Complement dysregulation and disease: from genes and proteins to diagnostics and drugs.

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ABSTRACT

During the last decade, numerous studies have associated genetic variations in complement components and regulators with a number of chronic and infectious diseases. The functional characterization of these complement protein variants, in addition to recent structural advances in understanding of the assembly, activation and regulation of the AP C3 convertase, have provided important insights into the pathogenic mechanisms involved in some of these complement related disorders. This knowledge has identified potential targets for complement inhibitory therapies which are demonstrating efficacy and generating considerable expectation in changing the natural history of these diseases. Comprehensive understanding of the genetic and non-genetic risk factors contributing to these disorders will also result in targeting of the right patient groups in a stratified medicine approach through better diagnostics and individually tailored treatments, thereby improving management of patients.

Keywords:

Complement, complotype, inflammation, alternative pathway, disease, mutation, polymorphism

The complement system

Complement is a major component of innate immunity with crucial roles in microbial killing, apoptotic cell clearance, immune complex handling and modulation of adaptive immune responses (Ricklin, et al., 2010). Complement works as an amplification cascade, initiated through either the classical pathway (CP), the lectin pathway (LP) or the alternative pathway (AP). Activation of complement results in the formation of unstable protease complexes, named C3 convertases (C3bBb in the AP; C4b2a in the CP/LP), which catalyze the central and most important step of complement activation: the cleavage of C3 to generate the activated fragment, C3b. When nascent C3b is generated, a reactive thioester is exposed which is attacked by nucleophiles (such as amine groups) on adjacent surfaces, resulting in covalent binding of C3b to the target surface, marking it for destruction and initiating inflammation at the site of activation.

The AP is critical for efficient activation regardless of the trigger because it amplifies the response. In contrast to the CP and LP where activation is triggered by immune complexes and bacterial mannose groups respectively, the AP is intrinsically active. Spontaneous activation of C3 in plasma occurs through the “tick-over” mechanism, which is initiated by the generation of a fluid phase C3b-like molecule, called C3i or C3(H_2O), by aqueous hydrolysis of the internal C3 thioester (Pangburn, et al., 1981). Activation of C3 may also occur by continuous low rate cleavage of C3 to C3b by plasma proteases. The balance between the rate at which the initial trigger is amplified and the degree to which C3b and the AP C3-convertases are inactivated determines the progression of the complement cascade to cell damage and death. Foreign

substances on microbial pathogens, antibodies, or mannan, tip the balance in favour of amplification, causing target opsonisation (Lachmann, 2009). The positive regulator, properdin, stabilizes the AP C3-convertase and serves as a focal point for promoting local amplification of complement (Kemper, et al., 2010). The terminal pathway fires when the AP C3-convertase incorporates one additional C3b molecule, which creates the C5-convertase and switches substrate specificity from C3 to C5, a C3 homologue that lacks the thioester. C5 cleavage triggers inflammation and leukocyte recruitment through production of C5a, and initiation of membrane attack complex (MAC) formation. The MAC contains C5b plus the complement components C6, C7, C8 and C9, and lyses cells by punching holes through the membrane (Muller-Eberhard, 1985).

Accelerated dissociation of the AP C3 convertase and inactivation of C3b are critical steps to maintain complement homeostasis and to prevent non-specific damage to self-cellular components when complement is activated. These activities are performed primarily by factor H (fH), in collaboration with the plasma serine protease factor I (fI) (Rodriguez de Cordoba, et al., 2004). FH is an abundant plasma protein (0.2-0.5g/l) with an elongated structure presenting several binding sites for C3b and glycosaminoglycans (GAGs) along its length. The GAG-binding domains are key to protection of self-tissues as they locate fH to cell membranes where it dissociates deposited AP convertases and inactivates C3b. Self-tissues are also protected by membrane-bound proteins that restrict complement activation in three ways: i) acting as cofactor for proteolytic inactivation of C3b by fI; ii) accelerating convertase dissociation; iii) inhibiting MAC formation (Figure 1). Thus, in health, spontaneous activation of C3 in plasma is kept at a low level and further

complement activation and C3b deposition is restricted to targets lacking surface regulators.

Genotype-phenotype correlations between complement and disease

The relationship between complement deficiencies and pathology has been recognized for over 50 years and complement is firmly implicated in a long list of diseases, responsible for sustaining a 'vicious cycle' of inflammation and perpetuating tissue damage. However, completion of the Human Genome Project during the last decade has catalysed expansion of this long list as studies have associated specific mutations and/or polymorphisms in complement components and regulators with predisposition to numerous rare and prevalent chronic inflammatory diseases, and with susceptibility to infection. Diseases associated with complement are genetically complex in that they are commonly associated with mutations and/or polymorphisms in more than one complement component. In addition, they are multifactorial in the sense that non-genetic risk factors and factors unrelated to complement also contribute to disease development and progression. Importantly, it is not unusual that the same gene or combination of genes is involved in several distinct pathological entities, perhaps because of the impact of non-complement drivers. Table 1 summarizes the complement genes associated with a number of different renal and ocular diseases to illustrate the complexities.

The functional characterization of disease-associated complement genetic variations has improved our understanding of these genetically complex diseases and, in some cases, unravelled the role that complement plays in pathogenesis. Complement dysregulation is a unifying pathogenic feature in

many of these diseases. Remarkably, functional analyses have shown that there is a strong correlation between mutations causing particular functional alterations in a complement protein and specific susceptibility to a particular disease. A comprehensive understanding of the genetic component predisposing to the pathology and its functional consequences at the protein level is therefore critical to guide appropriate diagnostics and effective treatment in complement-related disorders.

1) Mutations in the C-terminal region of fH are prototypical in aHUS.

Hemolytic uremic syndrome (HUS) is a rare, life threatening disease characterized by thrombocytopenia, Coombs test negative microangiopathic haemolytic anemia and acute renal failure. The commonest form of HUS follows a diarrhoeal prodrome and is associated with infections involving shiga-toxin producing *E. coli* strains (STEC-HUS; EHEC-HUS). However, five to ten percent of HUS patients lack an association with diarrhoea. This atypical form of HUS (aHUS), with an incidence of about $3/10^6$ per year and a prevalence of $1/10^5$ children in the whole European Union, has the poorest long-term prognosis. Recurrences in aHUS are common with a mortality rate that approaches 30% (Noris and Remuzzi, 2005).

