increased MASP-1[42] and MAp19[42] levels occur in lupus nephritis, so these changes are not specific to IgAN. Glomerular L-ficolin and MASP1/3 staining correlated with IgAN severity,[20] but circulating L-ficolin levels did not.[20] In fact, reduced circulating L-ficolin levels have been reported in lupus nephritis,[43] although this was not replicated in a Japanese cohort,[44] in addition to reduced M-ficolin levels.[29] M-ficolin[35] and L-ficolin[45] are capable of triggering lectin pathway activation, and MASP-1 is essential for MASP-2 and lectin complement activation,[46] so higher levels of these 3 components could be associated with greater complement activation within the kidney. The significance of the raised MAp19 is unclear. It has been shown to have a regulatory role on lectin pathway activation *in vitro*,[47] but the physiological relevance of this is unknown.[32]

MASP-3 levels are lower in patients with systemic lupus erythematosus with nephritis compared with those without,[42] so low MASP-3 levels are not specific to IgAN. When MASP-3 binds to pattern-recognition molecules, it can displace MASP-2 and MASP-1,[48] and, because it does not lead to convertase formation,[49] inhibit activation.[48,50] Low levels of MASP-3 could be associated with increased complement activation. However, MASP-3 activates pro-factor D to factor D,[51] a requirement for C3-convertase formation.[52] Whether or not glomerular activation of the alternative pathway in either lupus nephritis or IgAN directly influences MASP-3 levels is unknown.

We replicated the association between IgAN severity and glomerular C3b/iC3b/C3c[10] and C4d.[20,21] Our data also showed an association between progressive IgAN and glomerular C3d and C5b9. Glomerular C4d did not correlate with either C3b/iC3b/C3c or C5b9. This may reflect technical limitations (e.g., sample size, staining procedure), but also could be because the amount of C4d, compared with C3 and C5b-9, will be lower after glomerular complement activation. Previous studies have identified glomerular C3 and C5b9 in most C4d-positive and -negative cases, but did not record antigen correlations.[20,21]

The correlation of glomerular FHR5 deposition with progressive IgAN is a key finding of our study. Glomerular FHR5 correlated with C3b/iC3b/C3c, C3d, and C5b9 staining, as previously reported.[53] In a proteomic analysis, glomerular FHR5 was 1.79 times more abundant in patients with progressive versus stable IgAN.[54] FHR5 antagonizes the ability of fH to negatively regulate C3 activation.[55,56] Consequently, it was interesting that patients with progressive disease had more cases of glomerular FHR5 staining in the absence of fH. Notably, there are phenotypic similarities between IgAN and familial C3 glomerulopathy associated with mutant FHR5 proteins.[57,58]

Considering the genetic and serology associations between IgAN and FHR1,[11,12,59,60] it was surprising that glomerular FHR1 was absent in more than 50% of progressive IgAN biopsies. Unlike FHR5, glomerular FHR1 did not correlate with other complement antigens. This could be explained by differences in binding avidity of FHR1 and FHR5 to C3b, iC3b, and C3dg.[55] Nevertheless, our data indicate a more prominent role for FHR5 than FHR1 in complement activation in IgAN. Notably, 1 patient with progressive disease was deficient in FHR1.

The cause of tubular cell FHR1 staining is unclear. It did not correlate with proteinuria at the time of biopsy. It may result from changes to the tubular cell membrane as a consequence of nephron loss or changes in tubular fluid characteristics, such as acidity, or tubular epithelia or glycocalyx features.

Our complement staining data demonstrated the pathogenic heterogeneity of IgAN. For example, the co-deposition of FHR5, C3d, C3b/iC3b/C3c, and C5b9, especially in the absence of fH, implies FHR5-dependent fH deregulation and alternative pathway activation, and glomerular co-deposition of C4d with C3b/iC3b/C3c and C5b9 may reflect complement activation triggered by the lectin pathway. Interestingly, all 4 biopsies from patients with progressive disease and negative glomerular C4d had FHR5 staining. Identifying and understanding this heterogeneity of complement activity might be clinically important because we now have the ability to target complement activation at specific points in the activation sequence. For example, OMS721, a monoclonal antibody targeting MASP-2, was recently designated breakthrough therapy status for IgAN treatment; Eculizumab, a C5 inhibitor, has been used in recurrent[61] and progressive[62,63] IgAN; and factor D inhibitors are in clinical trials for C3 glomerulopathy.[64]

Although our observations require confirmation in larger IgAN cohorts, our data indicate (Figure 6) that (i) circulating MASP-3 is a potential biomarker of disease severity in IgAN; and (ii) glomerular FHR5 staining of diagnostic biopsies can identify those with severe disease who are at risk of progression to renal impairment.

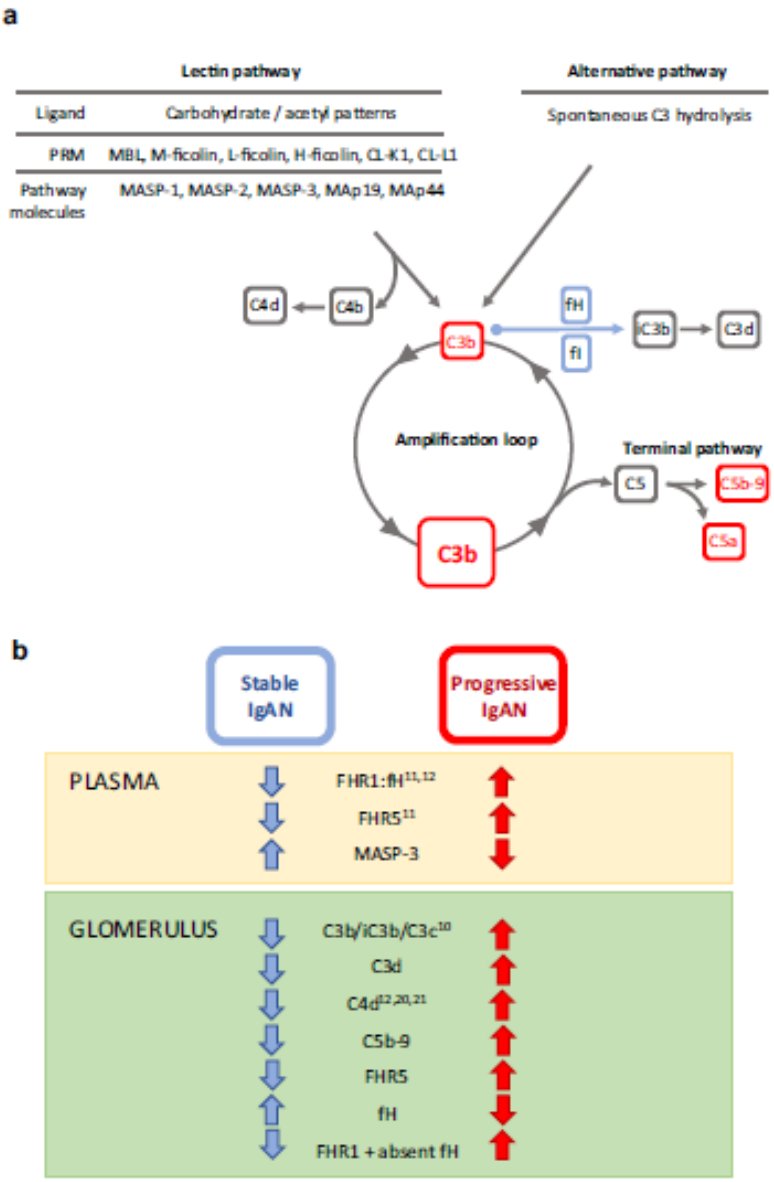

Figure 6. Complement and IgA nephropathy. (a) Schematic diagram depicting lectin and alternative pathway complement activation. Lectin pathway activation is triggered by the binding of pattern-recognition molecules (PRMs) to carbohydrate or acetyl molecular patterns. Alternative pathway activation happens through the spontaneous, constant generation of reactive forms of C3. Both pathways result in the generation of C3b. C3b can be proteolytically cleaved to iC3b and C3d by complement factor I in the presence of cofactors, such as complement factor H. Similarly, C4b produced during lectin pathway activation can be cleaved to C4d. C3b generation can be rapidly amplified through an amplification loop. This results in the generation of large amounts of the opsonin C3b and can trigger complement C5 activation. This leads to the generation of the anaphylatoxin C5a, and the membrane attack complex (C5b-9) through the terminal pathway. fH, factor H; fI, factor I; MAp, MBL-associated protein; MASP, MBL-associated serine protease; MBL, mannose-binding lectin. (b) Complement proteins and severity of IgA nephropathy. Within plasma, increased levels of FHR1,[11,12] FHR5,[11] and the FHR1:fH ratio[11,12] associate with progressive IgAN. Conversely, we found that reduced levels of MASP-3 associated with progressive disease. Within glomeruli, we replicated the association between increased C4d[12,20,21] and C3b/iC3b/C3c[10] with IgAN severity, and we showed that increased glomerular C3d, C5b-9, and FHR5 associated with progressive disease. The presence of FHR1 in the absence of fH was also more frequently seen in patients with progressive disease. FHR1, factor H–related protein 1; FHR5, factor H–related protein 5. (Continued)

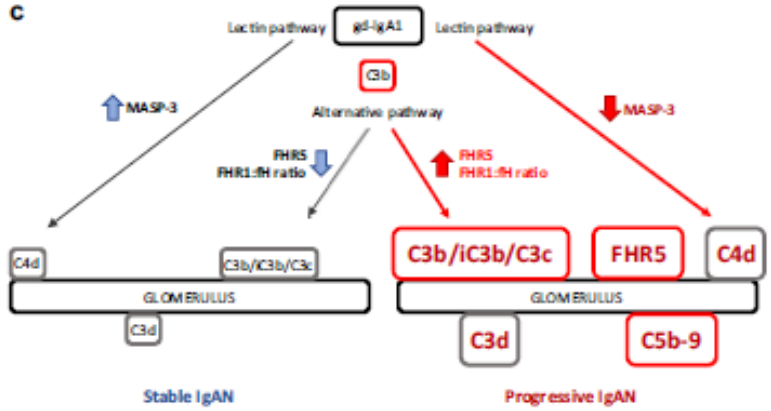

Figure 6. (Continued) (c) A hypothetical depiction of glomerular complement activation in IgA nephropathy. Galactose-deficient IgA1 (gd-IgA1) activates the lectin and alternative complement pathways in IgAN.[8] Glomerular complement deposition is enhanced in progressive disease. Glomerular complement activation is influenced by FHR5, and the FHR1-fH ratio and associated with changes in circulating MASP-3 levels. Changes in FHR1, FHR5, and fH influence complement activation through the alternative pathway. fH negatively regulates activation, whereas FHR1 and FHR5 promote activation through antagonizing fH ("fH deregulation"). The mechanism through which circulating MASP-3 levels fall in progressive disease are not understood but are presumed to be linked to lectin pathway activation. Red text highlights proteins demonstrated to associate with progressive IgAN, and the larger boxes indicate more deposition.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure S1. Table of IgA nephropathy and healthy control cohort characteristics.

Figure S2. Representative images of renal immunohistochemistry staining for complement pathway antigens: C3b/iC3b/C3c, C3d, C4d, and C5b-9. Original magnification ×400. Bar = 100 μm.

Figure S3. Representative images of renal immunohistochemistry staining for complement pathway antigens: FHR1, FHR5, and fH. Original magnification ×400. Bar = 100 μm.

Figure S4. Representative image of liver immunohistochemistry staining for fH. Original magnification ×400. Bar = 100 μm.

Figure S5. Graphs showing the (a) correlation of plasma MASP-3 levels and urine protein-creatinine ratio (PCR) in patients (n = 223) with IgA nephropathy (IgAN); (b) the correlation of plasma M-ficolin with white cell count (WCC) in patients (n = 108) with IgAN; and (c) the correlation between mannan-binding lectin (MBL)-associated serine protease (MASP)-3 with alanine aminotransferase (ALT) in patients (n = 108) with IgAN.

Figure S6. Graphs showing the correlation between estimated glomerular filtration rate (eGFR) and (a) M-ficolin, (b) mannan-binding lectin (MBL)-associated protein (MAp) 19, (c) MBL-associated serine protease (MASP)-3 levels, (d) L-ficolin, and (e) MASP-1 levels.

Figure S7. Table of circulating lectin pathway protein levels in stable and progressive IgA nephropathy.

Figure S8. Renal immunohistochemistry staining for factor H–related protein 1 (FHR1) in IgA nephropathy. Representative images of tubular staining intensity 3+ (a), 2+ (b), 1+ (c), and 0 (d–f). (f) Representative image from a patient with complete FHR1 deficiency. Bar = 100 μm. Correlation of tubular FHR1 staining intensity with estimated glomerular filtration rate (eGFR, g) at the time of biopsy, urine protein-creatinine ratio (UPCR) at the time of biopsy (h), the presence (T1 or T2) or absence (T0) of tubular

atrophy from the biopsy report (i); and disease severity at enrollment (j). No correlations reached statistical significance. Supplementary material is linked to the online version of the paper at www.kireports.org.

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Circulating complement factor H–related proteins 1 and 5 correlate with disease activity in IgA nephropathy

see commentary on page 790

OPEN

Nicholas R. Medjeral-Thomas[1,6], Hannah J. Lomax-Browne[1,6], Hannah Beckwith[1], Michelle Willicombe[2], Adam G. McLean[2], Paul Brookes[3], Charles D. Pusey[4], Mario Falchi[5], H. Terence Cook[1] and Matthew C. Pickering[1]

[1]Centre for Complement and Inflammation Research, Imperial College London, UK; [2]Renal and Transplant Centre, Imperial College Healthcare NHS Trust, London, UK; [3]Histocompatibility & Immunogenetics, Imperial College Healthcare NHS Trust, London, UK; [4]Renal and Vascular Inflammation Section, Imperial College London, UK; and [5]Department of Twin Research and Genetic Epidemiology, King's College London, UK

IgA nephropathy (IgAN) is a common cause of chronic kidney disease and end-stage renal failure, especially in young people. Due to a wide range of clinical outcomes and difficulty in predicting response to immunosuppression, we need to understand why and identify which patients with IgAN will develop progressive renal impairment. A deletion polymorphism affecting the genes encoding the complement factor H-related protein (FHR)-1 and FHR-3 is robustly associated with protection against IgAN. Some FHR proteins, including FHR-1 and FHR-5, antagonize the ability of complement factor H (fH), the major negative regulator of the complement alternative pathway, to inhibit complement activation on surfaces, a process termed fH deregulation. From a large cohort of patients, we demonstrated that plasma FHR-1 and the FHR-1/fH ratio were elevated in IgAN and associated with progressive disease. Plasma FHR-1 negatively correlated with eGFR but remained elevated in patients with IgAN with normal eGFR. Serum FHR5 was slightly elevated in IgAN but did not correlate with eGFR. Neither FHR5 levels nor the FHR-5/fH ratio was associated with progressive disease. However, higher serum FHR-5 levels were associated with a lack of response to immunosuppression, the presence of endocapillary hypercellularity, and histology scores of disease severity (the Oxford Classification MEST score). Thus, FHR-1 and FHR-5 have a role in IgAN disease progression.

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Correspondence: *Matthew C. Pickering, Centre for Complement and Inflammation Research, Imperial College London, Hammersmith Campus, Du Cane Road, London W12 0NN, UK. E-mail: matthew.pickering@imperial.ac.uk*

[6]NMT and HLB contributed equally to the research.

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IgA nephropathy (IgAN) is the most common primary glomerulopathy worldwide and is an important cause of chronic kidney disease, especially in young people.[1] IgAN is associated with a wide range of clinical outcomes. Although 40% of patients will have reached end-stage renal disease within 20 years of diagnosis, 20% of patients will have preserved renal function with only minor urinary abnormalities.[2] IgAN is characterized by dominant or codominant IgA-containing immune deposits on renal biopsy.[3] The pathophysiology of IgAN associated with characteristic galactose-deficient IgA1 (gd-IgA1)-containing immune deposits is considered to be a 4-hit mechanism.[4] However, the link between mesangial IgA deposition and the spectrum of clinical outcomes that are characteristic of IgAN is poorly understood. Therefore, we are limited in our ability to both identify patients for whom immunosuppression therapy is appropriate and develop novel therapeutic strategies. The current repertoire of clinical tools available to predict outcome and guide treatment strategies of IgAN, such as proteinuria or estimated glomerular filtration rate (eGFR), may reflect progressive glomerular scarring as well as active immunologically driven disease.

Systemic and renal complement activation in IgAN is well documented. However, the role of complement activation in IgAN pathogenesis remains poorly defined. Complement C3 accompanies IgA immunostaining in most diagnostic IgAN biopsies.[5] Evidence of complement alternative pathway activation was identified in the plasma of 30% to 75% of IgAN adults[6,7] and correlated with proteinuria and the rate of renal function loss in a cohort of 50 IgAN patients.[7] An association was demonstrated between mesangial C3 deposition, decreased serum C3 levels, and doubling of serum creatinine or reaching end-stage renal disease in a cohort of 343 IgAN patients.[8] This demonstrated a link between histology and serum markers of complement activation in IgAN pathogenesis. Complement activation by the mannose-binding lectin (MBL) complement pathway in IgAN has also been demonstrated. MBL and MBL-associated serine protease (MASP)-1 were detected in 24% of renal biopsies from IgAN

patients, but were identified in less than 3% of biopsies from patients with other forms of glomerulonephritis.[9] Deposited MBL and MASP-1 were associated with C3b, C3c, and C5b9, which are markers of alternative and terminal complement pathway activity.[9] Although this study did not show associations with markers of clinical outcomes, glomerular MBL and other MBL complement pathway components, including MASP-1/-3 and MASP-2, were found in 25% of a separate cohort of 60 IgAN patients, and these findings correlated with proteinuria and histologic features of severity.[10]

The ability of IgA to activate complement *in vitro*, predominantly through the alternative pathway, has also been demonstrated. IgA isolated from pooled human plasma triggers complement-dependent lysis of, and properdin deposition on, erythrocytes coated with mouse monoclonal antihuman IgA.[11] Furthermore, a rat model of IgA-mediated glomerular inflammation demonstrated that polymeric but not monomeric IgA triggered mesangial C3 deposition and not C4 or C1q deposition.[12]

Recent genetic studies implicate a role for the complement factor H related (FHR) proteins in IgAN.[13–16] The FHR proteins may interfere with the regulatory functions of factor H (fH), the major negative regulator of complement C3 activation.[17,18] This process is referred to as fH deregulation.[19,20] The deletion polymorphism of the genes coding FHR-3 and FHR-1 (del*CFHR3-1*) is associated with protection from IgAN.[13,14,16] A meta-analysis of approximately 20,000 individuals of different ethnicities estimated that the inheritance of the minor A allele at single-nucleotide polymorphism rs6677604, which tags the del*CFHR3-1* allele, reduced the risk of IgAN disease by 26% in heterozygosity and by 45% in homozygosity.[14] Across populations worldwide, del*CFHR3-1* frequency exhibits marked differences in a pattern inverse to that of IgAN prevalence.[14] An association has also been demonstrated between rs6677604 and histologic IgAN markers. In Chinese patients with IgAN, the rs6677604-A allele was associated with reduced mesangial C3 deposition, high serum fH levels, and low complement C3a levels but was not associated with clinical outcomes.[15] Xie *et al.* showed an association of del*CFHR3-1* with reduced segmental sclerosis and tubular atrophy in IgAN.[16] Although this was independent of eGFR and proteinuria, no other associations with clinical parameters or outcomes were demonstrated.

By enhancing complement activation in response to mesangial gd-IgA1-containing immune complexes, we hypothesized that fH deregulation influences disease severity in IgAN. In this study, we assessed circulating fH, FHR-1, and FHR-5 levels in IgAN patients who were stratified into cohorts with stable and progressive disease.

RESULTS

Patient cohort

The patient cohort characteristics are summarized in Table 1. Using the criteria detailed in the Materials and Methods, 179 patients had progressive IgAN and 89 had stable IgAN. We had insufficient data to categorize 26 patients as having either progressive or stable IgAN. The cohort of patients meeting the criteria for progressive IgAN showed lower eGFR and higher systolic blood pressure than the stable IgAN cohort (Table 1). Consistent with previous reports,[21–23] the median serum IgA and gd-IgA1 levels were higher in patients than in controls (Table 1). The progressive IgAN cohort showed lower median serum IgA and higher median serum gd-IgA1 levels than stable IgAN (Table 1). Similarly, of the patients who had received immunosuppression therapy, serum gd-IgA1 levels were higher in patients with progressive IgAN than stable IgAN after treatment (0.54 units [AU] vs. 0.44 AU, $P = 0.04$, Supplementary Figure S1A). Consistent with these data, we detected a negative correlation between serum gd-IgA1 levels and eGFRs at the sampling time point (Supplementary Figure S1B). The del*CFHR3-1* allele is associated with protection from IgAN.[13] However, we did not observe any difference in *CFHR3* and *CFHR1* copy numbers between patients with stable IgAN and those with progressive IgAN (Table 1).

Plasma FHR-1 levels and the FHR-1:fH ratio were elevated in IgAN and were associated with progressive disease

While fH is the major negative regulator of C3 activation via the complement alternative pathway, FHR-1 is postulated to act as a positive regulator by antagonizing the effect of fH, a process termed fH deregulation.[17–19,24] To investigate fH deregulation in IgAN, we measured both plasma fH and FHR-1 levels. The median plasma FHR-1 level was increased in IgAN patients compared with that in healthy controls, whereas the plasma fH level did not differ (Table 1). Notably, the relative abundance of these proteins differed between patients and healthy controls, and the FHR-1:fH ratio was significantly increased in IgAN patients (Table 1).

Because the presence of the del*CFHR3-1* allele will influence circulating FHR-1 levels (most clearly the absence of the protein in del*CFHR3-1* homozygotes), we stratified patients according to the *CFHR1* gene copy number. Patients with 2 copies of the *CFHR1* gene had higher FHR-1 levels compared with genotype-matched healthy controls (Figure 1a). This difference was not observed in patients with 1 *CFHR1* gene copy number (Figure 1a). However, the FHR-1:fH ratio was significantly higher in patients than in healthy controls, irrespective of the *CFHR1* gene copy number (Figure 1b). FHR-1 was undetectable in deletion homozygotes ($n = 12$, including 9 patients). Plasma fH levels remained similar between patients and controls when stratified according to the *CFHR1* gene copy number (Figure 1c). As previously reported,[15,25] fH levels were higher in del*CFHR3-1* homozygotes (Figure 1c).

We next examined if FHR-1 levels and the FHR-1:fH ratio differed between patients with stable IgAN and those with progressive IgAN. Irrespective of the *CFHR1* gene copy number, compared with patients with stable IgAN, those with progressive IgAN had significantly elevated plasma FHR-1 levels (Figure 1d) and FHR-1:fH ratios (Figure 1e), whereas fH levels did not differ (Table 1). In addition, the FHR-1:fH

Table 1 | Cohort characteristics

Variable	IgA nephropathy patients			Healthy controls (n = 161)
	Entire IgAN (n = 294)	Stable IgAN (n = 89)	Progressive IgAN (n = 179)	
Clinical features				
Male/Female	195/99	53/36	120/59	
Caucasian/Non-Caucasian	244/50	78/11	144/35	
Median age (range), yr	48.2 (18–84)	47.5 (18–82)	48.3 (19–84)	
Median eGFR, ml/min per 1.73 m^2	52.7 (28.7–82.7)	74.8 (47.7–106)	45.8 (17.4–70.9)[a]	
Median urine PCR, mg/mmol	44 (16–117)	39 (13.5–89)	50 (17–144.5)	
Median antihypertensive drug classes per patient	1.6	1.4	1.6	
Patients with ACEi/ARB at enrolment, excluding dialysis patients, % (n)	73.7 (n = 199)	79.8 (n = 71)	77.3 (n = 109)	
Median systolic/diastolic blood pressure, mm Hg	134 (122–145)/79 (70–88) (n = 286)	128 (116–140)/77.5 (70–85) (n = 88)	136 (124–146)[b]/79 (70–88) (n = 174)	
Median follow-up duration, mo	55.0 (22.5–100.8)	71.4 (22.9–177.6)	50.7 (22.2–91.6)	
Reached ESRD, %	34.9 (n = 103)	0	57.5[c] (n = 103)	
History of macroscopic hematuria, %	27.8 (n = 82)	44.9 (n = 40)	20.1[c] (n = 36)	
Diagnosis of Henoch-Schonlein purpura, %	6.4 (n = 19)	0	8.9[c] (n = 16)	
Laboratory measurements				
Median serum IgA, g/l	3.4 (2.9–4) (n = 293)	3.7 (3.2–4.3) (n = 88)	3.3 (2.7–3.8)[d] (n = 178)	2.8 (2.2–3.2)[e] (n = 57)
Median serum gd-IgA1, AU	0.50 (0.41–0.58) (n = 293)	0.47 (0.37–0.55) (n = 88)	0.52 (0.42–0.59)[f] (n = 178)	0.42 (0.33–0.55)[g] (n = 57)
CFHR1–2 copies/1 copy/no copies[h]				
n	183/101/9 (n = 293)	58/28/3	111/63/4 (n = 178)	85/45/3 (n = 133)
%	62.4/34.5/3.1	65.9/31.8/3.4	62.4/35.4/2.2	63.9/33.8/2.3
CFHR3–2 copies/1 copy/no copies				
n	185/100/8 (n = 293)	59/27/3	112/63/3 (n = 178)	
%	63.1/34.1/2.7	67/30.7/3.4	62.9/35.4/1.7	
Median plasma FHR-1, µg/ml	126.8 (86.4–158.6)	112.5 (74.7–149.3)	132.0 (88.6–162.8)	94.4 (70.5–119.6)[i]
Median plasma fH, µg/ml	153.8 (130.6–187.6)	155.1 (137.4–187.8)	150.1 (126.0–186.9)	152.5 (122.9–189.8)
Median plasma FHR-1:fH ratio	0.85 (0.55–1.10)	0.77 (0.46–0.98)	0.89 (0.59–1.16)[j]	0.68 (0.40–0.86)[k]
Median serum FHR-5, µg/ml	2.74 (2.07–3.64)	2.80 (2.07–3.49)	2.79 (2.08–4.03)	2.46 (1.79–3.67)[l] (n = 158)

ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; AU, arbitrary units; CFHR, complement factor H-related; CI, confidence interval; ESRD, end-stage renal disease; fH, factor H; FHR-1/-5, factor H-related protein 1/5; gd-IgA1, galactose-deficient IgA1; PCR, protein-to-creatinine ratio; no, number.
Values within parentheses represent interquartile range and number analyzed if less than the respective cohort numbers.
[a] $P < 0.0001$ versus stable disease.
[b] $P = 0.0014$ versus stable disease.
[c] $P < 0.0001$ versus stable disease.
[d] $P = 0.0007$ versus stable disease.
[e] $P < 0.0001$ versus entire IgAN cohort.
[f] $P = 0.01$ versus stable disease.
[g] $P = 0.015$ versus entire IgAN cohort.
[h] 1 patient had 3 copies of *CFHR3* and was excluded from the analysis.
[i] $P < 0.0001$ versus entire IgAN cohort (difference between medians, 32.4 µg/ml; 95% CI, 19.9–37.6 µg/ml).
[j] $P = 0.019$ versus stable disease (difference between medians, 0.12; 95% CI, 0.02–0.2).
[k] $P < 0.0001$ versus entire IgAN cohort (difference between medians, 0.17; 95% CI, 0.12–0.24).
[l] $P = 0.041$ versus entire IgAN cohort (difference between medians, 0.28 µg/ml; 95% CI, 0.01–0.48 µg/ml).

ratio was elevated in patients who had progressive compared with stable disease following immunosuppression treatment (Figure 1f).

Plasma FHR-1 was negatively correlated with eGFR but remained elevated in IgAN patients with normal eGFR

Given that we observed higher FHR-1 levels in patients with progressive IgAN, we next determined if FHR-1 levels were influenced by renal impairment. We stratified patients by the *CFHR1* gene copy number and assessed the correlation between FHR-1 levels and eGFR (Figure 2). We detected a negative correlation between plasma FHR-1 levels and eGFR in patients with either 1 (Figure 2a) or 2 (Figure 2b) *CFHR1* gene copy number. When we stratified patients into those with eGFR of <30 ml/min per 1.73 m^2 and those with eGFR of >60 ml/min per 1.73 m^2, FHR-1 levels were significantly higher in those with eGFR of <30 ml/min per 1.73 m^2 (Figure 2c and d, 1 and 2 *CFHR1* copy numbers, respectively). To determine if FHR-1 levels were higher in IgAN patients before the development of renal impairment, we compared FHR-1 levels between IgAN patients with normal eGFR and healthy controls (Figure 2e). Higher FHR-1 levels were observed in patients with 2 *CFHR1* gene copy numbers and normal eGFR than in genotype-matched healthy controls

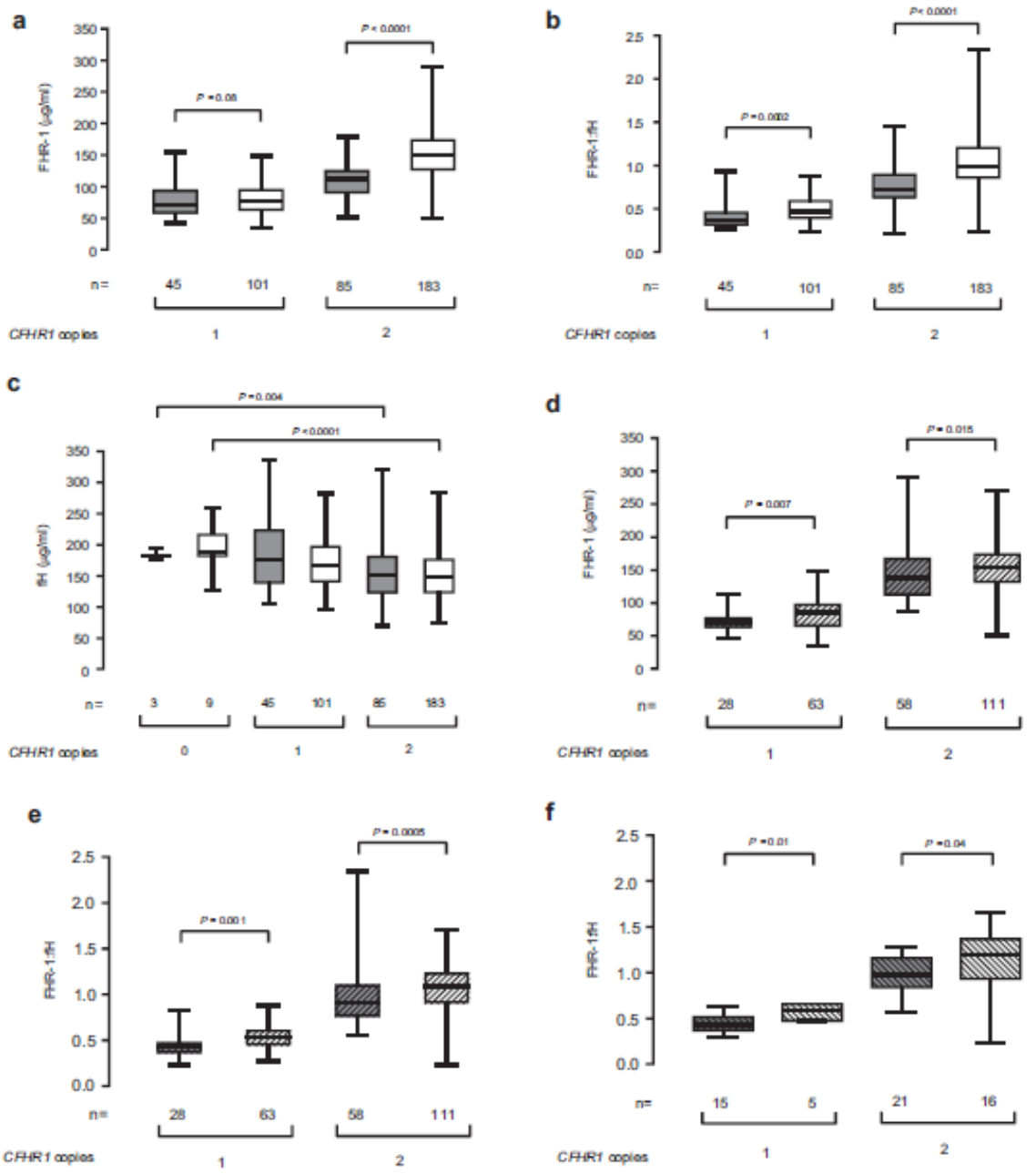

Figure 1 | The plasma factor H-related protein 1 (FHR-1) levels and the FHR-1:factor H (fH) ratio are elevated in IgA nephropathy (IgAN) and are associated with a progressive disease. (**a**) The plasma FHR-1 levels, (**b**) FHR-1:fH ratio, and (**c**) fH levels in healthy controls (gray boxes) and IgAN patients (white boxes) stratified according to the *CFHR1* gene copy number. Plasma FHR-1 was undetectable in patients with 0 *CFHR1* gene copy number. The plasma FHR-1 levels and FHR-1:fH ratio were significantly different ($P < 0.001$) between individuals with either 1 or 2 *CFHR1* gene copy numbers in both the control and IgAN cohorts. (**d**) Comparison of plasma FHR-1 levels and (**e**) FHR-1:fH ratio between patients with stable (dashed gray boxes) and those with progressive (dashed white boxes) IgAN stratified according to the *CFHR1* gene copy number. (**f**) Comparison of the FHR-1:fH ratio between patients with stable (dashed gray boxes) and those with progressive (dashed white boxes) IgAN after immunosuppression therapy. The bar represents the median value, box represents the interquartile range, and whiskers represent the range of values. *P* values derived using Mann-Whitney test.

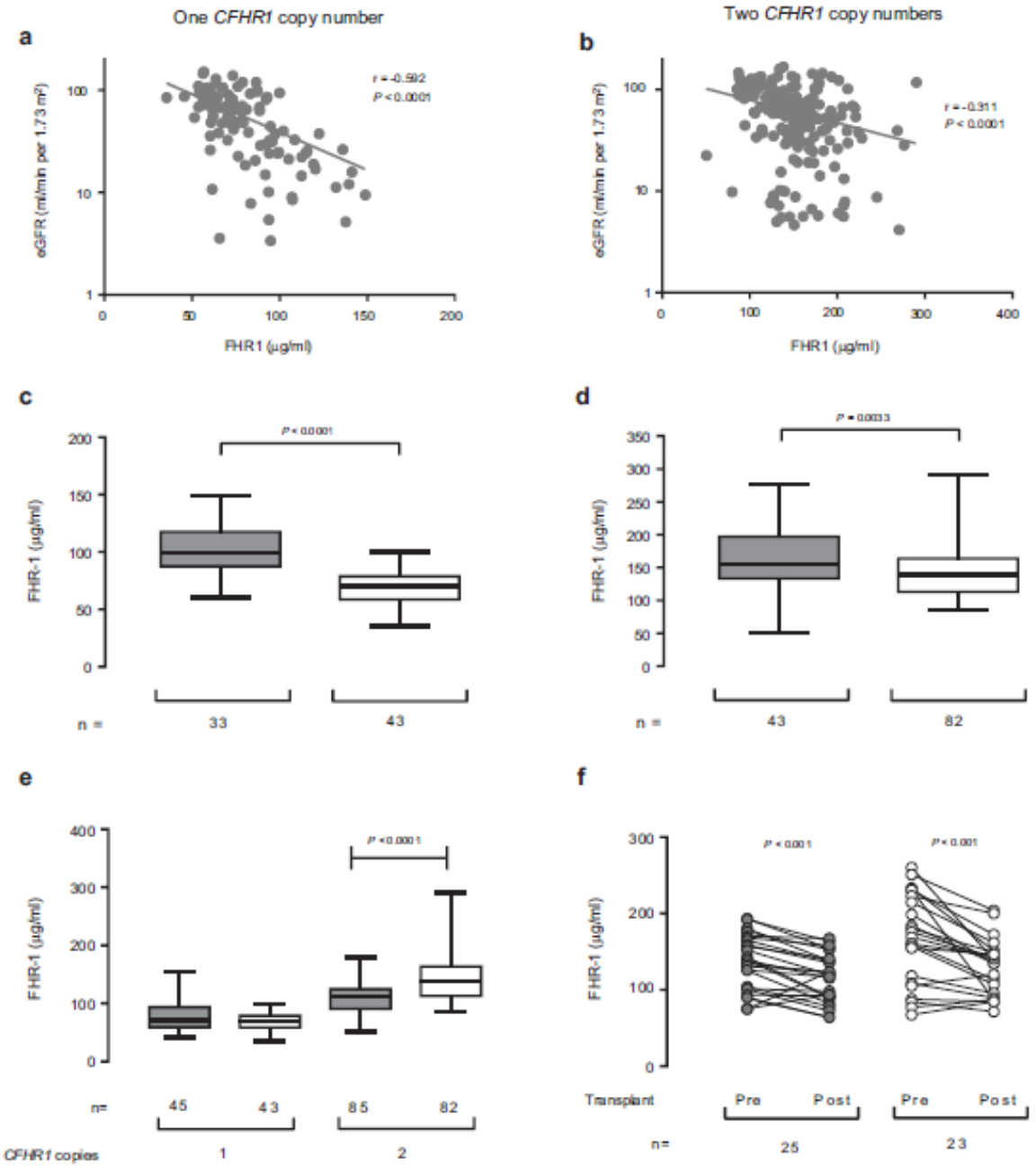

Figure 2| Factor H-related protein 1 (FHR-1) is negatively correlated with the estimated glomerular filtration rate (eGFR) but remains elevated in IgA nephropathy (IgAN) patients with normal eGFR. Correlation between plasma FHR-1 levels and eGFR after logarithmic transformation in IgAN patients with either 1 (**a**) or 2 (**b**) *CFHR1* gene copy numbers. *P* values derived from Spearman's rank correlation. Plasma FHR-1 levels in IgAN patients with eGFR of <30 (gray boxes) or >60 (white boxes) ml/min per 1.73 m^2 stratified according to 1 (**c**) or 2 (**d**) *CFHR1* gene copy number. (**e**) Comparison of the plasma FHR-1 levels between healthy controls (gray boxes) and IgAN patients with eGFR of >60 ml/min per 1.73 m^2 (white boxes) stratified according to the *CFHR1* gene copy number. The bar represents the median value, box represents the interquartile range, and whiskers represent the range of values. *P* values derived from Mann-Whitney test. (**f**) Paired FHR-1 levels before (Pre) and after (Post) renal transplantation in a cohort of patients with autosomal dominant polycystic kidney disease (ADPKD) (gray circles, n = 25) and IgAN (white circles, n = 23). All patients were homozygous for the major allele rs6677604, consistent with 2 *CFHR1* gene copy numbers.

(Figure 2e). This difference was not observed in patients with 1 *CFHR1* gene copy number (Figure 2e). We next assessed FHR-1 levels before and after renal transplantation in patients with either biopsy-proven IgAN or autosomal dominant polycystic kidney disease (ADPKD). The cohorts had comparable pretransplant characteristics (Supplementary Table S1), and no patients had a clinical diagnosis of delayed graft function, transplant rejection, or disease recurrence at the sampling time. Both groups showed significant reduction in serum FHR-1 levels after renal transplantation (Figure 2f). Altogether, our data indicate that both the diagnosis of IgAN and eGFR are independently associated with higher FHR-1 levels. Notably, there was no significant correlation between eGFR and plasma fH levels in our IgAN cohort (Supplementary Figure S1C).