Although fH deficiencies were implicated in the pathogenesis of aHUS in the early 1980's, it was the pioneering work by Warwicker et al., published in 1998, which identified the association of the RCA gene cluster with aHUS (Warwicker, et al., 1998). These findings triggered a number of seminal studies that delineated the genetic predisposition to aHUS and provided fundamental insights into its pathogenic mechanisms, changing the earlier perception that

aHUS was a pathology related to hypocomplementemia (lack of complement) to the realization that the disorder was caused by dysregulated complement activation (Caprioli, et al., 2001, Perez-Caballero, et al., 2001, Richards, et al., 2001).

aHUS is associated with mutations or polymorphisms in the genes encoding the complement regulatory proteins fH (*CFH*) (Caprioli, et al., 2001, Caprioli, et al., 2003, Perez-Caballero, et al., 2001, Richards, et al., 2001, Warwicker, et al., 1998) membrane cofactor protein (*MCP*) (Esparza-Gordillo, et al., 2005, Noris, et al., 2003, Richards, et al., 2003) and factor I (*CFI*) (Fremeaux-Bacchi, et al., 2004); (Kavanagh, et al., 2005), and with mutations in the genes encoding complement components factor B (*CFB*) (Goicoechea de Jorge, et al., 2007) and C3 (*C3*) (Fremeaux-Bacchi, et al., 2008). Mutations in thrombomodulin (THBD) have also been described in association with aHUS (Delvaeye et al, 2009). Missense mutations in *CFH* are prototypical of aHUS and the most prevalent genetic alteration, present in approximately 25% of the aHUS patients in all series. aHUS-associated *CFH* mutations cluster in the C-terminus of the protein, a region that is critical to the capacity of fH to bind cell surfaces and control local activation of complement (Figure 2). Carriers of these *CFH* mutations express fH molecules that possess normal regulatory activity in plasma but a limited capacity to bind and protect cells from complement lysis (Manuelian, et al., 2003, Perez-Caballero, et al., 2001, Sanchez-Corral, et al., 2004, Sanchez-Corral, et al., 2002). It is therefore the combination of an active complement system in plasma and a defective protection of cellular surfaces that determines the development of aHUS. These findings fit well with the identification of aHUS-associated loss-of-function mutations in *MCP* and *CFI*;

these mutations also lead to decreased protection of host cells from complement lysis without affecting significantly complement homeostasis in plasma (Figure 3) (Atkinson, et al., 2005).

The penetrance of disease in carriers of aHUS-associated mutations is approximately 50%, indicating that additional genetic and environmental factors contribute to disease development. In fact, it is now well documented that concurrence of different genetic risk factors greatly influences predisposition to aHUS in carriers of mutations in *CFH*, *MCP*, *CFI* and *CFB* (Esparza-Gordillo, et al., 2005, Esparza-Gordillo, et al., 2006, Goicoechea de Jorge, et al., 2007, Martinez-Barricarte, et al., 2008, Neumann, et al., 2003). Among these additional risk factors are two relatively frequent *CFH* and *MCP* haplotypes that may determine decreased expression of fH and MCP respectively (Caprioli, et al., 2003, Esparza-Gordillo, et al., 2005, Liszewski, et al., 2007).

Factor B and C3 gain-of-function mutations are characteristic of a subgroup of aHUS patients showing persistent activation of the AP in plasma (Fremeaux-Bacchi, et al., 2008, Goicoechea de Jorge, et al., 2007, Roumenina, et al., 2012). From a pathogenic point of view it is intriguing that these fB and C3 gain-of-function mutations, decreasing C3 plasma levels and causing different degrees of hypocomplementemia, are nevertheless associated with aHUS. One possible explanation is that increased complement activation caused by gain-of-function mutations, a situation that may be similar to that occurring during infection, coincides with an additional aHUS risk factor impairing surface protection. In support of this possibility, we note that carriers of fB or C3 gain-of-function mutations that develop aHUS are also carriers of the *MCP* risk haplotype (Goicoechea de Jorge, et al., 2007, Roumenina, et al.,

2012). In the case of mutations in C3 associated with aHUS, different experimental approaches have shown that these mutations alter the sensitivity of C3b to inactivation by fH and MCP and/or change the susceptibility of the AP C3 convertase to accelerated decay by fH and DAF ((Fremeaux-Bacchi, et al., 2008, Roumenina, et al., 2012); our unpublished results).

Taken together, genetic and functional analyses have established that aHUS involves complement AP dysregulation and develops as a consequence of defective protection of cellular surfaces from complement activation. Multiple hits, involving plasma and membrane-associated complement regulatory proteins as well as complement components, are likely required to cause dysregulation and significantly impair protection to host tissues. Environmental factors that activate complement likely modulate genetic predisposition and are also very important in aHUS. Infection, immunosuppressive drugs, cancer therapies, oral contraceptives, pregnancy and childbirth are important factors that trigger attacks of aHUS in some patients. In carriers of multiple strong aHUS genetic risk factors the contribution of the environment is probably minor. On the other hand, in those with a low genetic predisposition, strong environmental factors may still precipitate disease. Whether this scenario explains the severe or fatal outcome of a small proportion of individuals with the more common diarrhoea-associated STEC-HUS remains to be determined (Fang et al., 2008).

2) Complement fluid phase dysregulation and DDD

Dense deposit disease (DDD) is a rare form of glomerulonephritis which affects both children and young adults and frequently results in end-stage renal

disease (ESRD) (Smith, et al., 2011). The morphologic hallmark of DDD is the presence of electron-dense deposits within the glomerular basement membrane (GBM), as resolved by electron microscopy. The chemical composition of the dense deposits is largely unknown, although IgG is absent from them and other regions of the glomerulus, which excludes a role for immune complexes in dense deposit formation. DDD is associated with complement abnormalities which include persistent reduction of C3 serum levels and intense deposition of C3 fragments in GBM. The majority of DDD patients have C3 nephritic factors (C3NeF), autoantibodies against the AP convertase, in their plasma that may be causative. Familial cases of DDD are rare but extremely informative. The functional characterization of human DDD-associated fH and C3 mutations (Licht, et al., 2006, Martinez-Barricarte, et al., 2010) and previous studies in animal models (Hogasen, et al., 1995, Pickering, et al., 2002) have provided conclusive evidence that fluid phase complement dysregulation, resulting in the continuous activation of C3 in plasma, plays a major role in DDD pathogenesis (Figure 3).

Recently, the *CFH* genotype-phenotype correlation was formally established in a murine model. Factor H-deficient mice (*Cfh*$^{-/-}$) develop DDD as a consequence of massive activation of C3 (Pickering, et al., 2002). These mice present very low levels of C3 and complement activity in plasma. Introduction into *Cfh*$^{-/-}$ mice of a transgenic fH molecule (FH$\Delta_{16\text{-}20}$) that mimics the fH mutations found in aHUS patients restored the C3 levels and the complement activity in the plasma of these fH-deficient animals. As a result, *Cfh*$^{-/-}$ FH$\Delta_{16\text{-}20}$ animals switch their disease phenotype from DDD to aHUS (Pickering, et al.,

2007), validating the conclusion that the combination of active complement in plasma with a decreased protection of cell surfaces leads to aHUS.

Further studies are still necessary to unravel the precise molecular events that lead to the development of DDD. An important step has been taken with the creation of fH/fI double knockout mice; absence of fI protected the fH-deficient mice from developing DDD, demonstrating that fluid phase activation of C3 and generation of iC3b is critical in pathogenesis in DDD (Rose, et al., 2008).

While mutations at the C-terminus of fH are typical of aHUS and complete functional deficiency of fH is the prototypic mutation associated with DDD, there are both aHUS and DDD patients with partial fH deficiencies due to mutations in the *CFH* gene (Dragon-Durey, et al., 2004, Landau, et al., 2001, Levy, et al., 1986, Ohali, et al., 1998, Smith, et al., 2007). Overlap between syndromes illustrates their genetic complexity. Additional genetic and environmental factors likely provide the context that decides the pathological outcome in partial fH deficiencies. For example, concurrence of partial fH deficiencies with other mutations or polymorphisms that decrease protection to host cells will result in aHUS (Hakobyan, et al., 2010, Perez-Caballero, et al., 2001, Sanchez-Corral, et al., 2002), whereas the coincidence of partial fH deficiencies with strong complement activators like C3NeF will trigger DDD (Licht, et al., 2006). DDD is part of a large and heterogeneous group of kidney pathologies, the C3 glomerulonephropathies (C3-GN), with distinct patterns of glomerular inflammation, mediated by complement dysregulation and characterized by glomerular deposition of C3 (Fakhouri, et al., 2010). C3Nef are also found in some non-DDD C3-GN patients, often with mutations in CFI and

CFH (Servais et al., 2012), further illustrating the overlap between these complement dysregulation disorders and the requirement of particular combinations to trigger specific pathologies.

3) AMD is an exemplar complex genetic disorder involving common complement polymorphisms.

Age-related macular degeneration (AMD), the commonest cause of blindness in the western world, shows a very high (40-70%) and well characterized genetic component, including a large set of common polymorphisms in complement components and regulators (Gorin, 2012). Retinal drusen, the hallmark of AMD, was known to contain complement proteins based on immunohistochemical and biochemical analysis, a finding that initially had attracted little attention (Hageman and Mullins, 1999). A quantum shift in interest occurred in 2005 when several papers reported candidate region studies and whole-genome association analyses that implicated the region encoding fH at 1q31 as a major susceptibility locus for AMD (Edwards, et al., 2005, Hageman, et al., 2005, Haines, et al., 2005, Klein, et al., 2005). The CFH_{Y402H} allele (rs1061170) conferred a significantly increased risk of AMD with an odds ratio (OR) between 2.1 and 7.4. The association of a *CFH* polymorphism with AMD strengthened the implication of complement in the pathogenesis of AMD and prompted subsequent candidate gene studies that identified additional associations (protective and non-protective) of complement genes, including *CFI*, *C3*, *C2/CFB*, *CFHR3/CFHR1* and *C7*, with AMD (Dinu, et al., 2007, Fagerness, et al., 2009, Gold, et al., 2006,

Hughes, et al., 2006, Yates, et al., 2007). Together, these genetic data reinforce the concept that complement dysregulation is a major player in the pathogenesis of AMD.

The mechanism by which complement polymorphisms impact AMD risk is as yet unsolved, although it is clear that different polymorphisms can act additively to cause local complement dysregulation and pathology (Heurich, et al., 2011, Maller, et al., 2006). Some of them, like the fH_{62I} (rs800292), fB_{32Q} (rs641153) and $C3_{102G}$ (rs2230199) variants, alter AP convertase formation or regulation (Heurich, et al., 2011, Montes, et al., 2009, Tortajada, et al., 2009), while others like fH_{402H} (rs1061170) may result in failure to recruit fH to sites where complement is activated by the accumulation of endogenous complement-activating compounds. For example, it has been recently reported that the fH_{402H} variant fails to bind malondialdehyde, a common lipid peroxidation product found in the aged retina and implicated in AMD (Weismann, et al., 2011).

The association of AMD with the 1q31 locus is complicated by the large number of polymorphisms within these regions and the strong linkage disequilibrium between them (Figure 4A). As a consequence, it is often difficult to ascribe risk to a specific protein or polymorphism in the inherited set – the haplotype. For example, the FH_{Y402H} polymorphism (rs1061170) is part of the common H1 haplotype of the *CFH* gene (*CFH*) that encompasses several other linked polymorphisms in fH and fH-related (FHR) proteins (Hageman, et al., 2006). These include a recently described common allelic variation in the *CFHR1* gene; the *A variant of the FHR-1 protein, risk for AMD, differs from the *B variant by three amino acids (Abarrategui-Garrido, et al., 2009, Martinez-

Barricarte, et al., 2012). A second *CFH* haplotype, H2, is protective for AMD and includes the FH_{V62I} (rs800292; odds ratio (OR) 0.54) polymorphism and a non-coding intronic SNP, while a second AMD-protective haplotype, H4, includes the deletion, common in Caucasians, of the *CFHR1* and *CFHR3* genes encoding the proteins FHR-1 and FHR-3 (Hageman, et al., 2005, Hughes, et al., 2006). In order to prove that a particular protein variant in the haplotype is responsible for altered disease risk it is necessary to demonstrate that the polymorphism has an effect on protein function or production that is compatible with the observed change in disease risk. Although this has now been achieved for some of the disease-associated complement polymorphisms, the task is far from complete; for example, the functional effect of the FHR-1 polymorphism described above and its contribution to AMD risk in the H1 haplotype is unknown. Recently, the prevalent aHUS-associated FH_{R1210C} mutation (Martinez-Barricarte et al., 2008) has been associated with early onset of AMD (Raychaudhuri et al., 2011), a remarkable finding illustrating that AMD has links not only with DDD, but also with other complement dysregulation disorders, including aHUS. As discussed above for partial fH deficiencies, additional genetic and environmental factors likely provide the context that decides the pathological outcome in carriers of the FH_{R1210C} mutation.

4) CFHR genes, FHR proteins and disease risk

The family of fH-related proteins (FHRs), revealed over the last two decades predominantly by the work of Zipfel and co-workers (Jozsi and Zipfel, 2008) are somewhat neglected, in large part because they are compared to fH

itself (around 500mg/l in plasma), relatively minor plasma components with concentrations in the range 5 to 50mg/l. The genes *CFHR3*, *CFHR1*, *CFHR4*, *CFHR2* and *CFHR5* encoding these FHR proteins are located downstream and closely linked to the *CFH* gene (Figure 4A). Genomic and sequence analyses of the *CFH-CFHR1-5* gene region suggest that the CFHR genes originated from the CFH gene by tandem duplication events (Perez-Caballero, et al., 2001). All the CFHR genes retained, with different degrees of sequence conservation, the exons that encode the C-terminal region of fH (Figure 4B), explaining the capacity of most FHR proteins to interact with C3b, iC3b, C3dg and carbohydrates (Jozsi and Zipfel, 2008, Skerka and Zipfel, 2008, Zipfel, et al., 1999). None of the FHR proteins contain regions homologous with the complement regulatory SCRs 1-4 of fH; however, weak cofactor and/or decay accelerating activities have been reported for some of them. Although the physiological relevance of these interactions and activities is not yet fully clear, it is generally accepted that the FHRs influence complement activation and regulation via their interaction with C3b and C3dg fragments and carbohydrates on particular surfaces. How FHRs exert this influence is still controversial.

It has been suggested that some FHR proteins (like FHR3 and FHR1) modulate complement regulation, competing with fH for binding to C3b and interfering with fH complement regulatory activities (Fritsche, et al., 2010, Heinen, et al., 2009). An argument against this is the relatively low plasma concentrations of the FHR proteins compared to fH noted above. However, a differential binding specificity of the FHRs and fH proteins for C3b in the context of particular carbohydrates may increase the affinity of these FHR molecules locally to allow them to compete efficiently with fH. The hybrid *CFH::CFHR1*

genes found associated with aHUS may support this possibility ((Venables, et al., 2006); see below). An interesting, and contrasting alternative to the competition theory is the suggestion that FHR4A, FHR4B and FHR3 regulate complement activation by enhancing the cofactor activity of fH (Hebecker and Jozsi, 2012, Hellwage, et al., 1999).