Serum FHR-5 was slightly elevated in IgAN but was not correlated with eGFR

Abnormalities in FHR-5 have been shown to be associated with C3 glomerulopathy (C3G), a complement-mediated kidney disease with phenotypic similarities to IgAN.[26,27] In addition, FHR-5 is associated with fH deregulation *in vitro*.[19] We measured serum FHR-5 levels in our IgAN cohort. The median FHR-5 level was higher in IgAN patients than in healthy controls (Table 1), but the magnitude of the difference was small. The median FHR-5 levels were 2.46 and 2.74 μg/ml in the healthy controls and IgAN patients, respectively (difference between medians, 0.28 μg/ml, 95% confidence interval, 0.01–0.48 μg/ml). Furthermore, we did not detect any difference in FHR-5 levels between stable and progressive IgAN patients (Figure 3a and Table 1). However, patients with progressive disease following immunosuppression treatment had significantly higher FHR-5 levels than patients who improved to meet stable IgAN criteria after immunosuppression (Figure 3a). Unlike FHR-1, we did not find an association between serum FHR-5 levels and eGFR (Figure 3b and c). Moreover, we found no significant difference in the FHR-5:fH ratio between patients and healthy controls or between progressive and stable IgAN (Supplementary Figure S2).

Serum FHR-5 levels correlated with histologic markers of renal injury

To explore the significance of the changes in serum FHR-5 levels, we assessed the correlation between FHR-5 levels and validated markers of histologic injury in IgAN, according to the Oxford classification.[28] Serum FHR-5 levels at recruitment were significantly higher in patients with endocapillary hypercellularity score E1 at diagnosis than in those with no endocapillary hypercellularity (E0) (Figure 4a, right panel). Serum FHR-5 levels in IgAN patients with (M1) and without (M0) renal biopsy evidence of mesangial hypercellularity did not differ (Figure 4a, left panel). The total MEST score is calculated by adding the scores for mesangial hypercellularity (M), endocapillary hypercellularity (E), segmental sclerosis (S), and tubular atrophy (T). Serum FHR-5 levels were higher in IgAN patients with MEST score of 4 than in those with MEST score of 1 (Figure 4b). Unlike FHR-5, our data demonstrated that FHR-1 levels were influenced by eGFR. Therefore, we did not consider it valid to assess associations between histologic markers and plasma FHR-1 levels because eGFR would differ between the diagnostic renal biopsy and study plasma in many patients.

DISCUSSION

The mechanism underlying the robust association between the del*CFHR3-1* allele and reduced susceptibility to IgAN is unclear. While the role of fH as a critical negative regulator of C3 activation through the complement alternative pathway is well established, the biological roles of the FHR proteins have, until recently, been poorly understood.[17–19] In fact, the common occurrence of the complete absence of FHR-1 and FHR-3 in healthy individuals with the del*CFHR3-1* allele in homozygosity has suggested that these 2 FHR proteins are biologically redundant. Insights into key roles of FHR-1 and FHR-3 in diseases have been derived from the association between del*CFHR3-1* alleles and protection from not only IgAN but also age-related macular degeneration.[25] Furthermore, abnormal FHR proteins are associated with familial cases of C3G, a condition wherein complement-mediated renal injury is derived from abnormal regulation of alternative pathway activation.[20,26,29–31] *In vitro* data have shown that FHR-1[17] and FHR-5[19] compete with fH for binding to activated C3 (termed C3b). Unlike fH, interaction of C3b with either FHR-1 or FHR-5 allows continued complement activation, preventing the inhibitory actions of fH, a phenomenon referred to as fH deregulation. We considered that the association between del*CFHR3-1* alleles and IgAN can be explained by fH deregulation. We hypothesized that reduced (or absent) FHR-1 levels result in a reduction or absence of fH deregulation and consequently less complement-mediated renal injury.

In this study, we first explored the association between FHR-1 and fH levels in patients with either stable or progressive IgAN. We found that FHR-1 levels and importantly, the FHR-1:fH ratio, were higher in IgAN patients than in healthy controls. These data are replicated in a separate IgAN cohort (Tortajada A, Gutierrez E, Goicoechea de Jorge E, et al. Elevated factor H-related 1 and occurrence of factor H pathogenic variants in IgA nephropathy. *Kidney International*, submitted for publication). In addition, irrespective of the *CFHR1* gene copy number, patients with progressive IgAN had significantly elevated plasma FHR1/fH ratios compared to patients with stable IgAN. These data are consistent with our hypothesis that reduced fH deregulation is associated with favorable outcomes in IgAN. We categorized the patient cohort into those with progressive IgAN and those with stable IgAN. Our criteria were designed to enable us to identify patients with immunologically active disease. The criteria included eGFR loss without additional renal pathology and histologic features of glomerular inflammation, such as the presence of endocapillary hypercellularity and cellular crescents. Although this inevitably excluded a subset of patients

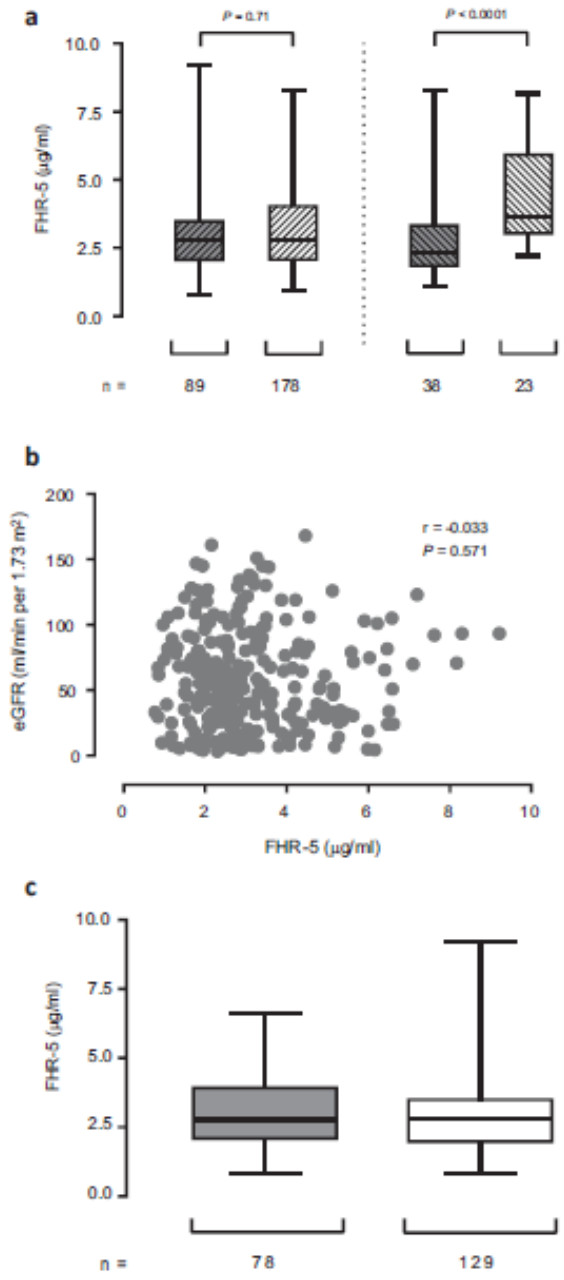

Figure 3| Serum factor H-related protein 5 (FHR-5) levels are associated with IgA nephropathy (IgAN) severity following immunosuppression therapy and are not correlated with estimated glomerular filtration rate (eGFR). (**a**) Left panel, Comparison of serum FHR-5 levels between stable (dashed gray boxes) and progressive (dashed white boxes) IgAN. Right panel, Comparison of serum FHR-5 between patients with stable (dashed gray boxes) and those with progressive (dashed white boxes) IgAN after immunosuppression therapy. (**b**) Correlation between serum FHR-5 levels and eGFR in IgAN patients. (**c**) Serum FHR-5 levels in IgAN patients with eGFR of <30 (gray boxes) or >60 (white boxes) ml/min per 1.73 m^2. The bar represents the median value, box represents the interquartile range, and whiskers represent the range of values. *P* values derived using Mann-Whitney test.

(those we could not reliably categorize as either stable or progressive at study entry), our robust classification enabled us to detect differences in FHR-1 levels and the FHR-1:fH ratio. Although changes in these parameters correlated with worse disease outcomes, the levels showed large overlap between the groups, precluding their use for patient stratification. In addition, our cohort included 9 individuals (3 with stable IgAN, 4 with progressive IgAN, and 2 who did not fulfil the criteria) with del*CFHR3-1* allele homozygosity, demonstrating that even in the complete absence of FHR-1 and FHR-3, renal injury that requires renal biopsy and hospital follow-up can occur. This is consistent with previous studies[16] and indicates that factors independent of fH deregulation can drive the disease.

We did not measure FHR-3 levels because of the lack of a reliable assay. FHR-3 is not clearly associated with fH deregulation, and its role remains unclear; it can interact with the meningococcus fH-binding protein and through competition with fH, influence the complement-mediated clearance of meningococcal strains and hence meningococcal disease severity.[32] Through interaction with its ligand C3d, FHR-3 may influence B-cell regulation through a B-cell receptor complex (CD19/CD21/CD81), but the association with IgAN is unclear.[33] Notably, despite the interaction with C3d, this effect was not observed for FHR-1.

We found a negative correlation between eGFR and plasma FHR-1 levels measured at study recruitment. When patients with normal eGFR were compared with genotype-matched healthy controls, patients with 2 *CFHR1* gene copy numbers and normal eGFR had higher FHR-1 levels. Both IgAN patients and ADPKD patients showed reduced FHR-1 levels coincidental with increased eGFRs after renal transplantation. This indicated that the diagnosis of IgAN and eGFR are independently associated with higher FHR-1 levels. Moreover, this conclusion is supported by the findings in a study that assessed FHR-1 levels in IgAN and polycystic renal disease (Tortajada A, Gutierrez E, Goicoechea de Jorge E, et al. Elevated factor H-related 1 and occurrence of factor H pathogenic variants in IgA nephropathy. *Kidney International*, submitted for publication). Although we consider the association to be robust, we currently do not know why FHR-1 increases as eGFR decreases. Nevertheless, this would be predicted to further enhance fH deregulation and aggravate disease. It is predicted that this could be applicable to any glomerular pathology in which there is complement-mediated injury and that there are more general implications beyond IgAN. Because FHR-1 levels were influenced by eGFR and diagnostic renal biopsy was not coincident with the timing of the study blood sample in our cohort, we did not analyze the association between FHR-1 and histologic changes

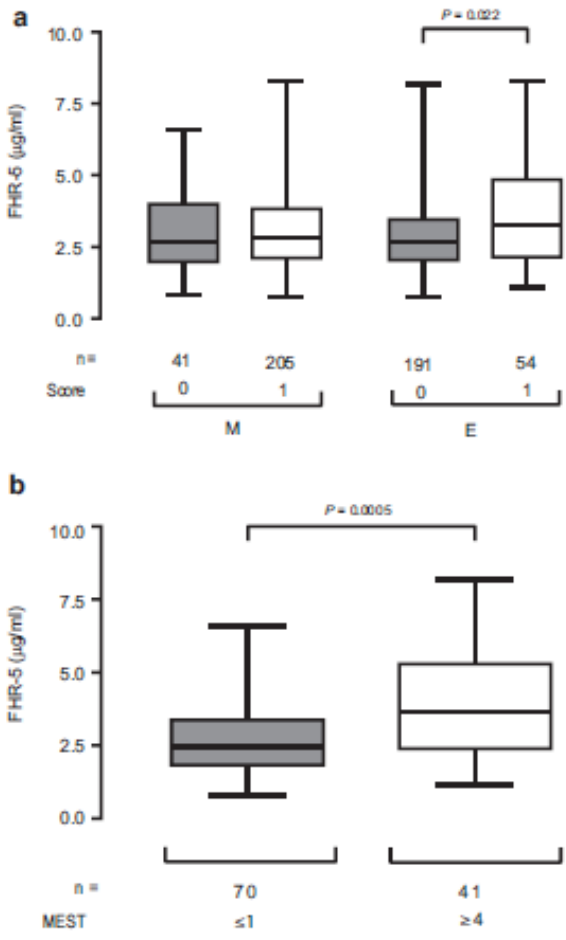

Figure 4| Serum factor H-related protein 5 (FHR-5) levels are correlated with histologic markers of disease severity in IgA nephropathy (IgAN). (**a**) Left panel, Serum FHR-5 levels in IgAN patients without (M0, gray box) and with (M1, white box) renal biopsy evidence of mesangial hypercellularity (denoted M). Right panel, Serum FHR-5 levels in IgAN patients without (E0, gray box) and with (E1, white box) biopsy evidence of endocapillary hypercellularity (denoted E). (**b**) Serum FHR-5 levels in IgAN patients with diagnostic renal biopsy MEST scores of 1 or less (gray box) or at least 4 (white box). The bar represents the median value, box represents the interquartile range, and whiskers represent the range of values. *P* values derived using Mann-Whitney test.

in the renal biopsy. A prospective study will be required to clarify the contribution of FHR-1 to histologic changes before decreases in eGFR.

As published data showed that FHR-5 can mediate fH deregulation[19] and studies indicated the strong association between FHR-5 mutation and C3G,[26,30,31] we measured serum FHR-5 levels and the FHR-5:fH ratio. Notably, there are strong phenotypic similarities between familial C3G associated with FHR-5 mutations and IgAN.[27] Despite a significant increase in serum FHR-5 levels between IgAN patients and healthy controls, the magnitude of the difference was very small and of doubtful biological significance. In addition, we did not identify any difference in FHR-5 levels between patients with stable disease and those with progressive disease. However, when we analyzed only patients treated with immunosuppression therapy, FHR-5 levels were higher in those with ongoing progressive IgAN. The significance of this is unclear and requires confirmation. Unlike the FHR-1:fH ratio, we did not detect any correlation between the FHR-5:fH ratio and disease outcome. Because eGFR did not influence the serum FHR-5 levels, we assessed the correlations between serum FHR-5 levels and validated histologic markers of renal injury in IgAN. Serum FHR-5 levels were associated with endocapillary hypercellularity independent of mesangial hypercellularity, a histologic marker of active inflammation.[34] They were also associated with higher overall Oxford classification of IgAN MEST scores.[35] To further understand these associations, it would be necessary to analyze the pattern and degree of FHR-5 deposition in renal tissues. In this respect, it is notable that FHR-5 was detected in association with complement C3 in patients with IgAN. Notably, the association between FHR-5 and C3 was also observed in membranous nephropathy, lupus nephritis, and postinfectious nephritis, indicating that this phenomenon is not specific to IgAN.[36]

The limitations of our dataset include the difference in timing between diagnostic renal biopsy and study sample collection and the lack of serial blood samples from patients. Additionally, it would be important to investigate the relative amounts of C3, fH, FHR-1, and FHR-5 in renal tissues in IgAN.

Current assessment of IgAN patients involves the use of nonspecific clinical and histologic markers to identify patients likely to improve with immunosuppression therapy. However, the benefit of the currently recommended 6-month corticosteroid treatment for persistently proteinuric, noncrescentic IgAN[37] has not been conclusively demonstrated.[38] Our data, together with those of a separate cohort (Tortajada A, Gutierrez E, Goicoechea de Jorge E, et al. Elevated factor H-related 1 and occurrence of factor H pathogenic variants in IgA nephropathy. *Kidney International*, submitted for publication), associate circulating FHR-1 levels and the FHR-1:fH ratio with IgAN severity. Our findings need corroboration with histologic data but nevertheless suggest that fH deregulation contributes to IgAN progression and support further investigation into this hypothesis. If it can be shown that complement-mediated kidney injury in IgAN is influenced by FHR-1 and mechanistically understood, the modulation of FHR-1 might be therapeutic in progressive IgAN.

MATERIALS AND METHODS

Study cohort and clinical measurements

The Causes and Predictors of Outcome in IgA Nephropathy study is a retrospective cohort UK study of patients with biopsy-proven IgAN ethically approved by the UK National Research Ethics Service Committee (14/LO/0155). The inclusion criteria were availability of the renal biopsy report and relevant clinical data. In this study, 334

patients were recruited, and after excluding 40 patients, 294 were analyzed. The reasons for exclusion were alternative diagnosis (n = 4), no biopsy immunostaining completed (n = 5), no biopsy report available (n = 3), and no creatinine measurement at enrollment (n = 28).

Progressive disease was considered if at least one of the following criteria was present: (i) progression to end-stage renal disease without histologic evidence of a second pathology causing renal impairment, (ii) renal biopsy evidence of endocapillary hypercellularity, (iii) renal biopsy evidence of cellular and/or fibrocellular crescents; (iv) treatment with immunosuppressants (including corticosteroids) for native IgAN, (v) clinical Henoch-Schonlein purpura, unless spontaneous resolution and >20 years of follow-up with "stable" criteria, (vi) 50% loss of eGFR or an average annual eGFR loss of >5 ml/min per 1.73 m^2 without evidence of a second pathology causing renal impairment. Stable disease was considered if none of the criteria for progressive IgAN were met and if all of the following criteria were met: (i) urine protein-to-creatinine ratio of <100 units or daily proteinuria of <1 g/24 h, (ii) combined Oxford classification MEST score of <3, and (iii) an average annual eGFR loss of <3 ml/min per 1.73 m^2.

All patients in the transplantation cohorts received a renal transplant and underwent clinical follow-up at Imperial College Healthcare NHS Trust. They received posttransplant immunosuppression therapy and clinical care as per local guidelines. All ADPKD patients had a radiological diagnosis. All transplant patients provided consent for the storage of serum and plasma samples that were surplus to clinical diagnostic requirements and their subsequent use for research. Blood samples were obtained within 4 weeks before and between 12 and 16 weeks after transplantation. Two transplant ADPKD patients, both of whom had the AA rs6677604 single-nucleotide polymorphism genotype, had undetectable FHR-1 levels before and after transplantation.

Healthy control samples were obtained from healthy volunteer donors and from members of the TwinsUK cohort,[39] a cohort of twins of Caucasian ethnicity. We randomly selected samples from only 1 individual of each pair of twins included in the cohort.

eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration Creatinine Equation.[40]

Assessment of *CFHR3* and *CFHR1* gene copy number

DNA was extracted from whole blood samples using QIAamp DNA Blood Mini Kits (Qiagen, Hilden, Germany). Quantitative real-time polymerase chain reaction (PCR) was performed using the ViiA Real-Time PCR System (Applied Biosystems, Foster City, CA). Copy number variation within the *CFHR3* and *CFHR1* genes was assessed using the Taqman Copy Number Real-Time Detection System (Applied Biosystems). Copy number variation calls were determined using the Copy Caller Software (Applied Biosystems). Assay readings were normalized to control samples, and the values were presented as mean ± SD. All probes were validated using genomic DNA from healthy controls with either heterozygous or homozygous polymorphic deletion of the *CFHR1* and *CFHR3* genes. The *CFHR1* gene copy number in the renal transplant cohort was inferred from the rs6677604 genotype, which is in linkage disequilibrium with the *CFHR1* and *CFHR3* gene copy number.[16] Genotyping was performed using the Taqman genotyping assays (Applied Biosystems).

Measurement of serum IgA and gd-IgA levels

Serum IgA levels were measured using enzyme-linked immunosorbent assay (ELISA) as previously described.[41] The capture antibody was the F(ab')2 fragment goat antihuman IgA (Jackson ImmunoResearch, West Grove, PA), and the detection antibody was the $F(ab')_2$ fragment biotinylated goat antihuman IgA1 (Jackson ImmunoResearch). Serum gd-IgA1 levels were measured using a lectin-based ELISA as previously described.[41] The capture antibody was a polyclonal rabbit antihuman IgA (Dako, Glostrup, Denmark). The detection involved *Helix aspersa* agglutinin-biotin (Sigma, Darmstadt, Germany), followed by poly-streptavidin horseradish peroxidase (Pierce, Waltham, MA). The intraclass correlation coefficient for the IgA assay was 0.74 (95% confidence interval, 0.63–0.83), and that for the gd-IgA1 assay was 0.89 (95% confidence interval, 0.73–0.95).

Measurement of plasma fH and FHR-1 levels and serum FHR-5 levels

We used a sandwich ELISA designed by Tortajada *et al.* (Tortajada A, Gutierrez E, Goicoechea de Jorge E, et al. Elevated factor H-related 1 and occurrence of factor H pathogenic variants in IgA nephropathy. *Kidney International*, submitted for publication) to measure the plasma fH and FHR-1 levels. Although FHR-1 and FHR-2 can form homodimers, heterodimers, and heterooligomeric molecules *in vivo*, the levels detected by this ELISA are referred to as FHR-1 levels because FHR-1 is the major component in these complexes. The capture antibody was a rabbit polyclonal antibody that recognizes both fH and FHR-1. fH was detected with a mouse monoclonal anti-fH antibody that recognizes SCR10 and SCR11 of fH. FHR-1 was detected using a mouse monoclonal antibody that recognizes an epitope within SCR1 and SCR2 of FHR-1 (both provided by Professor Santiago Rodriguez de Cordoba, Madrid). The interassay coefficient of variation was 8.7 for fH ELISA and 9.9 for FHR-1 ELISA.

FHR-5 levels were measured using ELISA. The capture antibody was a rabbit monoclonal anti-FHR-5 antibody (Abcam, Cambridge, UK). FHR-5 was detected using a mouse monoclonal anti-FHR-5 antibody (Abcam). The interassay coefficient of variation was 12.1.

Statistical analysis

Normally distributed continuous variables were tested using unpaired *t*-test, and pre- and posttransplant levels were compared using paired *t*-test. Continuous variables with skewed distribution were tested using Mann-Whitney *U* test, and confidence intervals for differences between medians were calculated using the Hodges-Lehmann method. Categorical data was tested using chi-square test and Fisher's exact test (samples, <10). We applied Pearson's correlations and simple linear regression to log eGFR and FHR-1, and Spearman's rank correlation to eGFR and gd-IgA1. A P value <0.05 was considered to be statistically significant. We used GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA) for all analyses.

DISCLOSURE

All the authors declare no competing interests.

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COLLABORATOR LIST AND AFFILIATIONS

Dr. A. Ahmed, Royal Preston Hospital, Lancashire, UK.
Dr. L. Baines, Freeman Hospital, Newcastle upon Tyne, UK.
Dr. C. Bingham, Royal Devon and Exeter Hospital, Exeter, UK.
Prof S. Bhandari, Hull Royal Infirmary, Hull, UK.
Dr. A. Bow, Cumberland Infirmary, Carlisle, UK.
Dr. A. Chan, Broomfield Hospital, Chelmsford, Essex, UK.
Dr. G. Cserep, Colchester General Hospital, Colchester, Essex, UK.
Dr. D. de Takats, Royal Stoke University Hospital, Stoke-on-Trent, UK.
Dr. S. Dickinson, Royal Cornwall Hospital, Truro, Cornwall, UK.
Dr. C. Goldsmith, Aintree University Hospital, Liverpool, UK.
Dr. S. Lawman, Royal Sussex County Hospital, Brighton, UK.
Dr. S. Mitra, Manchester Royal Infirmary, Manchester, UK.
Dr. R. Montero, Guys Hospital, London, UK.
Dr. A. Mooney, St James's University Hospital, Leeds, UK.
Dr. A. Power, Southmead Hospital, Bristol, UK.
Dr. C. Wroe, James Cook University Hospital, Middlesbrough, UK.

SUPPLEMENTARY MATERIAL

Figure S1. (**A**) Serum IgA (left panel) and galactose-deficient IgA1 (gd-IgA1; right panel) levels in patients with stable (gray dashed box) and progressive (white dashed box) IgA nephropathy (IgAN) after immunosuppression therapy. The progressive IgAN cohort shows higher median serum gd-IgA1 levels. (**B**) Correlation of estimated glomerular filtration rate (eGFR) and serum gd-IgA1 levels in IgAN patients ($n = 293$). A negative correlation was calculated with Spearman's rank correlation, and the black line represents the correlation equation. (**C**) Comparison of serum eGFR and plasma factor H (fH) at the same sampling point in IgAN patients ($n = 294$) shows a small and insignificant correlation between eGFR and plasma fH levels.

Figure S2. The plasma factor H-related protein 5 (FHR-5):factor H (fH) ratio is not associated with IgA nephropathy (IgAN). (**A**) The FHR-5:fH ratio in healthy controls and IgAN patients. We did not identify a significant difference in plasma FHR-5:fH ratio between healthy controls (gray box) and IgAN patients (white box). (**B**) The FHR-5:fH ratio in stable and progressive IgAN. There was no significant difference in the plasma FHR-5:fH ratio between patients with stable (gray boxes) and those with progressive (white boxes) IgAN for the same *CFHR1* genotype.

Table S1. Cohort characteristics for patients with IgA nephropathy (IgAN) or autosomal dominant polycystic kidney disease (ADPKD) who received a renal transplant. Values within parentheses represent interquartile range. ESRD, end-stage renal disease. *$P < 0.001$ compared with the pretransplant levels. *rs6677604 tags the *CFHR3-1* deletion.

Supplementary material is linked to the online version of the paper at www.kidney-international.org.

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INVITED REVIEW

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The complement factor H-related proteins

Nicholas Medjeral-Thomas | Matthew C. Pickering

Centre for Complement and Inflammation Research, Imperial College, London, UK

Correspondence
Nicholas Medjeral-Thomas, Centre for Complement and Inflammation Research, Imperial College, London, UK.
Email: matthew.pickering@imperial.ac.uk

Summary

The role of the complement factor H-related (FHR) proteins in homeostasis, pathogen defense, and autoimmune disease has recently attracted considerable interest. We highlight the exciting research that has contributed to our understanding of the FHR protein family. Unlike factor H, a potent negative regulator of complement C3 activation, the FHR proteins appear to promote C3 activation. These data have important implications for understanding complement-mediated diseases because, depending on the context, the balance between the actions of factor H and the FHR proteins determines the degree of complement activation.

KEYWORDS
complement, inflammation, infection, kidney

1 | INTRODUCTION

The complement system is a powerful and important component of innate and adaptive immunity.[1] It forms an intricate network of activating and regulating mechanisms that can discriminate between healthy and damaged or foreign surfaces.[2,3] Regulation of complement is required to target activation appropriately. There are many important diseases that result from an inability to regulate complement activation. This has lead to a greater understanding of the biology and pathophysiological roles of complement regulators. Complement factor H (fH) is a major negative regulator of complement activation. Variation within fH is associated with susceptibility to both rare (e.g. C3 glomerulopathy) and common (e.g. age-related macular degeneration) diseases. fH is part of a family of proteins that include the factor H-related (FHR) proteins. Although originally thought to be non-essential, the role of the FHR proteins in homeostasis, pathogen defense, and autoimmune disease has recently attracted considerable interest. We highlight the exciting research that has contributed to our understanding of the FHR protein family. Unlike fH, a potent negative regulator of complement C3 activation, the FHR proteins appear to promote C3 activation. These data have important implications for understanding complement-mediated diseases. Depending on the context, it is the balance between the actions of fH and the FHR proteins that determines the degree of complement activation.

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2 | OVERVIEW OF THE COMPLEMENT SYSTEM

Complement is an integral component of innate and adaptive immunity. It is important in host defense, the generation of immune responses, and in the physiological removal of immune complexes. The complement system is made up of more than 30 proteins either bound to cell surfaces or soluble in plasma.[2] These proteins are collectively abundant; they constitute 15% of the globulin fraction.[1] Many complement proteins are inactive enzyme precursors, or zymogens. The activated enzymes often cleave the substrate from which they originated, leading to self-amplification. Complement may be activated through at least three pathways, the classical, the alternative, and the lectin pathway (Fig. 1), which results in the activation of the central component of the pathway, complement C3. The classical pathway is initiated by IgM or IgG bound to antigen (immune complexes). However, the hexameric surface-binding component of the C1 complex that initiates classical pathway activation, C1q, is a versatile pattern recognition molecule (PRM) that can also recognize structures on viruses, Gram-negative bacteria, and endogenous molecules, for example C-reactive protein

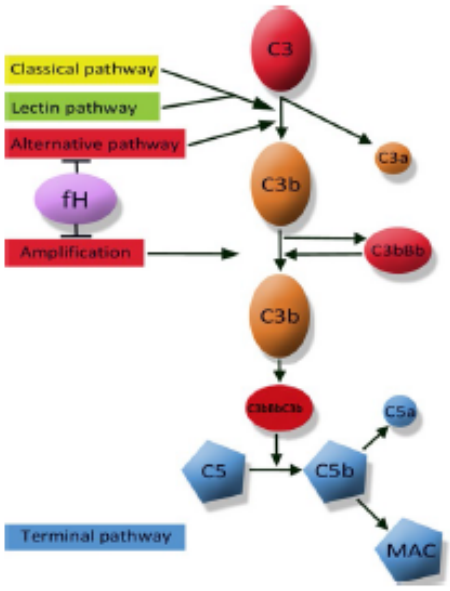

FIGURE 1 The Complement system. Three activation pathways can trigger complement activation: the classical pathway, the lectin pathway, and the alternative pathway. Regardless of the trigger and initial pathway, complement activation results in the formation of C3 convertases that cleave C3 molecules into C3b. C3b may itself interact with other complement molecules to produce more C3 convertase, with resultant amplification of complement C3 activation.[40] The C3 convertase amplifies complement activation by rapidly converting many C3 molecules to C3a, a potent anaphylatoxin, and C3b that opsonizes and binds at sites of complement activation. Some of the C3b binds to C3 convertases and to form complexes (C5 convertase) that can activate complement C5.[88] The C5 convertases cleave C5 to initiate the terminal complement pathway, releasing the anaphylatoxin C5a, and producing C5b, which initiates the formation of C5b-9, the membrane attack complex (MAC).[1] Complement factor H (fH) inhibits alternative pathway activation and the C3b amplification pathway. Due to the important role of the C3 convertase in complement amplification regardless of the original activating pathway, fH plays a major role in regulating complement amplification

(CRP).[4] The C1 complex consists of C1q and two molecules each of the proteases, C1s and C1r. Upon binding of C1q, C1r and C1s are consecutively activated. C1s subsequently cleaves C4 into C4a and C4b. This cleavage exposes a thioester group that enables covalent binding of C4b to structures in immediate vicinity of the activation. C1s also cleaves C2 into C2a and C2b. C4b binds the larger protease C2 fragment (historically referred to as C2a, but recently renamed C2b), forming the classical pathway C3 convertase, C4b2b.

The lectin pathway is activated by the interaction of mannan-binding lectin (MBL) with arrays of mannose groups, for example on bacterial cell surfaces. MBL associates with mannan-binding lectin-associated proteases 1 and 2 (MASP1 and MASP2). On binding of MBL to mannose, MASP2 is activated and, similarly to C1s, cleaves C4 and C2[5] to ultimately form the C3 convertase enzyme C4b2b.[2] In addition to MBL, the lectin pathway can be activated by other PRMs that include H-, M-, and L-ficolin, and CL-K1 and CL-L1.[6]

The alternative pathway is continuously activated by the production of activated C3 (termed $C3_{H2O}$) from the hydrolysis of an internal thioester bond in C3.[7,8] $C3_{H2O}$ binds the protease, factor B (fB). fB within the $C3_{H2O}B$ complex is cleaved by factor D (fD) to form the active serine esterase Bb. The $C3_{H2O}Bb$ complex (referred to as a C3 convertase) cleaves C3 into two biologically active fragments: C3a and C3b.[7] C3a is an anaphylatoxin and chemoattractant. C3b is a major opsonin.[9]

Irrespective of the activating pathway, all surface-bound C3b can associate with fB to generate additional C3 convertase (C3bBb), thereby amplifying the initial deposited C3b.[10] This pathway is referred to as the C3b amplification loop (Fig. 1). Properdin functions to stabilize the C3 convertase,[11] and may also direct surface complement activation by interacting with pathogen- or damage-associated molecular patterns.[12] As C3 amplification increases the density of deposited C3b, the C3 convertases C4b2b and C3bBb form multimeric complexes with additional C3b molecules, producing C4b2b3b or C3bBbC3b.[8] These complexes are capable of cleaving C5 and are referred to as the C5 convertases. The C5 convertases cleave C5 to the anaphylatoxin C5a and fragment C5b. C5b binds C6 and C7. The resulting complex inserts into cell membranes and associates with C8, followed by 10–16 C9 molecules to form the C5b-9 terminal complement complex or membrane attack complex (MAC).[13] The MAC forms a pore-like structure in the membrane's outer lipid bilayer that promotes lysis of non-nucleated cells, and induces activation and injurious pathways in nucleated cells.[14]

Effective regulation of complement activation is essential to modify reaction severity and control cell injury. Surface-bound and soluble regulators act on all three complement pathways to limit progression of complement activation. Control of the C3b amplification loop is particularly important and the key regulator of the pathway is fH.[10]

fH is an abundant plasma protein that negatively regulates the alternative pathway and the C3b amplification loop. It acts in the fluid phase and on cellular surfaces. fH and the fH-like protein 1 (FHL1) are alternative transcripts derived from the *CFH* gene on chromosome 1q32.[15] The biological role of FHL1 is unclear. FHL1 is approximately 10 times less abundant than fH in plasma. fH and FHL1 are composed of 20 and 7 short consensus repeat (SCR) domains, respectively. SCR domains are globular units of approximately 60 amino acids. fH contains a surface-binding region within its two C-terminal domains (SCR19 and SCR20) and a complement regulatory region within its N-terminal four SCR domains. Its complement regulatory domains prevent C3 activation through multiple mechanisms. These include (i) accelerating decay of the alternative pathway C3 convertase; (ii) cofactor activity for factor I-mediated proteolytic inactivation of C3b; and (iii) binding to C3b preventing the formation of C3bB complexes.[16–18] The molecular detail of this regulatory role has recently been described: Bound fH interferes with contact areas of fB and Bb on C3b to accelerate convertase decay, and also forms a binding pocket with C3b that allows factor I to bind and cleave C3b.[18]

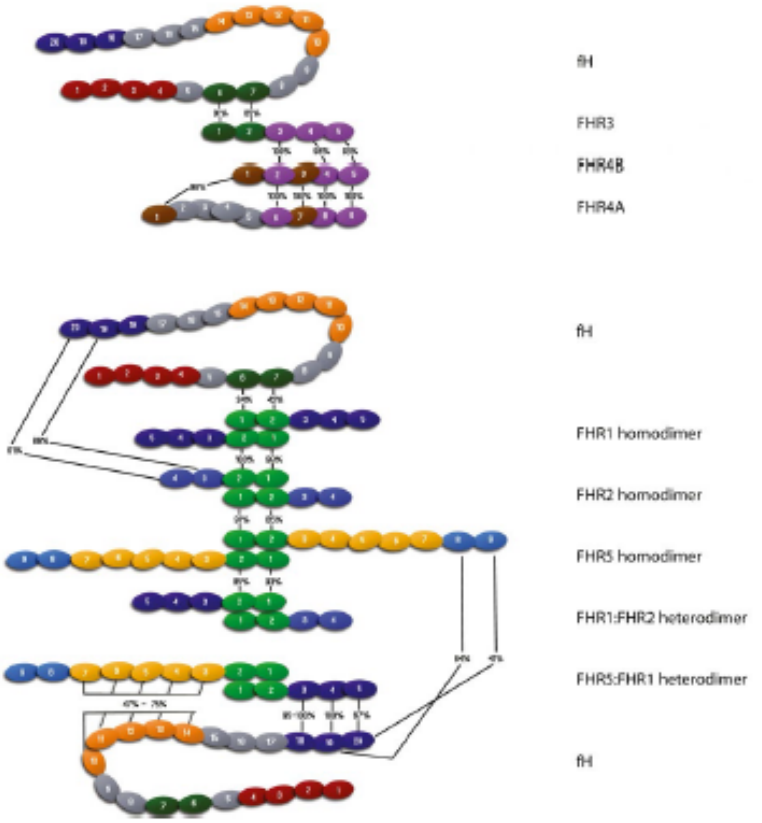

FIGURE 2 The factor H (fH)-related protein (FHR) family: Structural organization and domain similarity. High-resolution structural studies show fH to adopt a compact hinge-like arrangement in circulation.[18] Short consensus repeat (SCR) domains of fH and the FHR proteins are represented by ovals and numbered from the N-terminus. Color is used to depict amino acid sequence similarity between the protein SCR domains. Black numbers with lines linking SCR domains denote percentage amino acid similarity. For example, there is high sequence similarity between SCR1 and 2 of FHR3 and SCR6 and 7 of fH (see text). FHR1, FHR2, and FHR5 are found exclusively as dimers in the circulation, and heterodimers of FHR1 and FHR2 (FHR1:FHR2 heterodimer) and FHR5 and FHR1 (FHR5:FHR1 heterodimer) have been identified[87,88]

The surface-binding domains of fH mediate interaction with surface C3b and surface polyanions, such as glycosaminoglycans.[15] The role of fH on surfaces such as the glomerular basement membrane [GBM], which lack membrane-bound complement regulators, for example decay accelerating factor [CD55] or membrane cofactor protein [CD46], is of particular pathological relevance as in these circumstances it is the critical regulator.

3 | THE FACTOR H-RELATED PROTEINS—OVERVIEW

The initial characterization of the complement FHR proteins derived from molecular cloning experiments in the late 1980s.[19] cDNA clones for fH extracted from human liver were noted to include, in addition to clones for the entire fH protein, a clone for a fragment of fH. This fragment clone encoded most of the fH N-terminal and C-terminal but not the region in between. When the human fH fragment cDNA clone was used as an mRNA probe, multiple fH-specific mRNAs were identified[20]; the corresponding proteins have since been described as the FHR proteins. There are five FHR proteins: FHR1, FHR2, FHR3, FHR4, and FHR5. They are produced primarily in the liver and secreted in the plasma, mostly in glycosylated forms.[21] The FHR genes are located on human chromosome 1q32 within the regulator of complement activation (RCA) gene cluster.[22] The genes are located downstream of the *CFH* gene in the order *CFHR3*, *CFHR1*, *CFHR4*, *CFHR2*, and *CFHR5*.[23,24] The region contains several large regions of sequence similarity and is susceptible to genomic duplication and deletion events.[15] For example, deletion of a region containing *CFHR3* and *CFHR1* results in alleles containing only the *CFHR4*, *CFHR2*, and *CFHR5* genes (del*CFHR3-1*) and is a common polymorphism.[25]

The FHR proteins, like fH and FHL1, are composed of SCR domains. The C-terminal regions of fH and the FHR proteins show high levels of sequence identity (Fig. 2). For example, the FHR1 C-terminal SCR domains, SCR3-5, differ from the equivalent fH C-terminal domains, SCR18-20, by only two amino acids in FHR1 SCR5 and fH SCR 20; L290 is substituted for S1191 and A296 for V1197.[26] The C-terminal of fH is key for surface binding[27] and facilitates cell surface glycosaminoglycan, heparin, complement C3b [28], and in vitro C3d binding.[29] The similarity between the C-terminal sequences of fH and the FHR proteins suggests that FHR proteins can bind similar surface ligands as fH. Consistent with this, albeit with a lower affinity than fH, FHR1 binds to C3b, an interaction that depends on SCR3-5.[30] Recombinant

FHR1 also binds plasma-incubated HUVEC cells, epithelial cells, and C3b-treated rabbit erythrocytes.[50] The sequence uniformity of the C-termini has an additional implication on investigating the fH protein family; antibodies that react with the fH C-terminal are also likely to detect at least some of the FHR proteins.

Another region of high amino acid identity similarity is between fH SCR6 to SCR8 and FHR3 SCR1 to SCR3, and fH SCR6, SCR8, and SCR9 with FHR4 SCR1 to SCR3 [26] (Fig. 2). Specifically, the amino acid identities of FHR3 SCR1 are 91% similar to fH SCR6, FHR3 SCR2 shows 85% identity with fH SCR7, and FHR3 SCR3 shows 62% amino acid similarity with fH SCR8. FHR4 SCR1 shares 71% amino acid identify with fH SCR6, FHR4 SCR2 shares 62% identity with fH SCR8 and FHR4 SCR3 shows 68% amino acid identity with fH SCR9.[29] This region of fH SCR6 to SCR9 binds heparin and modified/monomeric C-reactive protein. The lack of complement regulatory domains similar to fH (fH SCR1-4) in any of the five FHR proteins indicates that they do not possess direct complement regulatory activity.