Experimental evidence has accumulates in recent years supporting the contention that some FHRs have endogenous complement regulatory functions. FHR1 is reported to act downstream of fH and inhibit the C5-convertase, while FHR3 has cofactor activity for factor I-mediated inactivation of C3b (Fritsche, et al., 2010, Heinen, et al., 2009). Cofactor activity has also been reported for the FHR4B isoform (Hellwage, et al., 1999) and for FHR5 (McRae, et al., 2005); however, these activities are weak and their physiological relevance has been questioned (Hebecker and Jozsi, 2012). Finally, it was recently suggested that the FHRs may promote complement activation; the A isoform of FHR4 enhanced complement activation via its interaction with C3b (Hebecker and Jozsi, 2012).

The *CFH-CFHR1-5* gene region shows significant genetic variability. In addition to conventional polymorphism due to sequence variations, the presence within the region of large genomic duplications (ranging in size from 1.2 to 38 kb) makes the region highly prone to genomic rearrangements through gene conversion and non-homologous recombination (Figure 4A) (Perez-Caballero, et al., 2001). These rearrangements are readily identified by MLPA (Multiplex Ligation-dependent Probe Amplification) technologies (Venables, et al., 2006), CNV microarrays or western blots (Abarrategui-Garrido, et al., 2009, Zipfel, et al., 2007). Notably, several rearrangements have been identified in

recent years associated with different pathologies involving complement dysregulation. They are remarkable “experiments of nature” that may help to clarify some of the current uncertainties regarding the function of the FHR proteins (Table 2).

A very prevalent rearrangement in this region, a true common polymorphism in humans, is the deletion of the *CFHR1* and *CFHR3* genes, likely resulting from a single non-homologous recombination event between a duplicated region downstream of the *CFH* and *CFHR1* genes, which became fixed early in human evolution, likely implying a selective advantage (Hageman, et al., 2006). The deletion of the *CFHR1* and *CFHR3* genes is included in a single extended *CFH-CFHRs* haplotype, H4, that associates with lower risk of AMD (Hughes, et al., 2006) and IgA nephropathy (Gharavi, et al., 2011) and increased risk of SLE (Zhao, et al., 2011). This remarkable finding suggests that binding of these FHR proteins, and capacity to regulate complement, directly or by complementing or competing with fH, can be beneficial or detrimental depending on the circumstances.

The deletion of the *CFHR1* and *CFHR3* genes is likely also protective in aHUS (Abarrategui-Garrido, et al., 2009, Martinez-Barricarte, et al., 2012); however, the frequency of homozygosity for the *CFHR3-CFHR1* deletion is increased in aHUS as a consequence of the association between complete deficiency of the FHR1 protein and the generation of anti-fH autoantibodies (Abarrategui-Garrido, et al., 2009, Dragon-Durey, et al., 2009, Jozsi, et al., 2008, Moore, et al., 2010, Zipfel, et al., 2007). This is an intriguing association for which there is no clear explanation. Importantly, the anti-fH autoantibodies recognize the C-terminus of fH, the region critical for the development of aHUS

that is nearly identical in FHR1; not surprisingly, the autoantibodies cross-react with FHR1. It has been suggested that deficiency of FHR1 may result in a failure of central and/or peripheral tolerance to the homologous region in fH, but there are other possibilities, including cross-reactivity with microbial antigens.

Another relatively frequent rearrangement in the *CFH-CFHR* region involves an unequal crossover between homologous regions in the 3' ends of *CFHR3* and *CFHR4* genes that specifically removes the *CFHR1* and *CFHR4* genes. This deletion is also found in aHUS patients in association with anti-fH autoantibodies for the reasons indicated above (Abarrategui-Garrido, et al., 2009), and is also associated with C3-glomerulopathies (C3-GN).

Most interesting are the various genomic rearrangements between the 3' end exons of *CFH* and the homologous regions in *CFHR1 or CFHR3*, which have been associated with aHUS (Francis, et al., 2012, Venables, et al., 2006). All result in the generation of *CFH::CFHR1 or CFH::CFHR3* hybrid genes that alter the C-terminal region of fH, further illustrating the remarkable correlation between C-terminal region deficits in fH and aHUS. On the other hand, the *CFH::CFHR1* hybrid gene in which the C-terminal exons of fH have been replaced by those in FHR1 demonstrates that, despite their sequence similarities, the C-terminal regions of fH and FHR1 have distinct binding specificities and do not compete - at least not for substrates that are relevant in aHUS.

A unique set of genomic rearrangements in the *CFHRs* gene region are found associated with C3-GN. The most remarkable of these involves the duplication of the first two SCRs of the FHR5 protein; this by itself defines a monogenic disorder endemic in Cyprus denominated FHR5 nephropathy

(Athanasiou, et al., 2011, Gale, et al., 2010, Gale and Pickering, 2011). Other rearrangements associated with C3-GN include a hybrid *CFHR3::CFHR1* gene (Malik, et al., 2012) and an internal duplication of the FHR1 protein (Abarrategui-Garrido, et al., 2010). The functional implications of these rearrangements are currently being investigated; it is unclear whether they represent loss of function or gain of function mutations.

Apart from these genomic mutations and polymorphisms, other disease-associated polymorphisms in FHR proteins include the FHR-1*A/*B variant described above; possession of the *B variant is risk for aHUS, while possession of the *A variant is risk for AMD (Abarrategui-Garrido, et al., 2009, Martinez-Barricarte, et al., 2012), further emphasizing the dichotomy of complement regulation in these disorders. The *A and *B variants differ by three amino acids with *B having higher homology with fH (Figure 4B); how these differences affect function remains unclear. Five different polymorphisms in the *CFHR5* gene have been described and associated with risk of aHUS, DDD and AMD (Abrera-Abeleda, et al., 2011, Monteferrante, et al., 2007, Narendra, et al., 2009).

The *CFH-CFHRs* genetic hotspot is an area in which we anticipate much more activity in the near future. It seems inevitable that other disease associated polymorphisms in this complex gene family will emerge and their functional characterization will resolve the mechanisms by which in health fH and the FHR proteins collaborate to control complement on different surfaces.

The complotype

1) A predictor of disease risk.

The complotype describes the repertoire of inherited common polymorphisms in genes encoding complement proteins and regulators. This term was used originally to describe haplotypic combinations of genetic variants of MHC-linked complement genes (Alper, et al., 1986), but has been expanded recently to include variants in the whole complement system (Harris, et al., 2012). Functional analyses of polymorphic variants within the AP demonstrate that they work together to dictate the balance between activation and regulation, and thereby set the complement activating capacity of an individual (Heurich, et al., 2011, Montes, et al., 2009, Tortajada, et al., 2009). Inheritance of more active variants of AP components (C3, fB) or less active variants of regulators (fH, fI, MCP) swings the balance in favour of AP activation and inflammation, while inheritance of less active variants of components and more active regulators dictates less AP activation and inflammation. The functional effect of each AP polymorphic variant is small, as might be expected from a common polymorphism. However, their combined effects can be striking, particularly when they directly influence the AP amplification loop, the means by which small activation triggers are massively amplified. Common non-coding polymorphic variation also influences expression levels of complement proteins, adding an extra dimension to the concept of the complotype.