3.1 | FHR1

FHR1 was identified in 1991 when human plasma was analyzed by immunoblotting with a goat antiserum against human fH.[51] In addition to the fH signal at 150 kDa, two antigens corresponding to molecules of 42 and 37 kDa (termed FHR Ag h42 and h37) were identified.[51] Enzymatic deglycosylation transformed h42 and h37 to molecules of the same mass at 33 kDa, suggesting h42 and h37 are glycosylation isoforms of the same protein, FHR1. Unlike fH, the newly described protein did not accelerate either the dissociation of C3bBb or demonstrate factor I cofactor activity.[52]

FHR1 consists of five SCR domains with variable similarity to fH (Fig. 2). As mentioned above, FHR1 SCR3-5 have high amino acid sequence similarity to the fH C-terminal domains SCR18-20, with subsequent functional consequences as FHR1 shares some binding characteristics with the fH C-terminus.[26] FHR1 has been confirmed as existing as two glycosylated forms in human plasma[53]; FHR1α displays one carbohydrate side chain; FHR1β has two.[50] Two allelic variants of FHR1 have been identified, FHR1A and FHR1B. FHR1A (the acidic isoform) expresses the amino acids HLE in SCR3. FHR1B (the basic isoform) expresses YVQ in SCR3.[53,54]

There is a common deletion polymorphism of the *CFHR1* and *CFHR3* genes (del*CFHR3-1* allele). The del*CFHR3-1* allele frequency varies with ethnicity. The highest frequency of the deleted allele is seen within African populations. For example in sub-Saharan Africa, the frequency is approximately 54.7%, while in European Caucasoid populations the frequency is approximately 15–25%.[25] The frequency is lowest in East Asian and South American and almost absent in Japanese populations. This equates to del*CFHR3-1* homozygosity frequencies of around 30% in Nigeria, 3–10% in Europe, and almost 0% in East Asia and South America.[25] The FHR1 plasma concentration has been measured at between 70 and 100 μg/mL,[50] although there is significant population variation. Besides the presence of del*CFHR3-1* alleles, factors that may influence its circulating concentration include its ability to form heterodimers with FHR2 and to interact with high-density lipoprotein particles.[55,56] fH also shows a wide range of plasma concentration with levels between 110 and 615 μg/mL found in healthy individuals.[15] Given the twofold higher mass of fH compared to FHR1, the molar ratio of the FHR1 dimer to fH has been estimated at approximately 0.3:1.[56]

The two FHR1 N-terminal SCR domains (SCR1-2) show remarkable sequence identity with the corresponding initial two N-terminal SCR domains of both FHR2 and FHR5 (Fig. 2). These domains have been shown to mediate obligate "head to tail" dimerization.[57] As discussed below, dimerization has implications for ligand avidity and implies that heterodimers could exist. To date, FHR1, FHR2, and FHR5 homodimers have been demonstrated from plasma.[57,58] Due to the inability of FHR2 to bind heparin in the absence of FHR1, the FHR1-FHR2 heterodimer has been readily identified in human plasma using heparin chromatography. The FHR1-FHR5 heterodimer has been less clearly identified, and the FHR2-FHR5 heterodimer was detected in small amounts in one study,[58] but could not be detected in a second.[59]

3.2 | FHR2

FHR2 exists in human plasma in two glycosylated forms with molecular masses of 24 kDa and 29 kDa.[52] Its plasma concentration is estimated to be around 50 μg/mL.[56] It consists of four SCR domains. Its C-terminal SCR domains shows a high level of sequence similarity with fH SCR19-20 (Fig. 2). FHR2 SCR3 shows 89% and FHR2 SCR4 61% sequence identity with fH SCR19 and SCR20, respectively.[26]

3.3 | FHR3

FHR3 consists of five SCR domains. FHR3 SCR1 and SCR2 domains show amino acid sequence similarity of 91% to fH SCR 6 and 85% to fH SCR7 (Fig. 2).[56] Furthermore, FHR3 SCR3 demonstrates perfect sequence identity with SCR6 of FHR4A and SCR2 of FHR4B.[40] Very close amino acid sequence identity is also found with the other FHR3 and FHR4 SCRs; FHR3 SCR4 shows 98% amino acid sequence similarity with FHR4B SCR4 and FHR4A SCR8; FHR3 SCR5 shows 93% sequence similarity with FHR4B SCR5 and FHR4a SCR9.[40] The plasma concentration of FHR3 is estimated to be around 70–100 μg/mL.[41] Clearly the concentration will be influenced by the presence of del*CFHR3-1* alleles. FHR3 is detected in plasma in multiple bands of molecular mass range 35 kDa to 56 kDa, suggesting the existence of four differentially glycosylated forms.[56]

3.4 | FHR4

Two transcripts of the *CFHR4* gene have been identified: FHR4A and FHR4B. FHR4A is composed of nine SCR domains, while FHR4B is composed of five domains. FHR4A has an internal duplication: SCR1-4 show sequence identity to SCR5-8.[42] With reference to the SCR domains in FHR4A, the FHR4B protein consists of SCR1 and SCR6-9.[43] CFHR4A has a molecular mass of 86 kDa and CFHR4B has a mass of 42 kDa.

3.5 | FHR5

FHR5 is composed of nine SCR domains.[36] FHR5 is glycosylated and has a molecular mass of 62 kDa. Through a dimerization domain in the initial two N-terminal domains, it exists as an obligate dimer (Fig. 2).[37,40] FHR5 plasma concentrations are approximately 5 μg/mL.[44] It has been detected in complement-containing kidney deposits.[45] In fact FHR5 was first detected using an antibody raised by immunizing mice with glomerular basement membrane preparation from glomerulonephritic human kidneys. The FHR5 staining pattern within the kidneys was similar to the pattern seen for both C3 and C5b9 staining and confirmed in a prospective study of 100 consecutive patient kidney biopsies stained with anti-FHR5 monoclonal antibody.[44]

4 | THE FACTOR H-RELATED PROTEINS—FUNCTIONAL ASSESSMENT

The biological functions of the FHR proteins have been elusive. The presence of the del*CFHR3-1* allele among healthy individuals indicates that both FHR1 and FHR3 are biologically non-essential. Likewise, the presence of a del*CFHR4-1* allele, although much less frequent that the del*CFHR3-1* allele, indicates CFHR4 also lacks an essential biological role.[34]

The structural and sequence similarity with fH domains lead to speculation as to whether the FHR proteins could replicate or interfere with functions of fH.[46] Because none of the FHR proteins contained domains analogous to the complement regulation domains of fH (SCR1-4), the FHR proteins were not expected to replicate this particular activity. Consistent with this, fH regulatory activity was not demonstrated for FHR1 and FHR2.[51] The most consistently identified binding was to heparin or components of lipoprotein complexes, although the relevance of this was unclear.[35] We first summarize some of the published data, the majority of which is derived from in vitro assays, reporting complement regulatory roles for the FHR proteins. Secondly, we review data indicating a role for these proteins in promoting complement activation.

4.1 | FHR proteins as regulators of complement activation

FHR1 has been reported to inhibit the C5 convertase.[50] Using a sheep erythrocyte lysis assay, FHR1 reduced generation of C5a, but not C3a, suggesting FHR1 has an inhibitory effect on the C5 convertase.[50] This was confirmed by the lack of C5 deposition on sheep erythrocytes and inhibition of C5a production in an in vitro C5 convertase activity assay. Furthermore, FHR1 was shown to bind C5 and the activation product C5b6, and inhibited hemolysis in a C5b9-dependent erythrocyte lysis model.[50] However, further studies have not confirmed this activity.[37]

FHR2 has been reported to inhibit the C3 convertase and terminal complement complex assembly in vitro.[47] Using human sera, FHR2 inhibited the production of C3a from C3 in a dose-dependent manner, and reduced terminal complement complex surface deposition on sheep red blood cells.[47] FHR2 also inhibited in vitro C3 convertase assembly but did not demonstrate factor I cofactor activity.[47]

Using surface plasmon resonance techniques, recombinant FHR3 and FHR4 were shown to bind the C3d region of C3b and, in the presence of factor H, promote cleavage of the C3b alpha chain.[48] These data suggested that FHR3 and FHR4 have factor I cofactor activity. However, FHR3 and FHR4 factor I cofactor activity in the absence of factor H was very low and only detectable at non-physiological FHR concentrations.[48] When added to fH-depleted, complement active plasma, FHR3 reduced complement-mediated hemolysis of sheep erythrocytes by approximately 15%. This compared with approximate 80% inhibition by fH.[41] At higher than estimated physiological concentrations, FHR3 also reduced production of C5a and C5b from C5 in an in vitro C5 convertase model.

Recombinant FHR5 bound C3b in a dose-dependent and saturable manner.[49] The addition of FHR5 to C3b and fI lead to C3b cleavage, suggesting that FHR5 has fI cofactor activity.[44] Furthermore, using a C3 convertase assay, FHR5 appeared to inhibit C3 activation.[44] However, 10 μg of FHR5 was needed to achieve a similar level of fI cofactor activity as 50 ng of fH, and 5 μg of FHR5 had a similar C3 convertase inhibition effect as 1 μg of fH. The dependence on very high FHR5 concentrations to observe these effects questions the biological relevance of these in vitro activities.

4.2 | FHR proteins as promoters of complement activation

Under some circumstances, FHR proteins appear to increase complement activation. This can be due to an ability to compete with fH for surface ligand binding. The result is a reduction in the ability of fH to negatively regulate surface C3b activation. In this setting, this process has been termed fH deregulation.[37,38] We will first discuss experiments that demonstrate direct complement activation by the FHR proteins, and then highlight data showing FHR interaction with fH binding and subsequent complement deregulation and activation.

As opposed to regulating C3b activation, investigation of the significance of FHR4 binding to C3b suggested that the FHR4-C3b interaction actually promoted complement activation. FHR4B immobilized on microtiter plates bound C3b and C3d,[48] while FHR4A was also able to bind inactivated C3b (iC3b) but not C3c. FHR4A binding to fluid-phase and plate-immobilized C3b was stronger than FHR4B and comparable to fH.[50] Analysis of recombinant deletion FHR4A mutants showed that the C3b- and C3d-binding site was localized within the SCR8 and SCR9 domains. Importantly, no significant factor I cofactor or C3 convertase decay accelerating activity could be demonstrated for FHR4A. Addition of Mg-EGTA-buffered human serum, which restricts complement activation to the alternative pathway, to FHR4A and FHR4B lead to C3 activation and the deposition of C3b fragments. This activation was dependent on the FHR4A SCR8-9 domains and associated with factor B and properdin deposition.[51] This demonstrated that FHR4-C3b complex could promote C3 convertase formation and consequently C3 activation.

More recently, FHR5 has been shown to directly promote C3 convertase formation. When surface-immobilized FHR5 is incubated with C3b, purified factor B, factor D, and properdin, the C3 convertase can form on FHR5 and functions to produce C3a and C3b.[52] The direct activation of the alternative complement pathway can be replicated in the fluid phase; the addition of normal human serum to FHR5 immobilized on microtiter plates leads to C3, factor B, and properdin deposition.[52]

These data raise the question as to what are the ligands for FHR4 and FHR5 in vivo that would promote local complement activation? Recently, FHR4 and FHR5 have been shown to bind to pentameric CRP[50,58] and pentraxin-3, respectively[52] leading, via different mechanisms, to complement activation.

FHR4 interacts with C-reactive protein. Using recombinantly expressed FHR4 fragments, binding was localized to the SCR1 domain of FHR4. Sequence comparisons with fH and the other FHR proteins showed this site to be unique to FHR4.[50] FHR4 binds preferentially to the native pentameric form of CRP, while fH predominantly binds to the monomeric form. FHR4-bound CRP leads to enhanced opsonization of necrotic cells by CRP.[58] Furthermore, as demonstrated by C3 fragment deposition, the bound FHR4 was able to activate complement via C2- and C4-dependent pathways,[50] independent of fH. The ability for FHR4 to allow C3 convertase formation on bound C3b may contribute to enhanced damaged cell and pathogen opsonization.

Interestingly, although FHR5 appears to interact with pentraxin 3 (PTX3), the subsequent complement activation seems to involve fH deregulation. Pentraxins are a family of pattern recognition molecules of the innate immune system which include CRP. Under inflammatory conditions, PTX3 is produced locally by neutrophils, macrophages, dendritic cells, fibroblasts, and endothelial cells and can recruit, initiate, and control complement regulators and activation. FHR5 binds PTX3-coated wells in a dose-dependent manner, and is able to do this at lower concentrations than fH.[52] Recombinant FHR5 and serum-derived FHR5 are able to interfere with the binding of fH to PTX3 in an extracellular matrix model.[52] The interaction of fH and PTX3 is potentially important in downregulating PTX3-induced complement activation. These studies describe a potential functional interaction at physiological concentrations of FHR5 with pro-inflammatory molecules and the ECM. They also provide a mechanism for FHR5-driven local complement deregulation by competition with fH for C3b and other physiological ligands.

The ability of FHR proteins to interfere with fH-mediated regulation of C3b activation, leading to complement deregulation, derived from the observation that fH acceleration of C3 convertase decay was reduced in the presence of FHR3 and FHR1.[41] Furthermore, in complement-dependent hemolytic assays using plasma deficient in both FHR3 and FHR1, the addition of exogenous FHR3 and FHR1 significantly increased complement-dependent hemolysis.[41] This fH deregulation was confirmed in subsequent experiments using FHR1 and FHR5. FHR5, like the other fH protein family members, recognizes and binds to C3b on surfaces.[44] FHR5[57] and FHR1[58] have been shown to compete with fH for binding to surface C3b. In erythrocyte lysis assays using guinea pig erythrocytes, both FHR5[57] and FHR1[58] promote the lysis of cells in a fH-dependent manner.

The ability of FHR1, FHR2, and FHR5 to form dimers[57] and higher order complexes[58] results in increased ligand avidity.[57] This phenomenon is predicted to potentiate fH deregulation. Support for this in vivo derives from the observation that abnormal FHR proteins, in which there is duplication of the dimerization domains, are associated with C3 glomerulopathy, a condition characterized by abnormal C3 deposition within the kidney.[58,54–57] In vitro evidence that dimerization enhances fh deregulation includes the differential ability of FHR1 monomers and dimers to inhibit the binding of fH to surface C3b[57] and to interact with glomerular-bound C3 in the fH-deficient mouse model.[57] In both situations, dimeric proteins were superior to monomeric counterparts.

5 | THE FACTOR H-RELATED PROTEINS—ASSOCIATION WITH DISEASE

Understanding the associations and role of the FHR proteins in disease progression has increased our understanding of their biological functions.

5.1 | FHR proteins and interactions with pathogens

FHR protein binding to several microbes and microbial proteins has been demonstrated[58] including *Neisseria meningitidis*,[59,60] *Streptococcus pyogenes*,[61] *Borrelia burgdorfei*,[62] *Leptospira interrogans*,[63] *Pseudomonas aeruginosa*, [64] and *Candida albicans*.[65] However, few studies have described the functional mechanism and effect of these interactions. Where there is both a FHR-pathogen and fH-pathogen interaction, the FHR interaction may, by promoting complement activation, facilitate complement-mediated pathogen damage.[40] The FHR-pathogen interaction may also influence immune cell recruitment, as FHR1, as well as fH have been shown to bind complement receptor type 3 on neutrophils.[58]

N. meningitidis is an important human pathogen causing rapidly progressive septicemia and meningitis. As evidenced by the susceptibility of individuals with alternative or terminal complement pathway deficiencies to *N. meningitidis*, complement activation is critical for disease protection.[59] All the single nucleotide polymorphisms (SNPs) associated with meningococcal disease in a recent genome wide association study (GWAS) were located within the *CFH-CFHR* locus. Specifically, three SNPs in *CFH* and three in *CFHR3* showed repeated independent association with disease susceptibility through an original UK-based study population and two European replication populations.[59]

N. meningitidis binds fH with high affinity via fH-binding protein (fHbp), a surface lipoprotein with mimicry to host cell carbohydrates.[59] fHbp binds fH SCR6 and SCR7 domains. These are very similar to FHR3 SCR1 and SCR2 (Fig. 2). Using full-length FHR3, mutant FHR3 proteins lacking specific SCR domains, and serum from FHR3-deficient individuals, it was demonstrated that FHR3 interacted with fHbp in a manner that depended on SCR domains 1–2.[60] Furthermore, FHR3 was shown to compete with fH for both fHbp and C3b on the bacterial

surface and significantly influenced *N. meningitidis* survival in serum assays.[60] This important study demonstrated that susceptibility to an important infectious disease was governed by the relative abundance of fH and FHR3 through their shared affinity for the pathogen fHbp. Interestingly, *N. meningitidis* fHbp shows variation in fH and FHR3 binding affinity which may explain the prevalence of certain strains.[60] The findings also demonstrate how individuals' genetics and the consequent competition between two complement factors with opposing effects influence infectious disease susceptibility, and suggest the distribution of complement proteins, particularly the FHR proteins, should be considered in epidemiology disease mapping and immunization strategies.

5.2 | Atypical hemolytic uremic syndrome

Atypical hemolytic uremic syndrome (aHUS) is a renal thrombotic microangiopathy characterized by hemolytic anemia with schistocytes (microangiopathic hemolytic anemia), thrombocytopenia, and acute kidney injury. Although rare, it is serious and life-threatening and leads to end-stage renal disease in about 50% cases.[66] Although the typical form of the disease is associated with shigatoxin-producing *Escherichia coli*, the atypical form is most commonly caused by impaired regulation of complement activation on endothelial surfaces. aHUS is associated with mutations or variants of the complement system, commonly affecting fH.[66] Four *CFHR* gene rearrangements are associated with aHUS. Three of these rearrangements result in the generation of hybrid genes between *CFH* and *CFHR1* or *CFHR3* that affect the fH C-terminal SCR20.[67–70] The *CFH:CFHR1* hybrid gene involves the substitution of the C-terminal SCR 20 of fH with the C-terminal SCR of FHR1. The *CFH:CFHR3* hybrid gene substitutes fH SCR20 with the whole FHR3. The *CFHR1:CFH* hybrid gene results from the substitution of the two C-terminal SCRs of FHR1 with those of fH.[67–70]

Despite the aforementioned close sequence homology between fH SCR20 and the C-terminal FHR1 SCR5, the FHR1 SCR domain renders fH ineffective in endothelium binding and complement regulation. This demonstrates FHR1 lacks appropriate complement regulatory activity to substitute fH and the essential role of the fH C-terminal region in the protection of host surfaces from complement-driven injury. It is also a striking example that small changes in amino acid sequences result in remarkable differences in molecular binding and surface interaction.[70]

The fourth *CFHR* gene rearrangement associated with aHUS is with the common deletion *CFHR3-CFHR1* genetic polymorphism (del*CFHR3-1*), or the less common deletion of genes *CFHR1-CFHR4* (del*CFHR1-4*). As discussed below, although these deletion polymorphisms are associated with protection from other conditions, they are also associated with and may contribute to, the presence of anti-fH autoantibodies.[54] fH autoantibodies are seen in about 9% of aHUS cases, and the majority, but not all, of these patients have homozygous deletion of *CFHR1*.[71] Using recombinant fH fragments, the binding sites of fH autoantibodies from five unrelated aHUS patients has been mapped and localized to recognize the fH C-terminal SCR19 and SCR20 of fH.[72] The autoantibodies mimic C-terminal fH mutations by impairing fH cell surface recognition, binding, and complement regulation, leading to the development of aHUS. The FHR3-FHR1 deletion and associated generation of anti-fH autoantibodies is highly prevalent in hematopoietic stem cell transplantation-related thrombotic microangiopathy.[73] Interestingly and potentially because of a predisposition to produce anti-fH antibodies, the polymorphism is also a risk factor for systemic lupus erythematosus (SLE).[74]

5.3 | Age-related macular degeneration

Age-related macular degeneration (AMD) is a heritable and progressive retinal disease that leads to loss of central vision. AMD is a common cause of blindness in older individuals. The early stage of the disease, known as age-related maculopathy, is characterized by the formation of drusen. Drusen are small yellow deposits, external to the outer retina between the retinal pigment epithelium and Bruch's membrane. Large numbers of drusen and damage to the retinal pigment epithelium increase the risk of progressing to atrophy or aberrant neovascularization of the outer retina, with associated vision loss.[75] AMD is associated with polymorphisms in *CFH* protein family genes and variants in fH and the FHR proteins. GWAS have identified multiple SNPs associated with AMD disease risk, many of which are located within the regulation of complement activation locus on chromosome 1.[75] A polymorphism in the *CFH* gene affecting amino acid 402 in SCR7, known as Y402H, is major risk factor for AMD and has been confirmed in several cohort studies.[41,76] The FHR proteins are also implicated in AMD pathogenesis. The homozygous deletion polymorphism of FHR3 and FHR1 is associated with a lower risk for AMD; the deletion haplotype is present in 20% of healthy controls and 8% of individuals with AMD.[76] Regression analysis confirms the del*CFHR3-1* remains significantly associated with the risk for AMD even without the effect of the Y402H fH.[41] The pathogenic role of complement deregulation in AMD is further suggested by the composition of the drusen that characterize the condition. The drusen contain a number of complement activation products and regulators and show remarkable similarities to the FHR-containing deposits found in C3 glomerulopathy, a complement-driven renal disease.