The inherited pattern of complement variants, and the expression levels of these proteins, have direct effects on systemic activity and thus alter risk for pathologies driven by complement activation. However, the contribution of the complotype to disease risk is complex – in addition to overall complement activity, the pattern of inherited variants influences development of pathology in different ways depending on the underlying disease mechanism (Table 1).

Genetic studies in AMD, DDD and aHUS described above, show that often multiple hits are needed to cause disease. For example, in aHUS, disease requires not only the disease-associated mutation in an AP protein, but also altered expression of another AP protein or a risk complotype (Esparza-Gordillo, et al., 2005, Hakobyan, et al., 2010). Protein variants which are risk for aHUS, a disease caused by complement dysregulation at the cell surface, may not be risk for other pathologies caused primarily by dysregulation in the fluid phase.

2) Molecular complotyping in complement dysregulation disorders.

Molecular analyses provide most of the information required to define an individual's complotype, although some aspects, for example plasma concentrations of components, can be ascertained only by protein assays. Complement molecular diagnostics contribute to identification and understanding of complement dysregulation disorders by providing basic information on complement mutations and polymorphisms, and by building from these datasets algorithms that are predictive of disease susceptibility, progress or response to therapy.

In the simplest situation, a single complement gene or set of genes is demonstrated to be associated with development of a particular pathology; identification of the responsible complement protein mutations/polymorphisms and their effects on protein function then provides direct help in tailoring management and therapeutic strategies. Even this superficially simple task is often one of significant complexity. In the case of aHUS, for example, complotyping will include a search for mutations not just in the *CFH* gene, but also in *MCP*, *CFI*, *CFB* and *C3* genes, by PCR exon amplification and DNA

sequencing, together with copy number variation (CNV) analysis of the CFH-CFHRs gene region to identify genomic rearrangements, deletions and duplications. Thereafter, interpretation of the findings is not always straightforward, frequently requiring mutation segregation analysis between affected and unaffected family members, searches in the general population to identify and eliminate rare polymorphisms, and an analysis of the impact of the mutation on either protein expression or function (Tortajada, et al., 2012). Current technologies are limiting because of cost and time constraints in analysis of these numerous genes and interpretation of the variability of the complement components; however, recent advances in DNA sequencing technologies are generating enormous expectations in the community. Next-generation sequencing and the continuously falling cost and increasing speed of sequencing will solve some of these problems by making it possible to undertake a complete characterization of genetic variability in all complement components in an individual at reasonable cost. These data will only be of value if linked to advanced informatics to identify relevant patterns and rationalize mutation analysis of the whole set of complement genes. There will be a need to develop predictive algorithms, likely different for each disease, to estimate risk based on complotype, other genetic factors and environmental or other exogenous factors. An excellent example of this is the case of AMD where genetic variations in complement proteins are additive in their effects on disease susceptibility and interact strongly with other genetic and non-genetic factors (for example, smoking) (Hecker, et al., 2010, Heurich, et al., 2011, Martinez-Barricarte, et al., 2012, Reynolds, et al., 2009, Scholl, et al., 2008).

In the future, we predict that analysis of an individual's complotype will be widely used, in conjunction with environmental and other known risk factors, to predict disease risk and course. Complement quantitative traits must also be incorporated into the disease-predictive algorithms as complement protein levels show important inter-individual variability and are relatively stable characteristics with a high heritability (Buil, et al., 2010, Esparza-Gordillo, et al., 2004, Sanchez-Corral, et al., 1995).

A particular challenge is the identification of biomarkers that may help to anticipate development of the disease in predisposed individuals and be useful in monitoring disease activity and response to treatment in patients. Measurement of complement activation products is likely to be an important add-on to the genetically determined complotype in such analyses; even in isolation, measures of complement activation have been shown to be predictive of disease course in diseases as diverse as AMD and multiple sclerosis (MS) (Ingram, et al., 2012, Reynolds, et al., 2009, Scholl, et al., 2008, Smailhodzic, et al., 2012). Assay automation, multiplexing and other recent advances in -omics technologies has opened a window of opportunity for rapid and substantial progress in this area.

3) The role of the complotype in patient stratification and choice of therapy.

We are now in the age of stratified medicine, the stated goal of which is to deliver the right drug or other intervention to the right patient at the right time. The concept is built on understanding that the genetic makeup of an individual impacts their risk of developing a particular disease, their disease course and their response to therapies. Stratifying patients into groups based on likely

outcome and responsiveness to treatment will deliver better outcomes at lower cost because those most likely to respond are identified early and treated appropriately. For complement-mediated diseases, defining the complotype will be a key component of patient stratification and personalization of therapy. To take AMD as an example, individuals presenting with early disease would be complotyped using appropriate genetic (outlined above) and/or protein analyses, including measures of plasma levels of key components and activation products. Those with “high risk” complotypes, defined as carriers of multiple risk alleles (*CFH-402H*, *CFH-62V*, *CFHR1*A*, *CFB-32R*, *C3-102G*, others), particularly accompanied by evidence of complement activation, would be stratified for therapies targeting complement activation. Those with a low risk complotype and no evidence of ongoing complement activation would be stratified to other therapeutic options.

Defining the complotype will also help evolve new therapeutic strategies in complex diseases where complement may play a role in a subset of patients. For example, MS is a highly complex disease that is currently impossible to treat (Compston and Coles, 2002). The disease is heterogeneous, likely representing a number of overlapping clinical entities, and the numerous failures of initially promising agents in clinical trials likely reflects this fact – some respond but most don’t. Better stratification of MS patients into disease subgroups is essential for future drug development, not least because it will enable selection of appropriate patients into trials, and is attracting much effort. A role for complement in MS has been suggested for more than thirty years based on studies in animal models (Ingram, et al., 2009, Linington, et al., 1989, Morariu and Dalmasso, 1978); however, data from patients has been

inconsistent, likely reflecting disease heterogeneity (Lassmann, 2004). Based upon pathological and clinical evidence, Lassmann described four subgroups in MS that differed in the underlying pathology; one of these, Type II – some 30% of patients, involved abundant complement activation, but the other subgroups did not (Lucchinetti, et al., 2000). Because this stratification required access to brain tissue – biopsy or autopsy – it has been of little value clinically. However, several recent reports describing measurement of complement proteins and activation products, in CSF or plasma, as biomarkers of disease opens up the real prospect of identifying those patients most likely to benefit for inclusion in trials of anti-complement drugs (Ingram, et al., 2012, Ingram, et al., 2010, Ingram, et al., 2010). Although some individual complement polymorphisms have been explored as potential susceptibility factors, there has been no broad brush analysis of complement genetic associations with MS; a combination of proteomic and genomic complotype analyses might provide a tractable way of identifying the complement-driven subset, enabling targeted trials of anti-complement therapies. Similar issues pertain in many other chronic inflammatory diseases. In AD, complement has been implicated both as a protective and destructive player (Bonifati and Kishore, 2007, Veerhuis, et al., 2011). Evidence for complement activation is found in tissues, CSF and plasma but no clear picture of how complement influences the disease has emerged. Complotyping, particularly in early disease or in those at high risk of developing disease (mild cognitive decline for example), may be helpful in identifying subgroups or stages of the disease that might benefit from anti-complement therapy,

4) The complotype defines disease-driving complement pathways.