5.4 | C3 glomerulopathy

C3 glomerulopathy (C3G) is a group of kidney diseases caused by complement deregulation. The deregulation may be in the form of abnormal control of complement activation, deposition, or degradation.[77] Cases of C3G are characterized by glomerular deposits composed of C3 as the dominant antigen, and electron-dense deposits on electron microscopy. Although a rare condition, C3G often progresses to end-stage renal failure and recurs in renal transplants. A number of studies of familial cases of C3G have identified mutant FHR proteins associated with and likely pathogenic in the development of C3G.[58,54–57] These include a heterozygous mutation of *CFHR5* involving internal duplication of exons 2 and 3 that encode FHR5 SCR1 and SCR2, respectively, in a large Greek Cypriot cohort[54]; the identical FHR5 with

duplicated SCR1 and SCR2 caused by a novel intronic genetic mutation in a British family without Cypriot ancestry[55]; a heterozygous mutation in a large Irish kindred whereby deletion of *CFHR3* exons 4–6 and *CHFR1* exon 1 produced a hybrid protein with SCR1 and SCR2 of FHR3 linked to full-length FHR1[56]; a Spanish family with a heterozygous mutation causing duplication of FHR1 SCR1 to SCR4[58]; and a German sibship with heterozygous deletion of *CFHR2* exons 4 and 5, producing a hybrid protein of FHR2 SCR1 and SCR2 linked to full-length FHR5, which is structurally similar to the abnormal FHR5 identified in the aforementioned Cypriot and British families.[57]

The pathogenic FHR proteins are mostly characterized by duplication of their dimerization domains (the initial N-terminal two SCR domains). The abnormal dimerization domains likely increase avidity and the current hypothesis is that this results in enhanced fH deregulation within glomeruli resulting in abnormal C3 accumulation. One example of data to support this derives from the detailed study of a mutant FHR1 associated with familial C3 glomerulopathy in which the mutant protein formed higher order oligomers, likely tetramers, of FHR, and large multimeric complexes.[58] The mutant FHR1 contained a duplication of the two N-terminal SCR1 and SCR2; the location of the dimerization motif. The mutant protein bound to C3b, iC3b, and C3dg immobilized on a Biacore chip with greater affinity than native FHR1. It also showed enhanced competition with fH in fH-dependent complement-driven hemolysis assay using guinea pig erythrocytes.[58] A comparable assay assessed the ability of FHR1, FHR2, and FHR5 from individuals with familial C3G, and either a mutant CFHR5,[54] or a FHR3-1 hybrid protein[56] to activate complement.[57] The mutant FHR proteins from individuals with either mutation are characterized by duplicated dimerization motif SCR1 and SCR2. When added to a fH-dependent complement-driven guinea pig erythrocyte hemolysis assay, the serum-derived preparations from patients with mutant FHR proteins showed significantly greater hemolysis and complement deregulation than healthy controls.[57]

Investigations of the mutant FHR1 also demonstrated the potential for native FHR proteins to form heterodimers, likely via binding at the dimerization motif; in addition to FHR1, the elution fraction from heparin chromatography purification for FHR1 of fH-depleted human plasma showed the capture of FHR2 and FHR5.[58] FHR2 was not detected from individuals with a *CFHR1* deletion polymorphism, suggesting FHR1 interacts with FHR2 and FHR5. No interaction of FHR1, FHR2, and FHR5 was detectable with FHR3 and FHR4A/B, members of the fH protein family lacking the dimerization domain.[58]

An important observation that demonstrates our understanding of the FHR-driven complement deregulation is incomplete is the effect of a hybrid protein with SCR1 and SCR2 from FHR2 added to a full-length CFHR5 protein. Although structurally similar to a mutant FHR5 with duplicated SCR1 and SCR2, unlike other FHR mutants it causes severe plasma C3 depletion. This effect is similar to C3 nephritic factor, suggesting this mutant FHR may have C3 convertase stabilizing properties.[59]

5.5 | IgA nephropathy

IgA nephropathy (IgAN) is the most common primary glomerular disease and a common cause of renal failure, especially in young people.[78] Historically, an important role for complement in IgAN pathogenesis has been suspected but poorly defined. Series of IgAN pathology studies from the 1980s demonstrated glomerular immunostaining of C3 co-deposited with the defining IgA in almost all cases, predominantly in the absence of classical pathway components such as C1q.[79] Interestingly, C3G associated with mutant FHR proteins shares many clinical, phenotype, and histology features with IgAN. Recent genetic studies specifically implicated a role for the FHR proteins in IgAN pathogenesis. An initial GWAS of 1194 cases and 902 controls of Chinese Han ancestry identified a strong association between IgAN and SNP rs6677604 that tags the delCFHR3-1 deletion.[80] The latest meta-analysis of more than 20000 individuals of different ethnicities confirmed this variation to have a protective effect in IgAN, with inheritance of a single allele calculated to reduce disease risk by 36%, while two alleles reduce the disease risk by 45%.[81] Interestingly, the allelic frequency of the deletion exhibits marked differences across worldwide populations, with the highest frequency of the protective alleles in ethnicities of African ancestry (55%), in whom the prevalence of IgAN is least common, while people from South America and East Asia, where IgAN is most common, have the lowest prevalence of protective alleles (0–5%). Subsequently, an association was demonstrated between the same SNP rs6677604, serological and histopathology evidence of complement deregulation, and histology markers of disease severity, although no association was established between complement deregulation with markers of disease outcome.[82]

6 | CONCLUSIONS

Despite sharing significant sequence homology with fH, the FHR proteins lack the fH N-terminal complement regulatory domains. A common deletion polymorphism of the *CFHR3* and *CFHR1* genes, and less frequently, a combined deletion of the *CFHR1* and *CFHR4* genes, result in significantly reduced or absent levels of the respective proteins in healthy individuals. These observations suggested that FHR proteins lack fH-like complement regulatory ability and were, in the case of FHR1, -3, and -4, biologically non-essential. However, genetic studies in both rare and common diseases indicated that these proteins have important roles in complement-associated diseases. The appreciation of two further structural characteristics has been key in developing theories to explain their function. First, the FHR proteins are characterized by C-terminal domains that share sequence similarity with the C-terminal domains of fH. They therefore display similar surface binding to fH and can act as competitive antagonists. Second, FHR1, -2, and -5 share a conserved dimerization domain within their first two N-terminal domains which, through dimerization, enhances ligand avidity. The characterization of rare conditions caused by FHR gene mutations, in particular aHUS and familial C3G, has revealed important clues as to their function. Data derived from these conditions suggests that FHR proteins antagonize the ability of FH to negatively regulate surface complement C3b within the kidney. It is important to note that to date fH deregulation has not been directly demonstrated in vivo on surfaces like the GBM. This

will be important to investigate as the regulation of C3 along surfaces is likely to be determined not only by the relative concentrations of fH and FHR proteins in the vicinity but also the affinity of the proteins for a given surface. Studies of *N. meningitidis* illustrate that both protein availability influenced, for example by the presence of del*CFHR3-1* alleles, and the surface upon which complement activation is occurring, influenced for example by the type of fhbp on the Neisserial surface, is biologically relevant to disease susceptibility. A similar imbalance is likely to play a pathogenic role in IgAN. In conditions where there is marked complement dysregulation, such as C3 glomerulopathy, it may be appropriate to fully block effector functions. However, in chronic complement-associated disease like IgAN and AMD, modulation of complement activation may be sufficient. Manipulation of the FHR protein family may be a feasible means to achieve complement modulation and provide a safe long-term therapy for complement-associated disease.

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Review

The role of complement in IgA nephropathy

Agustin Tortajada[a,1], Eduardo Gutierrez[b,1], Matthew C. Pickering[c], Manuel Praga Terente[b], Nicholas Medjeral-Thomas[c,*,1]

[a] Department of Immunology, Ophthalmology and ENT, Complutense University School of Medicine and 12 de Octubre Health Research Institute (imas12), Madrid, Spain
[b] Department of Nephrology, Research Institute Universitary Hospital 12 de Octubre (imas12), Madrid, Spain
[c] Centre for Inflammatory Disease, Imperial College London, United Kingdom

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ABSTRACT

IgA nephropathy (IgAN) is common and often progresses to end stage renal disease. IgAN encompasses a wide range of histology and clinical features. IgAN pathogenesis is incompletely understood; the current multi-hit hypothesis of IgAN pathogenesis does not explain the range of glomerular inflammation and renal injury associated with mesangial IgA deposition. Although associations between IgAN and glomerular and circulating markers of complement activation are established, the mechanism of complement activation and contribution to glomerular inflammation and injury are not defined. Recent identification of specific complement pathways and proteins in severe IgAN cases had advanced our understanding of complement in IgAN pathogenesis. In particular, a growing body of evidence implicates the complement factor H related proteins 1 and 5 and lectin pathway as pathogenic in a subset of patients with severe disease. These data suggest complement deregulation and activity may be dominant drivers of renal injury in IgAN. Thereby, markers of complement activation may identify IgAN patients likely to progress to significant renal impairment and complement inhibition may emerge as an effective method of preventing and reducing glomerular injury in IgAN.

1. Introduction

1.1. IgA nephropathy overview

IgA Nephropathy (IgAN) is the most common primary glomerulonephritis in the world. A diagnosis of IgAN is associated with an average reduction in life expectancy of 6–10 years (Hastings et al., 2018; Jarrick et al., 2019). Approximately 40% of IgAN patients older than 30 years age at diagnosis develop end stage renal disease (ESRD) over 20 years (D'Amico, 2004). However, IgAN is characterized by a broad range of clinical presentations and courses. This translates into different presentations in children and adults. Furthermore, clinical severity differs markedly with gender, ethnicity and race. It is estimated that in 5.8% of cases there may be a family history or genetic predisposition for the disease (Julian et al., 1985). Hence, it is thought that IgAN can encompass different biological processes, which presents the question of whether IgAN is a single pathogenic entity. Stratification of patients on clinical and histology features is currently difficult, which greatly complicates the therapeutic approach.

IgAN is an autoimmune disease characterized by abnormal IgA1 synthesis and glycosylation, resulting in imbalanced increase of circulating galactose deficient IgA1 (gd-IgA1). This anomalous IgA1 can form complexes with specific autoantibodies, forming gd-IgA1 immune complexes (gd-IgA1-IC). The gd-IgA1-IC deposit in glomeruli, a process that leads to mesangial cell proliferation and matrix expansion by mechanisms that are not completely understood (Heineke et al., 2017). Recurrence of this glomerular entity after renal transplantation suggests systemic immune dysregulation, rather than local renal abnormalities, is pathogenic in IgAN. Consistent with this, peripheral blood mononuclear cells are hyperactivated in IgAN, showing decreased regulation of the cellular machinery involved in antigenic processing (Floege, 2011; Wyatt and Julian, 2013).

The most consistent clinical finding in IgAN is the presence of hematuria; approximately half of patients present with outbreaks of macroscopic gross hematuria (MGH) and most other patients have microscopic hematuria. However, the pathogenesis and clinical significance of MGH are not understood. Bouts of macroscopic hematuria are more common in children and early stages of adult IgAN and are concomitant with mucosal infections, usually in the respiratory and, occasionally, gastrointestinal tracts (Haas et al., 2008). Interestingly,

* Corresponding author at: Centre for Inflammatory Disease, Imperial College London, Hammersmith Campus, London W12 0NN, United Kingdom.
E-mail address: n.medjeral-thomas@imperial.ac.uk (N. Medjeral-Thomas).
[1] AT, EG and NMT contributed equally to the manuscript.

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mesangial IgA deposition also coincides with these infections (Robert et al., 2015), indicating that dysregulation of the mucosal immune system and mucosa-kidney axis may be important in the pathogenesis of haematuria in IgAN (Suzuki et al., 2011a). In adult IgAN patients, MGH can precipitate acute renal failure and 25% of patients over 50 years age may not recover renal function if MGH is prolonged beyond 10 days (Gutierrez et al., 2007). Classically, recurrent episodes of macroscopic haematuria were considered an infrequent clinical manifestation after 40 years age. However, the degrees of haematuria and proteinuria in IgAN are wide-ranging. Furthermore, the demographic of IgAN patients seems to have changed; recent series have found a significant increase in the average age of IgAN patients and the proportion of patients over the age of 65 years at presentation (Gutierrez et al., 2018). Acute kidney injury, MGH and nephrotic range proteinuria are more common in patients older than 65 years, and the reasons for this are not known (Gutierrez et al., 2018).

Proteinuria, the presence or development of arterial hypertension and reduced glomerular filtration at diagnosis are the clinical factors that influence the development of chronic kidney disease (CKD).The amount of proteinuria is particular relevant in IgAN. Presenting proteinuria greater than 0.5 g/day is significantly associated with worse renal outcomes (Reich et al., 2007). Consequently, therapeutic approaches aim for proteinuria reduction. The use of renin-angiotensin-aldosterone system blockers is widely recognized as the best therapeutic approach in patients with IgAN and proteinuria greater than 0.5–1 g/day and arterial hypertension (Chapter 10, 2011).

IgA Nephropathy can manifest diversely, ranging from isolated microscopic hematuria to rapidly progressive glomerulonephritis. The natural history and clinical outcomes of IgAN are also diverse and include spontaneous remissions (5–15%, less common in adults) and rapidly progressive glomerulonephritis (< 10%), although slowly progressive CKD (30–40%) and "benign" evolution (40–50%) are the most common (Bartosik et al., 2001; Yoshikawa et al., 1990). Inherent variation in the rate of IgAN progression makes randomized controlled trials difficult to design and interpret. Therefore, risk scores are needed that predict and stratify patients to fast or indolent courses (Coppo, 2018).

About 7%–15% of IgAN patients present with malignant hypertension (Sevillano et al., 2015; El Karoui et al., 2012). IgAN with malignant hypertension is characterized by severe renal impairment at presentation and commonly progresses to ESRD. Renal biopsy demonstrates mild to moderate glomerular sclerosis and interstitial fibrosis in more than 80% of cases. Interestingly, renal biopsy also demonstrates thrombotic microangiopathy (TMA) in 30%–100% patients, although laboratory evidence of TMA, such as thrombocytopenia or schistocytes on blood film, is much less common (Sevillano et al., 2015; El Karoui et al., 2012). TMA is characteristic of microangiopathic syndromes in which complement dysregulation seems to play an important pathogenic role, including atypical haemolytic uraemic syndrome (aHUS) (Noris et al., 2012). IgAN patients with malignant hypertension and TMA demonstrate the potential for complement to drive glomerular injury in IgAN.

1.2. IgA nephropathy pathogenesis

IgAN is an autoimmune disease influenced by multiple genetic, ethnic and environmental factors. Although the protagonist seems to be gd-IgA1, multiple events contribute to IgAN and renal damage. The currently accepted model of pathogenicity in IgAN is the *"four-hit"* model (Fig. 1) (Suzuki et al., 2011b).

1.2.1. First hit: elevated serum gd-IgA1 levels

IgA is the most synthesized antibody in humans, abundant on mucosal surfaces and fundamental to mucosal antigen responses and host-commensal homeostasis. There are two IgA subclasses, IgA1 and IgA2. About 80% of serum IgA is IgA1 (Breedveld and van Egmond, 2019). The subclasses differ mainly by the structure of the heavy chain, specifically in the hinge region (HR). IgA1 contains numerous *O*-linked glycans and two *N*-linked glycosylation sites per heavy chain, whereas IgA2 does not possess *O*-glycans and contains two additional heavy chain *N*-glycan sites (Woof and Russell, 2011). Also, the IgA1 HR is 13 amino acids longer than IgA2. The structural differences make IgA1 susceptible to bacterial proteases. In contrast to other immunoglobulins, IgA is found in monomeric (mIgA1) and polymeric (pIgA1) forms. Monomeric IgA1 predominates in blood whereas pIgA1 is found mainly in external secretions (Woof and Mestecky, 2005). Most serum mIgA is produced in bone marrow; secretory pIgA is synthesized locally in mucosal tissues. Polymeric IgA1 composition is diverse and includes dimeric, secretory and immune complexes of IgA (Oortwijn et al., 2006).

IgAN patients show greater proportions of poorly O-galactosylated IgA1 in circulation compared to healthy individuals (Hiki et al., 2001; Coppo and Amore, 2004; Moldoveanu et al., 2007). These glycosylation defects seem to have a high component of heritability. The gd-IgA1 glycoforms are polymeric, and normally produced at mucosal surfaces as part of innate immune responses (Boyd et al., 2012). Therefore, abnormal innate immunity responses to mucosal infections or antigens may be involved in the gd-IgA1 production in IgAN. Supportive of this, a role for Toll-like receptors (TLR), which cause polyclonal lymphocyte proliferation (Meng et al., 2014; Nishikawa et al., 2000) and formation of circulating immune complexes (Robert et al., 2015; Barratt et al., 2007), may be involved in IgAN pathogenesis. Coppo R et al. demonstrated overexpression of TLR4 in circulating mononuclear cells from patients with IgAN (Coppo et al., 2010). Mucosal TLR9 activation induces B cell activation factor (BAFF) overexpression in dendritic cells, B cell expansion and increased IgA synthesis (Kajiyama et al., 2011) all of which may influence IgAN pathogenesis (Suzuki et al., 2011b). Recently, the potential anti-proteinuric benefit of hydroxychloroquine, an immunomodulator that inhibits TLR as well as the production of cytokines and chemokines, has been demonstrated in IgAN (Liu et al., 2019).

1.2.2. Second hit: anti-gd-IgA1 antibodies

Gd-IgA1 is the target for antiglycan autoantibodies (Maixnerova et al., 2019). The glycan-specific autoantibodies are often characterised by the replacement of serine with alanine in the complementarity-determining region 3 of the heavy chain variable region (Suzuki et al., 2009). This change increases the affinity of binding to O-linked glycans, particularly exposed N-acetylgalactosamine (GalNac), on gd-IgA1. This alteration arises from somatic mutation during active immune responses, perhaps following exposure to viruses or bacteria that express GalNac (Wyatt and Julian, 2013). Anti-gd-IgA1 IgG levels correlate with disease severity, specifically with the amount of proteinuria. Also, their presence in renal biopsies correlates with mesangial and endocapillary proliferation (Bellur et al., 2011).

1.2.3. Third hit: Increased circulating gd-IgA1- immune complexes (IC)

IgAN is characterised by mesangial deposition of IgA1 and complement proteins. The IgA1 deposits derive from pathogenic circulating IC and contain aberrantly glycosylated pIgA1, which shows galactose deficiency in HR O-linked glycans (Hiki et al., 2001; Allen et al., 2001; Mestecky et al., 2013). IgA complexed with soluble Fcα receptor (CD89) can be detected in serum from IgAN patients but not other diseases, and a mouse model of IgAN implicates soluble CD89-IgA complexes in disease pathogenesis (Launay et al., 2000). However, other studies have found soluble CD89-IgA1 complexes are neither specific nor relevant to IgAN development (van der Boog et al., 2003; Vuong et al., 2010). The mesangial cells represent the primary target of these pathogenic immune deposits and the unique anatomy of the mesangium, located between the fenestrated endothelium and the glomerular basement membrane, makes it a deposit focus. Gd-IgA1-IC deposition can be increased during episodes of macroscopic hematuria,

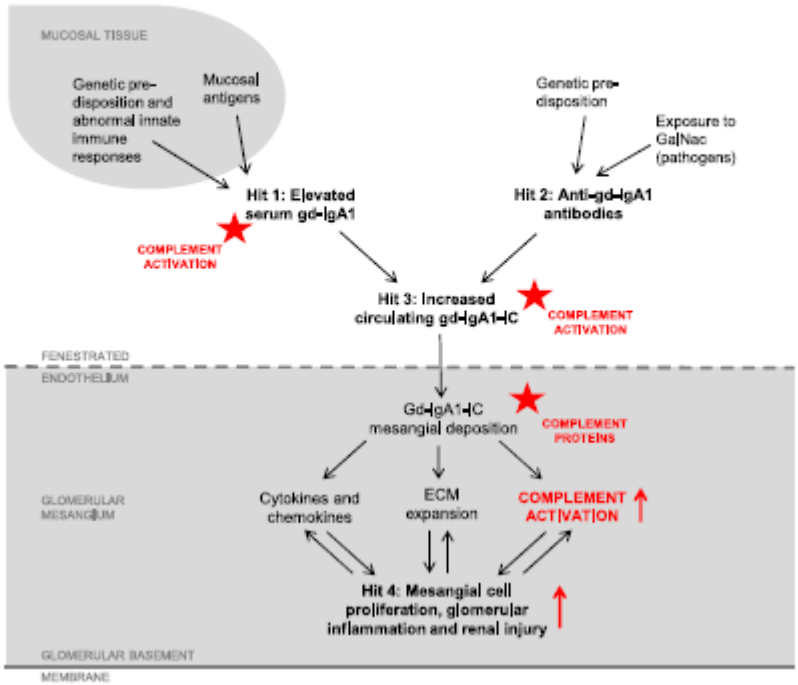

Fig. 1. Complement in the multi-hit pathogenesis of IgA nephropathy. IgA Nephropathy (IgAN) pathogenesis involves multiple hits (Wyatt and Julian, 2013; Yeo et al., 2018). Genetically predisposed individuals mount abnormal immune responses to common and environmental pathogens. This leads to Hit 1, elevated serum levels of galactose-deficient IgA1 (gd-IgA1), Hit 2, the overproduction of autoantibodies with heavy chain variable region characteristics that recognise gd-IgA1, and Hit 3, the formation of circulating immune complexes of gd-IgA1 and anti-gd-IgA1 autoantibodies (gd-IgA1-IC). Presumably by traversing glomerular fenestrated endothelium, gd-IgA1-IC deposit in mesangium and trigger cellular responses, cytokine release, complement activation, and extracellular matrix (ECM) expansion. The result is Hit 4; mesangial cell proliferation, glomerular inflammation and renal injury. The multi-hit theory does not currently explain why glomerular IgA1 and circulating gd-IgA1 and gd-IgA1-IC are detected in healthy individuals (Suzuki et al., 2003; Gale et al., 2017). Evidence of complement activation has been identified at multiple points in IgAN pathogenesis (indicated with red stars). For example, polymeric gd-IgA1 can trigger alternative and lectin pathway activation *in vitro*. Alternative pathway activation proteins have been identified in patient-derived circulating gd-IgA1-IC (Hiemstra et al., 1987; Roos et al., 2001). The ability for gd-IgA1-IC to induce mesangial cell responses *in vitro* is dependent on a heat-labile factor, which could be complement (Yanagihara et al., 2012). And immunohistology evidence of glomerular alternative, lectin and terminal pathway activation correlate with IgAN severity (Medjeral-Thomas et al., 2018; Roos et al., 2006). Due to its self-amplifying nature, complement activity at any step in IgAN pathogenesis would amplify (red arrows) glomerular complement activation, inflammation and renal injury. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

perhaps after binding receptors that recognize the pathogenic IC (Novak et al., 2005).

1.2.4. Fourth hit. Mesangial cell proliferation, glomerular inflammation and renal injury

This is characterized by extracellular matrix expansion, complement activation and cytokine release. Some cytokines can injure podocytes, alter podocyte gene expression and glomerular filtration and induce proteinuria (Lai et al., 2009; Trimarchi and Coppo, 2019). However, how gd-IgA1 interacts with and damages glomerular cells is not clear. Mesangial cells have IgA receptors and are candidates for IC clearance, initiation of injurious mechanisms, and localizing complement activation. The binding of pIgA1 to mesangial cells increases the production of IL-8 *in vitro* (Oortwijn et al., 2006). But direct binding of IgA-containing immune complexes to mesangial cells has been difficult to demonstrate. Mesangial cells from IgAN patients, but not healthy controls, overexpress the transferrin receptor (CD71), which binds pIgA1 and colocalizes with IgA1 immune deposits. However, its blockade does not completely prevent IgA binding to mesangial cells (Moura et al., 2014). Recently β-1,4 galactosyltransferase was identified as a potential receptor for the Fc portion of IgA (Molyneux et al., 2017). Tissue deposition of IgA1-IC can trigger local complement activation, although how this occurs is unclear. One challenge is to identify mechanisms that link glomerular IgA1 deposition with complement activation, inflammation and the spectrum of clinical features encompassed by IgAN.

recent data suggests IgAN could be a podocytopathy, which may force current pathogenesis models to be modified. Experimental assays indicate mesangial cell stress, as seen in IgAN, can influence podocyte behavior. Mesangial cells are fundamental to maintain glomerular stability. In IgAN, mesangial expansion can be accompanied by capillary prolapse that could cause podocyte detachment (Kriz, 2018; Bellur et al., 2017). This mesangium-podocyte cross-talk may explain the occurrence of proteinuria and tubulo-interstitial injury in IgAN; the release of cytokines such as TNF, IL-6 and Angiotensin II could induce

inflammation and subsequent glomerulosclerosis. Thereby, hematuria could be involved in mesangial expansion and podocyte damage through mechanisms including mesangial architecture modification.

Many components of IgAN pathogenesis have been described. However, we do not understand the spectrum of glomerular responses to deposited gd-IgA1 seen in IgAN. This ranges from apparent immunological tolerance and absent glomerular reaction to progressive CKD and ESRD. Consequently, we are unable to consistently identify patients who would benefit from immunosuppression, leading to a number of negative randomized clinical trials in IgAN (Lv et al., 2017). A growing body of evidence suggests the alternative, lectin and terminal complement pathways influence glomerular inflammatory and injurious responses to deposited IgA1 and contribute to IgAN disease severity.

2. Alternative pathway in IgAN

The earliest descriptions of IgAN by Jean Berger noted that C3 deposits often accompanied mesangial IgA (Berger, 1969). Mesangial co-deposition of C3 and IgA is characteristic of IgAN, being present in at least 90% of biopsies (Evans et al., 1973). The alternative pathway (AP) is the main complement cascade activator in IgAN and is principally responsible for C3 deposition. Mesangial C4, and particularly the activation fragment C4d is frequently identified in IgAN biopsies. This, together with the almost universal absence of C1q in IgAN deposits indicates that lectin pathway (LP) also activates complement in IgAN, and that the classical pathway (CP) is unlikely to contribute to pathogenesis (Rauterberg et al., 1987). In other words, the glomerular presence of C3 alone implies AP activity; the presence of C4 and C3 in the absence of C1q implies LP activity, possible with AP dependent amplification; and CP activity would be evidenced by glomerular C3, C4, C1q and IgG. A summary of the evidences supporting the association of AP and LP complement components in IgAN disease is depicted in Table 1.

Regarding the AP, multiple well-established associations (Bene and Faure, 1987; Wyatt et al., 1987) and recent evidence of factor H related protein (FHR)1 and FHR5 involvement indicate a role for the AP in IgAN pathogenesis.

2.1. Established associations

Typically, components of the AP found in the renal biopsies of IgAN patients include C3, in more than 90%, properdin in 75%–100% and factor H (FH) in 30%–90% of cases (Bene and Faure, 1987; Maillard et al., 2015; Zhang et al., 2000). Other AP regulators, such as the FHR proteins, have also been identified (Murphy et al., 2002; Paunas et al., 2017). C3 mesangial co-deposition with IgA1 correlates with the severity and progression of IgAN, suggesting activation of complement particularly *via* AP contributes to glomerular injury (Maillard et al., 2015; Kim et al., 2012). Glomerular C3 deposits may help differentiate IgAN from isolated lanthanic IgA deposits found in up to 4–16% of the population (Waldherr et al., 1989; Suzuki et al., 2003), which are especially common in East Asian populations (Li and Yu, 2018; Kiryluk et al., 2012).

Evidence of complement activation is not limited to kidneys. Decreased plasma C3 levels with increased C3 activation products (iC3b and C3d) were observed in some IgAN patients and indicate that systemic complement activation and subsequent regulation through the AP may also occur in the fluid phase (Wyatt et al., 1987; Kim et al., 2012; Zwirner et al., 1997). Supporting this, *in vitro* assays using plate-bound, or aggregates of, IgA showed IgA1 were able to trigger the complement cascade and C3 cleavage *via* the AP; and proteomic analyses of circulating gd-IgA1-IC revealed the presence of C3 breakdown products like iC3b, C3c, and C3dg (Hiemstra et al., 1987; Roos et al., 2001; Knoppova et al., 2016). This evidence of AP dependent complement activation and regulation on the essential effector molecule of IgAN suggests gd-IgA1-IC act as complement activating surfaces. Importantly, the ability of IgA-containing IC formed from cord blood gd-IgA1 and either anti-glycan IgG or patient-derived gd-IgA1-specific IgG to activate mesangial cells is dependent on a heat-sensitive serum factor, presumably complement (Yanagihara et al., 2012). The presence of IgA1 receptors such as CD71, which is overexpressed by mesangial cells in IgAN patients, makes the mesangium primary target for the pathogenic IC deposition (Moura et al., 2014).

C3 mesangial co-deposition, C3 consumption and increased plasma C3 fragments correlate with unfavorable IgAN histopathology features and outcomes, supporting the relevant contribution of AP to the pathogenesis of glomerular injury (Wyatt et al., 1987; Zwirner et al., 1997). In the following sections we aim to summarize key recent advances that further support the involvement of complement activation in IgAN pathogenesis, and shed light on different mechanisms of AP dysregulation.

2.2. delCFHR3-R1

Perhaps the most striking recent links between IgAN and complement system activity were the genome wide association studies that identified protective associations for IgAN within the *CFH* locus on chromosome 1q32. These large, replicated genetic studies implicated a role for the FHR proteins in IgAN pathogenesis. The protective allele identified tags the deletion polymorphism of the FHR1 and FHR3 genes (*delCFHR3-R1*) (Gharavi et al., 2011). This allele associates with protection from IgAN. The risk of developing IgAN is reduced by 26% by the presence of the allele in heterozygosity (odds ratio (OR) = 0.74) and by 45% in homozygosity (OR = 0.55). Histopathology studies showed that *delCFHR3-R1* was associated with reduced mesangial C3 deposition and tubulointerstitial injury in Chinese cohorts (Zhu et al., 2014; Yeo et al., 2018). *delCFHR3-R1* is the most common rearrangement among the FHR family, with a wide range of allelic frequencies in different populations: 0% to 5% in East Asians and Native Americans, 20% in Europeans, and up to 50% in African populations. Interestingly, allele frequencies correlate with IgAN epidemiology; the protective *delCFHR3-R1* allele is more prevalent in African populations where there is lower disease prevalence (Holmes et al., 2013; Yeo et al., 2019).

By interacting with the same ligands, such as C3b and glycosaminoglycans, members of the FHR family can modulate FH regulatory functions on surfaces. The *CFHR1–5* genes likely originated from the *CFH* gene by tandem duplication events. While all FHR proteins conserve homology with the ligand recognition sites of FH, they lack the regulatory region of FH (Jozsi et al., 2015). Thus, FHR proteins modulate FH function by competition with ligands and deregulate complement activation on cell surfaces. Conversely, the inappropriate function of FHR proteins may determine susceptibility to complement mediated injury. This mechanism may explain the protective association of *delCFHR3-R1* with IgAN; reduced or absent FHR1 and FHR3 increase the regulating capacity of FH, reducing AP activity and preventing the potential risks of complement activation. Interestingly,*delCFHR3-R1* is in strong linkage disequilibrium with a *CFH* haplotype that produces higher levels of plasma FH, (Zhu et al., 2014) further supporting the protective effect that *delCFHR3-R1* mediates in IgAN.

2.3. Circulating FH and FHR1 levels

Recent analyses of circulating FH and FHR1 levels highlight the importance of AP dysregulation to IgAN. Two independent studies showed FHR1 plasma levels were significantly higher in IgAN patients than controls irrespective of the *delCFHR3-R1* allele carriage (Medjeral-Thomas et al., 2017; Tortajada et al., 2017). The authors also observed a negative correlation between FHR1 levels and eGFR. Higher FHR1 associated with disease progression to CKD. Notably, while FH levels did not associate with disease status, the relative abundance of these

Table 1
Evidence of complement activity in IgA nephropathy (IgAN). Associations between complement variants and activation markers and IgA nephropathy risk and severity.

Complement Protein		Evidence in IgA Nephropathy	References
Lectin Pathway	C4d	Glomerular deposits associate with IgAN severity and worse renal outcomes.	(Roos et al., 2006; Espinosa et al., 2014)
	MBL	Glomerular deposits associate with IgAN severity.	(Roos et al., 2006; Endo et al., 1998)
		Low of high plasma levels associate with IgAN severity.	(Guo et al., 2017; Ouyang et al., 2019)
		Altered levels in urine.	(Roos et al., 2006; Shi et al., 2015)
	L-ficolin	Found in glomerular deposits.	(Roos et al., 2006)
		Increased plasma levels	(Medjeral-Thomas et al., 2018)
	M-ficolin	Increased plasma levels.	(Medjeral-Thomas et al., 2018)
	MASP-1	Found in glomerular deposits.	(Roos et al., 2006)
		Increased plasma levels.	(Medjeral-Thomas et al., 2018)
	MASP-2	Found in glomerular deposits.	(Roos et al., 2006)
	MASP-3	Found in glomerular deposits.	(Roos et al., 2006)
		Reduced plasma levels correlate with IgAN severity	(Medjeral-Thomas et al., 2018)
	Map19	Increased plasma levels correlate with IgAN severity	(Medjeral-Thomas et al., 2018)
Alternative Pathway	C3 and C3 activation fragments	C3 deposits correlate with IgAN severity and progression.	(Berger, 1969; Evans et al., 1973)
		Low C3 in plasma correlates with worse renal outcomes.	(Kim et al., 2012)
		Increase plasma of iC3b and C3d correlate with worse outcomes.	(Wyatt et al., 1987; Zwirner et al., 1997)
		iC3b, C3c, and C3dg observed in IgA1-IC complexes.	(Knoppova et al., 2016)
	Factor H	Found in glomerular deposits (30%-90%).	(Berne and Faure, 1987; Zhang et al., 2009)
		Partial deficiencies. Variants in 4 of 5 cases affect expression.	(Wyatt et al., 1991; Tortajada et al., 2017)
		Increase levels in urine correlate with worse renal damage.	(Zhang et al., 2009)
	FHR1	Found in glomerular deposits.	(Murphy et al., 2002; Paunas et al., 2017; Medjeral-Thomas et al., 2018)
		Absence confers protection (del*CFHR3-1*).	
		Elevated plasma levels correlated with IgAN progression	(Gharavi et al., 2011; Zhu et al., 2014) (Medjeral-Thomas et al., 2017; Tortajada et al., 2017)
	FHR3	Absence confers protection (del*CFHR3-1*).	(Gharavi et al., 2011)
	FHR5	Found in glomerular deposits	(Murphy et al., 2002; Paunas et al., 2017; Medjeral-Thomas et al., 2018)
		FHR-5 variants resulting in increased binding to C3b.	
		Elevated plasma levels correlated with worse renal outcome	(Zhai et al., 2016)
		Glomerular FHR5 deposition associate with IgAN progression	(Medjeral-Thomas et al., 2017; Zhu et al., 2018) (Medjeral-Thomas et al., 2018)
	Properdin	Found in glomerular deposits (30%-90%).	(Berne and Faure, 1987)
		Anecdotal cases with partial deficiency.	(Wyatt et al., 1991)
	Factor I	Anecdotal cases with partial deficiency.	(Maillard et al., 2015; Tortajada et al., 2017)
	C3a	Induce secretory phenotype in mesangial cells.	(Wan et al., 2007)
Terminal Pathway	C5b9 (MAC)	Found in glomerular deposits	(Rauterberg et al., 1987; Paunas et al., 2017)
		Correlation with severity of renal injury and tissue inflammation.	(Stangou et al., 2008; Qiu et al., 2014; Zhang et al., 2014)
	C5a	Mesangial activation and histological IgAN markers associated with C5aR activation.	(Zhang et al., 2017)

MBL, mannan-binding lectin; MASP, MBL associated protease; Map, MBL associated protein; FHR, factor H related protein; MAC, membrane attack complex.

two proteins varied with severity; elevated FHR1/FH ratio also associated with worst clinical features independent of the *delCFHR3-R1* allele carriage (Medjeral-Thomas et al., 2017; Tortajada et al., 2017). In addition, four FH variants associated with decreased plasma FH levels were identified in the Spanish cohort of 106 IgAN patients (Tortajada et al., 2017). Another case of partial deficiency of FH in association with IgAN has been described (Wyatt et al., 1991). The *delCFHR3-R1* is associated with higher circulating C3 and FH levels, lower plasma C3a levels and less mesangial C3 deposition (Zhu et al., 2014). Thereby, the protective effect of *delCFHR3-R1* is likely mediated by greater FH regulatory activity (Zhu et al., 2014), while unbalanced FHR1/FH ratio from elevated FHR1 or decreased FH plasma levels aggravate the disease by compromise FH regulatory activity causing AP dysregulation.

Circulating FHR1 levels may be related to renal function. In the non-complement-mediated autosomal dominant polycystic kidney disease (ADPKD), higher FHR1 or FHR1/FH ratio also associated with disease progression. Furthermore, renal transplantation in both IgAN and ADPKD patients was followed by reduction of FHR1 plasma levels (Medjeral-Thomas et al., 2017; Tortajada et al., 2017). In IgAN, the presence of complement activating surfaces, like the gd-IgA1-IC in circulation or mesangial deposits, makes FH competition critical, and unbalanced FHR1/FH ratio following decreased renal function may exacerbate AP dysregulation leading to progressive renal function impairment. Mesangial C3 deposition contributes to sustained FHR1/FH competition because activated fragments iC3b and C3dg are better ligands for FHR1 than for FH (Tortajada et al., 2013). Remarkably FHR1 has been observed in renal biopsies of IgAN with complement activating products, but correlations with disease progression were not clear (Paunas et al., 2017). In ADPKD there is likely no such complement activating surfaces. Consequently, associations between complement activity and ADPKD disease occurrence or severity have not been identified.

2.4. *FHR5 levels and variants*

FHR5 is also able to deregulate complement by competing with FH. Gain-of-function *CFHR5* mutations cause complement dysregulation and strongly associate with a type of familial C3 glomerulopathy, CFHR5 nephropathy. CFHR5 nephropathy shows phenotypic similarities with IgAN, like C3 glomerular deposits and mesangial proliferation (Goicoechea de Jorge et al., 2013). Rare *CFHR5* gene variants were identified in IgAN. Functional assays demonstrated the variants affect FHR5 surface binding regions to increase C3b binding capacity, suggesting the variant FHR5 impairs FH regulation, resulting in greater complement-mediated injury and IgAN susceptibility (Zhai et al., 2016). FHR5 protein plasma levels were higher in IgAN patients than controls in a UK and large Chinese IgAN cohort. In addition, serum FHR5 levels correlated with histologic markers of renal injury in both cohorts (Medjeral-Thomas et al., 2017; Zhu et al., 2018). In the larger, Chinese cohort, the differences in FHR5 levels between IgAN patients and controls was more pronounced and FHR5 levels correlated positively with proteinuria and hypertension, and negatively with eGFR

(Zhu et al., 2018). Glomerular FHR5 deposition was present in all IgAN cases from a series of glomerular disease biopsies, and was found in mesangial locations similar to IgA and C3 (Murphy et al., 2002). Recently, glomerular FHR5 deposition has been associated with IgAN progression and correlated with deposition of the C3 fragments C3b, iC3b, C3c and C3d as well as C5b-9, but not C4d. Importantly, glomerular FH staining was negative (Medjeral-Thomas et al., 2018). This is remarkable because it suggests FH competition takes place at the site of renal injury, where complement is intensely activated. Unexpectedly, the same study found no correlation of FHR1 glomerular staining with IgAN status. Together, these data demonstrate FHR5 is an important and independent player in AP dysregulation in IgAN.