Analysis of the complotype will, by identifying which complement pathway is most important in driving dysregulation and pathology in a particular disease, aid choice of therapy. Diseases of AP dysregulation, linked to mutations in AP components or regulators, are most likely to be ameliorated by drugs that target the AP, while diseases in which the CP is the driver will be best treated using CP inhibitory drugs. Hereditary angioedema (HAE) provides the perfect example of the latter scenario. Mutations in the *C1inh* gene result in deficiency of C1inh and dysregulation of the CP; replacement therapy using purified C1inh restores homeostasis (Cicardi and Zanichelli, 2010). Surprisingly, despite its long and successful record of use in HAE, C1inh therapy has not been used for other disease of complement dysregulation – this may in part be because it has not been clear which diseases are CP-driven. In contrast, a much more recently developed complement therapeutic is finding broader use. Eculizumab™, a blocking monoclonal antibody against human C5 that inhibits C5a and MAC generation (Rother, et al., 2007), was first successfully used for treatment of paroxysmal nocturnal hemoglobinuria (Brodsky, 2009), a hemolytic and thrombotic disorder caused by deficiency of GPI-linked proteins (including complement regulators CD55 and CD59) on blood cells (Takeda, et al., 1993). Erythrocytes, deficient in surface regulators, activate complement, probably via the AP, and are lysed by MAC formation; blocking C5 prevents MAC formation and rescues patients from dependence on transfusions. Eculizumab was first suggested as a therapy for DDD in 2007 (Smith, et al., 2007), and soon after tested with good effect in aHUS patients to prevent relapses of the disease and recurrences after transplantation (Mache, et al., 2009, Nurnberger, et al., 2009).

Based on the excellent results obtained in phase II clinical trials during 2009-10, Eculizumab was approved by the US Food and Drugs Administration and the European Medicines Agency and has rapidly become the accepted therapy in patients with aHUS, both as a rescue therapy in acute episodes and as prophylaxis in labile patients and following renal transplant.

Isolated case reports describe a heterogeneous response in cases of C3-GN, including DDD, indicating that further research is needed to define the subgroup of C3-GN patients in whom Eculizumab therapy may be effective (Bomback, et al., 2012, Daina, et al., 2012, Vivarelli, et al., 2012). A Phase II study of systemic Eculizumab therapy in dry AMD is scheduled to report July 2012.

It is at first sight surprising that a therapy targeting the terminal pathway would be effective in diseases of AP dysregulation. C3 convertase assembly and C3 fragment deposition will continue unhampered, provoking the conclusion that C5a and MAC are major drivers of pathology in these diseases. It is not yet clear whether deposition of C3 fragments and other early activation products causes residual injury in Eculizumab-treated aHUS and DDD patients; however, in PNH patients treated with Eculizumab, C3 fragment opsonised erythrocytes are removed in spleen and liver, reducing circulating half-life (Risitano, et al., 2009). There would thus be some advantage in using therapies that target the AP convertase and switch off activation. A recombinant human CR2/factor H fusion protein, TT30, has been developed that fits the bill and is in Phase I trials in PNH (Fridkis-Hareli, et al., 2011); its use in other AP dysregulation diseases, either alone or in combination with Eculizumab, is anticipated.

5) The complotype also impacts on infectious diseases.

So far we have focused on the relevance of the complotype to acute or chronic inflammatory diseases; however, the principle role of complement is to kill invading bacteria so a role in infection is obvious. While a complotype that provides a more active complement system will increase risk in inflammatory diseases, the opposite is true in infection where a more active complement system will more efficiently target pathogens and is thus protective. Evidence in support of this is provided from comparison of complement polymorphisms in different populations; in communities at high risk of death from infection, for example, in Sub-Saharan Africans, there is a much higher frequency of polymorphisms that increase complement activity, a more active complotype, compared to low infection risk communities (Fridkis-Hareli, et al., 2011). A direct effect of the complotype on infection risk was revealed by a recent meningococcal disease GWAS (Davila, et al., 2010). A SNP in *CFH* (rs1065489; E936D) was identified where the D allele was protective for survival after infection. The functional effect of this SNP is not yet clear, in part, because it is in strong LD with other SNPs along the *CFH-CFHR3-CFHR1* region within CFH haplotype H3 (see above). Nevertheless, it is clear that polymorphisms in fH or FHRs influence risk of meningococcal disease by altering complement activation either in plasma or on the bacterial surface, possibly by altering function or relative expression levels of these key components. Although GWAS is a powerful way of identifying disease associations when allele frequency is high, linkage to less common polymorphisms can be missed and are only found when sought in a targeted manner. For example, a regulator element

polymorphism in *CFH*, found by a targeted approach to be associated with meningococcal disease, caused altered plasma fH levels (Haralambous, et al., 2006), while a polymorphism in complement C5 (rs17611;V802I), of unknown functional effect, was linked to poor outcome in bacterial meningitis (Woehrl, et al., 2011).

Restoring homeostasis in diseases of complement dysregulation.

Inter-individual differences in overall complement activity caused by the polymorphisms comprising the complotype are subtle, and even the disease-linked complement protein mutations often cause relatively small changes in activity. Impact of these small changes builds over time and hence many of the associated pathologies are chronic, evolving over years. Using drugs that completely block complement activation or effectors in these circumstances seems excessive. Except in acute episodes, complete inhibition of complement is not only unnecessary but is also potentially harmful, increasing risk of infection and other pathologies (Asghar and Pasch, 2000). The identification and characterization of protective disease-associated complement polymorphisms (Heurich, et al., 2011, Montes, et al., 2009, Tortajada, et al., 2009) suggests that down-modulation without causing complete inhibition would be a measured and effective approach to an “over-active” complotype. In the case of HAE, drugs that cause relatively modest increases in plasma levels of C1inh, either by enhancing synthesis (anabolic steroids) or reducing consumption (protease inhibitors) are remarkably effective at preventing attacks (Cicardi and Zanichelli, 2010). Perhaps similar strategies to modestly increase plasma fH concentrations by enhancing production, supplementing with

exogenous protein or re-balancing the complex interplays with fHR proteins, will be effective, low-risk and affordable as prophylactic therapy for diseases like aHUS, DDD and AMD. Knowledge of the complotype will guide such “fine tuning” approaches to the treatment of complement dysregulation.

Conclusions.

The complotype is a concept that will radically alter the way we consider the complement system and the way that complement contributes to disease. It starts from the knowledge that individuals inherit very different complement systems that impact on their susceptibility to infections and risk of developing particular diseases. Advances in genomics and proteomics make it possible to assess an individual’s complotype and use this information for prediction of disease risk or outcome, counselling on behaviour modification and making decisions on therapies. This patient-directed approach will enable better targeting of available drugs and guide future development of drugs tailor-made to redress the balance when complement is dysregulated.

Acknowledgements.

The work of the authors described in this review was supported by the Wellcome Trust (068823; to CLH) and the MRC (G0701298; to CLH and BPM). SRdeC is supported by the Spanish Ministerio de Economia y Competitiviad (SAF2010-26583), the Communidad de Madrid (S2010/BMD-2316) and the Fundación Renal Iñigo Alvarez de Toledo.

Disclosures.

SRdeC has undertaken consultancy work for Alexion and is listed as a co-inventor on a patent held by Secugen SL, the Agencia Estatal Consejo Superior de Investigaciones Cientificas and the University of Navarra, regarding a method for the prediction of risk of developing AMD in the Spanish population. BPM has undertaken consultancy work for Baxter, Viropharma and Alexion. BPM and CLH are named on a patent held by Cardiff University that protects monoclonal antibodies specific for the Y402H variants of factor H. None of these interactions has influenced the results and interpretations in this manuscript.

Figure Legends.

Figure 1. Activation and regulation of the complement system.

Cleavage of C3 to generate C3b is the critical event in complement activation. The classical (CP) and lectin (LP) pathways involve recognition of target-bound antibody through C1q binding (CP), or pathogen-specific carbohydrates by MBL (LP). The associated protease units (C1s and MASP-2 respectively) then cleave C4 and C2, leading to formation of the C3 convertase C4b2a. In the alternative pathway (AP), continuous, low level, activation of C3 by spontaneous hydrolysis of the internal C3 thioester or cleavage by plasma proteases generates $C3(H_2O)$, a C3b-like molecule, or C3b, which can bind fB to form an AP convertase which cleaves C3 to C3b. In the AP amplification loop (circle arrows), C3b, either membrane-bound or in the plasma, binds factor B (fB) generating additional AP C3 convertases that cleave many C3 molecules into C3b amplifying complement activation. The AP C3/C5-convertases are very labile enzymatic complexes (half-life of about 1min). However, binding of properdin (P) stabilises the enzyme, extending its half-life more than 10-fold. Activation of C3 by a membrane bound C3 convertase causes cluster deposition of C3b in the vicinity of the convertase. Incorporation of an additional C3b molecule to the C3 convertase creates a new enzyme, the C5 convertase, C3bBbC3b or C4b2a3b, which cleaves C5 to C5b and C5a. C5a is a highly proinflammatory polypeptide, whereas C5b initiates the formation of the lytic pore, the membrane attack complex (MAC) (terminal pathway). Complement homeostasis and protection of self-tissues from accidental complement attack is provided by proteins present in plasma and on cell membranes (boxes) which

either catalyze proteolytic inactivation of C3b/C4b by fI (MCP, CR1, fH, C4bp) or accelerate convertase dissociation (DAF, CR1, fH, C4bp).

Figure 2. Mutations in fH found in aHUS and DDD patients.

The location of most of the fH mutations thus far characterized in aHUS and DDD patients is indicated in a diagram of the structure of human fH showing the 20 SCR repeats. The mutations associated with DDD are highlighted with a black box. Note that mutations associated with aHUS are clustered in the C-terminus, the region of factor H that is critical for the control of C3b deposited on cell surfaces.

Figure 3. Complement activation in different glomerulopathies.

Schematic representation of the consequences of complement dysregulation in aHUS, where surface dysregulation results in strong C5 activation, and in DDD, where fluid phase activation primarily results in massive generation of C3b and iC3b. In other C3-GN, the situation may vary depending on the proteins involved.

Figure 4. CFHRs gene organization and structure of the FHRs proteins.

A) Genomic organization of the *CFH* and *CFHR1-5* genes. Arrows represent the genes with their names. Blue lines above the genes indicate the extent of linkage disequilibrium within three different SNP LD blocks. The coloured boxes underneath indicate the sequence repeats within this genomic region, labelled with the same letter (i.e., A, A', A''). The vertical lines indicate the positions of the exons of the *CFH* and *CFHRs* genes.

B) Structural organization of the fH and FHR proteins, including the *FHR1*A* and *FHR1*B* allotypes and the FHR4A and FHR4B isoforms. Short consensus repeats (SCRs) are represented by ovals and are numbered from the N-terminal end. Homologous SCRs are aligned and the amino acid differences from fH are indicated for SCR-3, -4, and -5 of FHR1*A and FHR1*B. Homologous SCRs are depicted with the same colour where they have greater than 80% amino acid identity. The colour code use in A to identify the different genomic sequence repeats has been maintained to correlate the SCR similarities with the genomic duplications.