The AP variants and risk factors described correlate with markers of IgAN severity, such as the Oxford classification, renal impairment, or the risk of progression to CKD. Considering the multiple risk factors and clinical heterogeneity associated with IgAN, a range of contributors and mechanisms may trigger AP dysregulation and modulate disease susceptibility and phenotype. Our appreciation of associations between FHR1 and FHR5 and IgAN coincided with insights in FHR protein biology, particularly the ability of FHR1 and FHR5 homodimers to interfere with the physiological actions of FH and deregulate complement activation (Tortajada et al., 2013; Goicoechea de Jorge et al., 2013). This allowed speculation of possible pathogenic mechanism linking variants in circulating and glomerular FHR proteins and IgAN incidence and severity; in response to a trigger, such as mesangial IgA1 deposition, imbalances or gain-of-function variants in the FHRs would increase alternative pathway activation and C3 cleavage leading to amplified complement-dependent inflammation and renal injury. Other associations with AP include partial deficiencies of regulators Factor I and Properdin, but these are anecdotal cases and the precise relationship with IgAN needs to be studied further (Maillard et al., 2015; Tortajada et al., 2017). In addition to AP dysregulation, pathogenesis since 17%–25% of IgAN patients show evidence of LP activation, we must consider the contribution of LP to mechanisms of IgAN (Maillard et al., 2015).

3. Lectin pathway in IgAN

The LP is activated by the binding of pattern recognition molecules (PRM) which include mannan-binding lectin (MBL), ficolins and collectins, to pathogen associated molecular patterns. The resultant complexes are then variably able to activate MBL-associated serine proteases (MASP) which consist of MASP-1, MASP-2 and MASP-3 (Axelgaard et al., 2013; Ma et al., 2013; Matsushita et al., 2000, 2002; Liu et al., 2005). MASP activation results in the formation of a C3 convertase, C4bC2b, and C3 activation (Garred et al., 2016). Immunohistological evidence of LP activation is the identification of C4d in the absence of C1q. Additional LP components found in renal biopsies include MBL, ficolins and MASP (Roos et al., 2006).

The LP is a plausible link between gd-IgA1 deposition and glomerular inflammation in IgAN pathogenesis. IgAN is characterised by disease flares following respiratory or gastrointestinal tract inflammation (Wyatt et al., 1987); both IgA and the LP are important mediators of innate immunity at these sites. Due to a deficiency in O-linked glycosylation, IgAN is associated with higher levels of IgA1 with exposed GalNac (Berthoux et al., 2012). GalNac may trigger LP activation due to interaction with ficolins (Thiel, 2007). *In vitro* data demonstrates IgA purified from pooled normal human serum binds MBL-MASP complexes. The presence of MBL-MASP leads to C3 and C4 deposition on immobilized IgA in the absence of CP activity, indicating MBL can bind pIgA and activate complement LP (Roos et al., 2001). Polymeric IgA fractions form IgAN patients can demonstrate strong MBL binding, but there is significant variability in IgAN patients and healthy controls (Roos et al., 2006). This *in vitro* data is supported by recent studies of genetic, serological and immunohistological LP variants in IgAN.

3.1. MBL variants

The influence of genetic and serology MBL variants in IgAN was investigated in a cohort of 749 IgAN patients and 489 controls from China (Guo et al., 2017). Circulating MBL levels are predominantly influenced by variants in exon 1, the promoter region, and the 5'-untranslated region of the *MBL2* gene (Madsen et al., 1995). These polymorphisms are in linkage disequilibrium, resulting in haplotypes associated with different MBL levels (Madsen et al., 1995, 1998; Madsen et al., 1994). The study found LYPB/LYPB and LXPA/LYPB *MBL2* genotypes were dominant determinants of deficient MBL levels (Guo et al., 2017). However, perhaps due to the large number of haplotypes limiting study power, no significant differences in haplotype frequencies between IgAN and controls were identified (Guo et al., 2017). Average plasma MBL levels were lower in controls than patients. Interestingly, associations between circulating MBL levels and IgAN severity were non-linear. First, MBL deficiency, defined as plasma levels less than 100 ng/ml, associated with poorer renal outcomes. About 25% MBL deficient (10 of 39) and 12% MBL sufficient (51 of 437) patients reached either 50% loss of eGFR or ESRD over median 47 months ($p = 0.01$) (Guo et al., 2017). This was statistically significant after adjustment for multiple risk factors known to influence IgAN progression. Second, markers of IgAN severity, in particular more severe proteinuria and the presence of cellular crescents, were also more common in the cohort of IgAN patients with MBL levels greater than 3540 ng/ml. Associations with high MBL levels were lost after multivariate adjustment, but may have retained statistical significance with longer duration of follow-up (Guo et al., 2017). Furthermore, there were more cases of prodromal infection and gross hematuria in the MBL deficient IgAN cohort. These suggest links between MBL deficiency, infection susceptibility, IgAN exacerbation frequency, and disease severity. Associations between IgAN severity and both high and low circulating MBL levels seem contradictory but are probably explained by multiple MBL-dependent mechanisms contributing to IgAN pathogenesis. For example, high circulating MBL levels might predispose individuals to LP triggered complement activation, renal injury and inflammation. And low circulating MBL levels might predispose to infections and associated IgAN exacerbations.

Associations between low MBL levels and IgAN severity have been replicated in other Chinese IgAN patient cohorts. Genetic sequencing of *MBL2* and *FCN2*, the gene that influences L-ficolin functional activity, in 50 Chinese IgAN patients revealed seven variants thought to influence protein expression. After adjustment for other ESRD risk factors, one *MBL2* variant, rs1800450, associated with ESRD in discovery and validation cohorts totaling 1007 IgAN patients. The at risk allele, rs1800450-A, is associated with reduced MBL levels; patients with homozygous AA at this SNP have no detectable circulating or glomerular deposited MBL, but more severe tubulointerstitial renal damage and increased ESRD risk (Ouyang et al., 2019). Associations between progressive IgAN and low serum and urine MBL levels were identified in another study of 131 IgAN patients from a separate center in China (Shi et al., 2015).

Associations between IgAN and genetic and circulating MBL variants have not been demonstrated in non-Chinese cohorts. MBL polymorphism analysis in 160 IgAN patients and 74 controls from Italy did not demonstrate differences in allelic and genotypic frequencies. There were also no associations with disease severity (Pirulli et al., 2001). Albeit in small cohorts, circulating MBL levels did not associate with IgAN patients compared to healthy controls (Roos et al., 2006), other primary glomerulopathies, or disease severity in non-Chinese populations (Lhotta et al., 1999; Ohsawa et al., 2012). However, MBL is excreted in large quantities in the urine of patients with progressive IgAN (Roos et al., 2006). Explanations for these inconsistent MBL associations could include differences in cohort sizes, ethnicities, effects of reduced glomerular filtration on circulating MBL levels and definitions of progressive disease.

3.2. Variants in other lectin pathway proteins

Ficolin PRMs can interact with GalNac acetyl groups exposed on gd-IgA1 and therefore could, in theory, bind and localize complement activation (Garred et al., 2016; Kjaer et al., 2013). Analysis of 323 IgAN patients from London, 16% of whom were non-Caucasian ethnicity, demonstrated increased circulating M-ficolin, L-ficolin, MASP-1 and MAp19 levels in IgAN patients compared to healthy controls. Also, MASP-3 levels were reduced in IgAN and associated with progressive disease, both in the whole cohort and following immunosuppression treatment. Low MASP-3 and high MAp19 plasma levels also correlated with histology features of IgAN severity (Medjeral-Thomas et al., 2018). This study investigated the influence of estimated glomerular filtration rate (eGFR) loss on circulating lectin pathway protein levels. IgAN patients with preserved eGFR showed significant differences in plasma lectin levels compared to healthy controls, suggesting the differences were secondary to IgAN activity. Furthermore, despite a marked change in eGFR MASP-3 was not influenced by transplantation in IgAN and ADPKD patients (Medjeral-Thomas et al., 2018).

3.3. Lectin pathway immunohistology

Glomerular LP protein deposition associates with IgAN severity. Endo et al detected MBL and MASP-1 in renal biopsies from 24% of IgAN patients and 3% of other glomerulopathies (Endo et al., 1998). The deposited MBL/MASP-1 associated with glomerular C3b/C3c and C5b9 deposition, but did not correlate with serology markers of complement activation or clinical markers of IgAN severity. Roos et al demonstrated glomerular MBL, L-ficolin, MASP1/3 and C4d deposition in 25% of a cohort of 60 IgAN patients (Roos et al., 2006). Glomerular deposition associated with features of IgAN severity, specifically proteinuria, higher serum creatinine and renal failure (Roos et al., 2006). Espinosa et al identified glomerular C4d and absent C1q in 38.5% of 283 patients from Spain with IgAN (Espinosa et al., 2014). C3 deposition was identified in an equal proportion of C4d positive and C4d negative cases. It was argued that patients could be categorized based on glomerular evidence of either or both LP and AP activation. After multivariate analysis, positive glomerular C4d staining was a significant predictor of ESRD (Espinosa et al., 2014). This was replicated in a second Spanish cohort of 102 IgAN patients, in whom C4d deposition on renal biopsy associated with progression to doubling of serum creatinine (Zhu et al., 2014). Although not significant after multivariate analysis in this cohort, an interesting association was identified between glomerular C4d deposition, raised circulating FHR1 levels and IgAN progression that may be mechanistically informative (Zhu et al., 2014).

4. Terminal pathway

Terminal complement pathway activity may also be important to IgAN pathogenesis. As mentioned, immunohistochemistry analysis of IgAN patient biopsies identified glomerular C5b9 deposition (Rauterberg et al., 1987). Significant amounts of C5 to C9 proteins have been identified by proteomic analysis of micro-dissected glomeruli from IgAN kidney biopsies (Paunas et al., 2017). C5b9 deposition is associated with renal inflammation and progression of glomerulosclerosis in IgAN (Stangou et al., 2008). In a rat model of human mesangioproliferative glomerulonephritis, C5b9 induced mesangial cell apoptosis and the production of immune stimulatory signals, such as IL-6 and TGF-β1 (Qiu et al., 2014; Zhang et al., 2014), supporting a role for C5b9 in IgAN pathogenesis. Recently, *in vitro* evidence has emerged of a role for C5a, the anaphylatoxin released during terminal pathway activation in IgAN. Cultured human mesangial cell stimulation induced by IgA1 from pooled human serum is reduced by C5a receptor (C5aR) antagonists. Using a Sendai virus model of IgAN, C5aR knockout mice had less proteinuria, reduced glomerular C3 and IgA deposition and improved renal histology changes (Zhang et al., 2017).

5. Complement inhibition in IgAN

Due to the strong association between glomerular complement activation and IgAN, complement inhibitors are being tested in clinical trials (see clinicaltrials.gov for up-to-date trial information).

5.1. Factor B inhibitor: LNP023

Factor B (fB) is a fundamental protease in AP activation and amplification. AP dysregulation predisposes individuals to the development of numerous diseases including C3 glomerulopathy and IgAN (Maillard et al., 2015). Serum levels of fB are increased in patients with IgAN, which correlates with the activation of B-cells and the role of mucosal immunity in this disease (van den Wall Bake et al., 1988). Given B-cell activation may be involved in the production of Gd-IgA1 and its antibodies and complement AP activation is important in IgAN pathogenesis, blocking fB has potential pathophysiological importance. IgAN proteinuria reduction after 90 days of treatment with LNP023, an oral small molecule reversible fB inhibitor, is being tested in a phase IIa / IIb trial (NCT03373461).

5.2. Anti C5 monoclonal antibody: eculizumab

Eculizumab is a recombinant humanized monoclonal antibody that selectively inhibits C5, thereby preventing C5a release and formation of the membrane attack complex (MAC, C5b9). Eculizumab has been used in two young patients with progressive IgAN (Rosenblad et al., 2014; Ring et al., 2015). Eculizumab use was associated with temporary renal function and proteinuria stabilization, and disease progression on withdrawal. The patients had previously received other immunosuppressive treatments. Eculizumab has also been used in aggressive relapses of IgAN after kidney transplantation with little consistent clinical success, although this may be partially related to the timing of treatment. Interestingly, deposits of C3, C4d and C5 have been seen in the biopsies of these patients, demonstrating close relationships between complement and recurrent IgAN pathogenesis (Wyld and Chadban, 2016; Herzog et al., 2017).

5.3. MASP-2 inhibitor: OMS721

OMS721 is a monoclonal antibody to MASP-2 that led to significant reductions in proteinuria in 4 IgAN patients (Presented as abstract: Block GA, Whitaker S. Maintenance of remission following completion of OMS721 treatment in patients with IgA nephropathy (IgAN). J Am Soc Nephrol 2017; 28: 749-50. SA-PO278). A phase III, double-blind, randomized and placebo controlled study (NCT03608033) is in recruitment and aiming to evaluate the safety and efficacy of OMS721 in patients with IgAN and more than 1 g/day proteinuria (Smedbraten et al., 2016).

5.4. Anti-C3: compstatin and APL-2

Compstatin inhibits the activation of C3. As described above, inhibitors of C3 activation may be potential candidates for IgAN treatment (Rops et al., 2018; Yamada et al., 2017). Specific pathophysiological justification is provided by the finding that C3 (and C5) receptor antagonists prevent the proliferation of cultured human mesangial cell stimulated by IgA1 (Zhang et al., 2017). Reduction in IL-6 up-regulation, a key mediator of mesangial cell activation and immune cell glomerular filtration, was also observed. APL-2 is a pegylated derivative of compstatin that binds C3 and prevents cleavage to C3a and C3b by C3 convertase. APL-2 is being evaluated in a phase 2 study as treatment for patients with IgAN, lupus nephritis, primary membranous nephropathy or C3 glomerulopathy (NCT03453619).

5.5. Anti-C5a receptor inhibitor: CCX168 (Avacopan)

Avacopan is a small molecule that antagonizes complement-dependent inflammation through its binding to the C5aR. C5a is an important inflammatory mediator in systemic vasculitis, for which Avacopan seems to be an effective alternative to glucocorticoids (Jayne et al., 2017). The aforementioned mesangial cell and animal model experiments suggest C5aR inhibition could be effective in IgAN (Zhang et al., 2017). A Phase II study (NCT02384317) to evaluate the safety, tolerability and efficacy of CCX168 in reducing proteinuria in IgAN patients receiving maximum tolerated RAAS blockade is being analyzed.

5.6. C5 suppression by a RNA interference (RNAi): ALN-CC5 (Cemdisiran)

Cemdisiran is a synthetic RNAi designed to suppress liver production of C5, which may reduce terminal complement pathway activation and subsequent inflammation. A Phase II, double-blind, randomized and placebo controlled study (NCT03841448) is in recruitment and aims to evaluate the safety and efficacy of Cemdisiran in patients with IgAN and more than 1 g/day proteinuria.

Successful application of these agents will depend on accurately selecting patients for who the benefit of complement inhibition outweighs the risks. Such personalized approaches to treatment decisions are particularly important in IgAN because of its wide range of clinical outcomes. We need to reliably identify cases with ongoing complement activation. We also need to identify theresponsible complement pathways and proteins. This necessitates the development of biomarkers of specific complement activation. We next need to identify cases with ongoing glomerular inflammation. Although the Kidney Disease Improving Global Outcomes (KDIGO) clinical practice guidelines consider that proteinuria greater than 1 g/day justifies treatment (Floege et al., 2019), proteinuria in IgAN may be the result of sclerotic hyperfiltration and tubular damage. Therefore, microscopic haematuria may clarify disease activity and ongoing inflammation (Sevillano et al., 2017). This is exemplified by IgA1 protease treatment that decreases IgA1 deposition, inflammation, mesangial expansion, and haematuria without significantly influencing proteinuria (Lechner et al., 2016). Additionally, histology can identify ongoing inflammation, such as with the presence of endocapillary hypercellularity and cellular crescents. However, using the Oxford MEST-C score to make therapeutic decisions is currently unproven (Trimarchi et al., 2019). This is being addressed with the Treatment of IgA Nephropathy According to Renal Lesions (TIGER) study (NCT031888887) (Coppo, 2018). Additionally, glomerular and circulating markers of complement activation may become useful biomarkers of ongoing inflammation that identify patients who would benefit from immunosuppression, even if therapeutic complement inhibition is unavailable.

6. Future research

Complement is in the spotlight of IgAN research. Unsurprisingly given the complexity and heterogeneity of IgAN pathogenesis, a number of questions require clarification.

i) Where is the specific site of complement activation? *In vitro* assays showed IgA can trigger both LP and AP, and circulating gd-IgA1-IC contain C3 the products iC3b, C3c and C3dg (Hiemstra et al., 1987; Roos et al., 2001; Knoppova et al., 2016; Tomana et al., 1999). However, it is not clear whether pathogenic complement activation occurs in the fluid phase on circulating complexes, in the glomeruli after mesangial deposition, or both. This may help place complement activation in the four-hit model of IgAN pathogenesis.
ii) A unique characteristic of IgAN is the presence of gd-IgA1-IC deposits in the mesangium, but does the O-glycan deficiency have a role in the activation of AP and LP? The results of experiments using laboratory IgA preparations were controversial. Removal of sialic acid or N-glycans from IgA1 and IgA2 isotypes with specific enzymes resulted in enhanced AP C3b fixation. This was not seen for O-linked galactose and galactosamine, which are important components of IgAN glomerular deposits (Nikolova et al., 1994). Conversely, another study using culture-generated deglycosylated IgA showed weaker C3b deposition (Zhang and Lachmann, 1994).
iii) How does complement cause glomerular damage in IgAN? It has been shown that deposition of C5b9 induces mesangial cell stress and that C3a stimulates mesangial cells leading to secretory phenotypes (Stangou et al., 2008; Qiu et al., 2014; Zhang et al., 2014; Wan et al., 2007). However, it is unknown if complement additionally alters glomerular cell responses to pathogenic gd-IgA1-IC. Accordingly, whether inflammatory responses to gd-IgA1-IC are amplified in individuals with underlying imbalances of complement regulation needs investigation.
iv) Which complement pathways are pathogenically dominant in IgAN? Why is LP activation only seen in some patients? Discrepancies in pathway associations may reflect the heterogeneity in IgAN clinical presentations and outcomes. In addition to looking for correlates with clinical outcome such as ESRD, given the increasing availability of inhibitors of specific complement pathways, it will also be important to establish whether particular LP and AP activation correlate with active glomerular inflammation. Although individual studies have looked at endocapillary hypercellularity in patients with complement variants such as raised FHR5 (Medjeral-Thomas et al., 2017), how LP and AP activity correlate with histology evidence of inflammation or scarring is unclear.
v) How does IgAN severity associate with both glomerular evidence of LP activity, raised circulating LP protein levels including MBL, and MBL deficiency? Predicting how reduced MASP-3 levels associate with IgAN severity is difficult because the physiological roles of MASP-3 are unclear. Although MASP-3 binds lectin PRMs, it cannot cleave complement convertases (Ouyang et al., 2019), and inhibits LP activation when added to MBL-MASP complexes (Shi et al., 2015; Pirulli et al., 2001). Conversely, MASP-3 can cleave pro-factor to fD thereby potentially linking lectin and alternative complement pathways.
vi) Which FHR1 and FHR5 mechanisms drive IgAN progression? It has been proposed that FHR1 and FHR5 competition with FH ligands cause AP dysregulation and influence IgAN and other complement-associated diseases. However, other mechanisms may be important. *In vitro* C3b-bound FHR4 and FHR5 have been shown to enhance complement activation by scaffolding AP convertase formation C3b (Hebecker and Jozsi, 2012; Csincsi et al., 2015). Also, FHR proteins may interfere with the uptake and metabolism of circulating or deposited C3, perhaps bound to gd-IgA1-IC, thus potentiating complex immunogenicity and amplifying inflammation.

7. Conclusion

IgAN remains a common disease without a treatment. Glomerular complement activation is strongly associated with IgAN (Table 1) and may be the dominant driver of renal injury. If this is the case complement inhibition may emerge as an effective method of preventing or reducing glomerular injury in IgAN. The outcome of trials of complement inhibitors in IgAN is eagerly awaited.

Declaration of Competing Interest

None.

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