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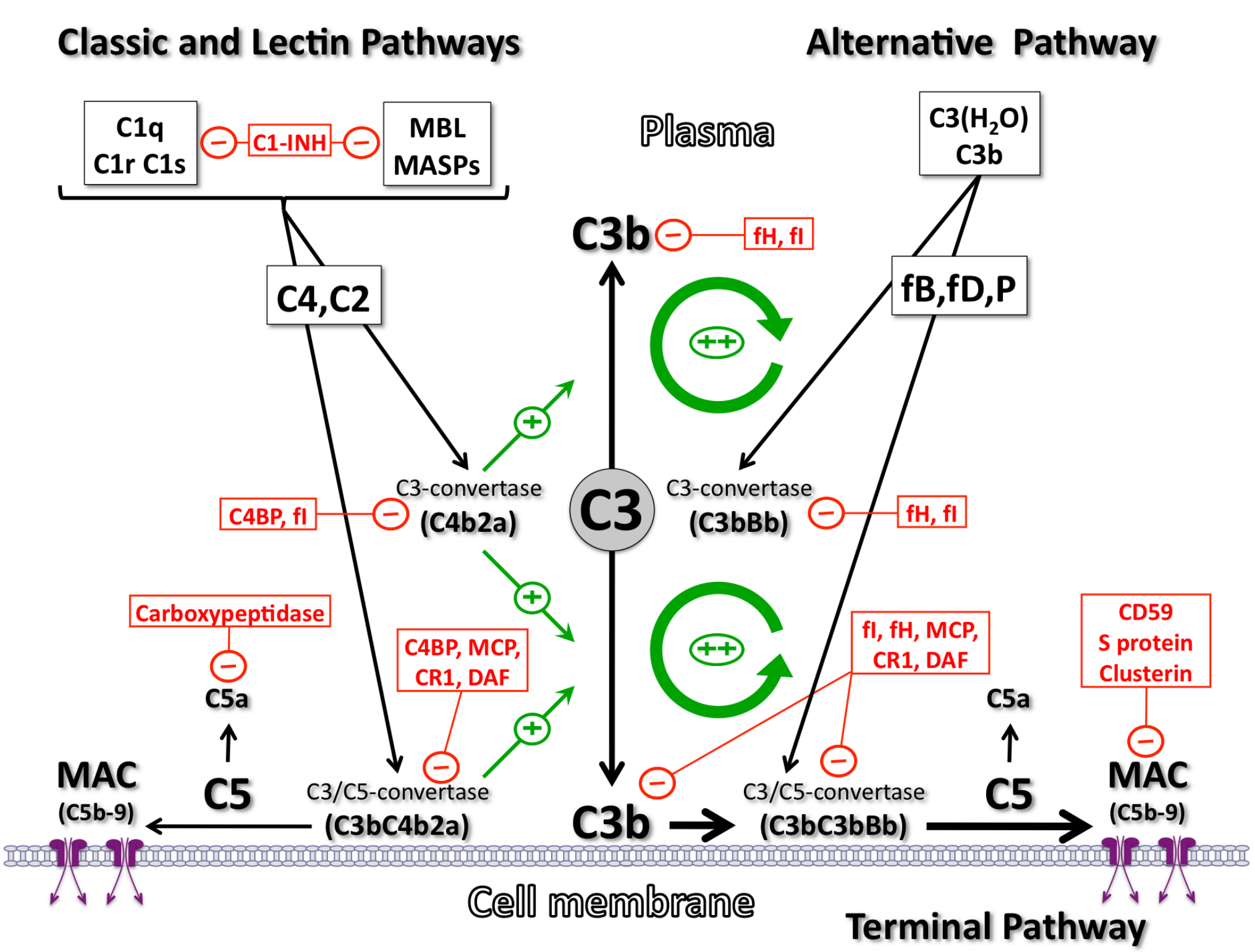
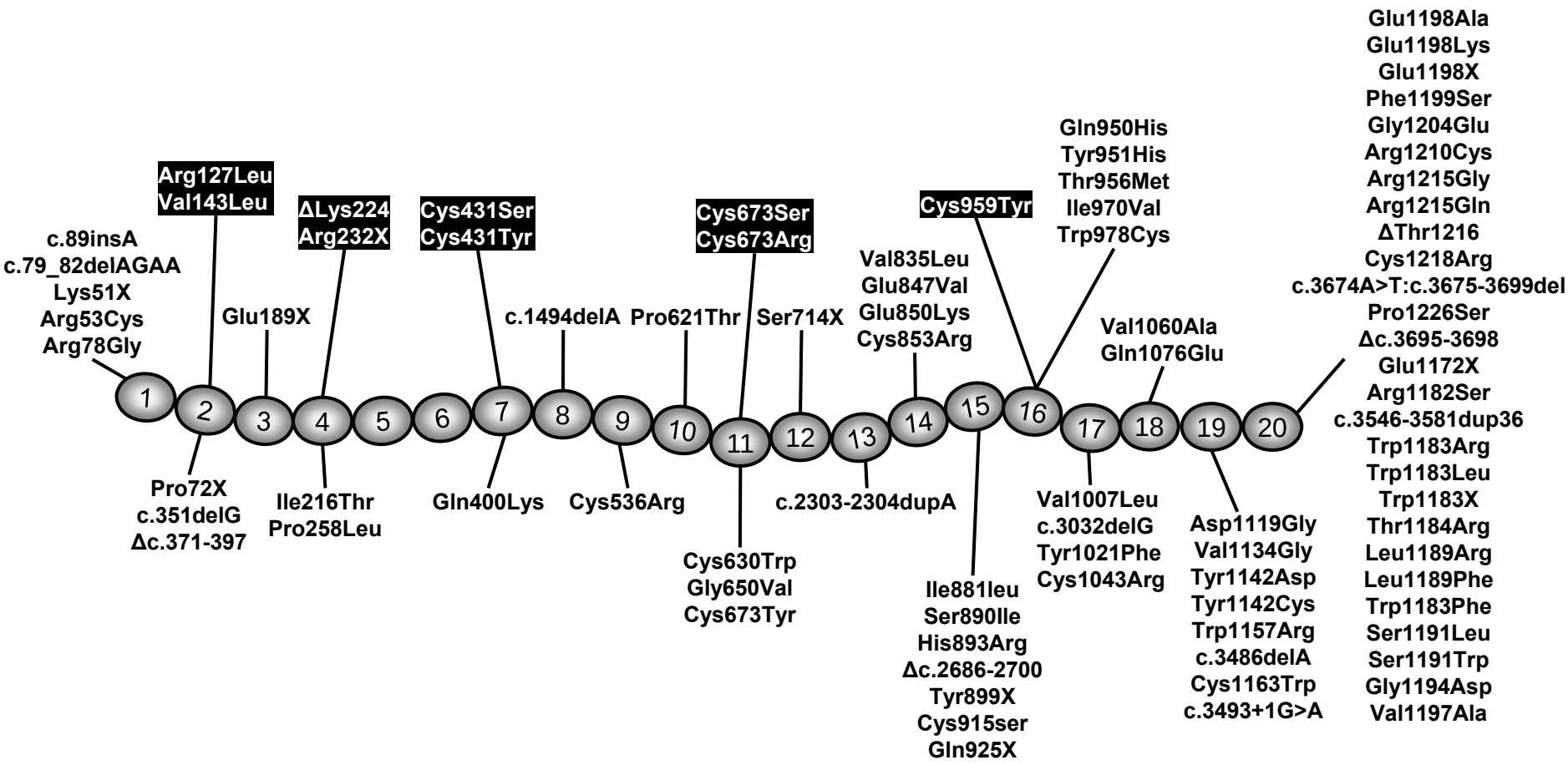

Classic and Lectin Pathways
Alternative Pathway
C1q
C1r C1s
C1-INH
MBL
MASPs
Plasma
C3(H2O)
C3b
C3b
fH, fI
C4,C2
fB,fD,P
++
C3-convertase
(C4b2a)
C3
C3-convertase
(C3bBb)
C4BP, fI
fH, fI
Carboxypeptidase
C5a
C4BP, MCP,
CR1, DAF
++
fI, fH, MCP,
CR1, DAF
CD59
S protein
Clusterin
C5a
MAC
(C5b-9)
C5
C3/C5-convertase
(C3bC4b2a)
C3b
C3/C5-convertase
(C3bC3bBb)
C5
MAC
(C5b-9)
Cell membrane
Terminal Pathway

Figure 1

Figure 2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c.79_82delAGAA
Lys51X
Arg53Cys
Arg78Gly
Arg127Leu
Val143Leu
Glu189X
ΔLys224
Arg232X
Cys431Ser
Cys431Tyr
c.1494delA
Pro621Thr
Cys673Ser
Cys673Arg
Ser714X
Val835Leu
Glu847Val
Glu850Lys
Cys853Arg
Cys959Tyr
Gln950His
Tyr951His
Thr956Met
Ile970Val
Trp978Cys
Val1060Ala
Gln1076Glu
Glu1198Ala
Glu1198Lys
Glu1198X
Phe1199Ser
Gly1204Glu
Arg1210Cys
Arg1215Gly
Arg1215Gln
ΔThr1216
Cys1218Arg
c.3674A>T:c.3675-3699del
Pro1226Ser
Δc.3695-3698
Glu1172X
Arg1182Ser
c.3546-3581dup36
Trp1183Arg
Trp1183Leu
Trp1183X
Thr1184Arg
Leu1189Arg
Leu1189Phe
Trp1183Phe
Ser1191Leu
Ser1191Trp
Gly1194Asp
Val1197Ala
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
Pro72X
c.351delG
Δc.371-397
Ile216Thr
Pro258Leu
Gln400Lys
Cys536Arg
Cys630Trp
Gly650Val
Cys673Tyr
c.2303-2304dupA
Ile881leu
Ser890Ile
His893Arg
Δc.2686-2700
Tyr899X
Cys915ser
Gln925X
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Tyr1142Cys
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Cys1163Trp
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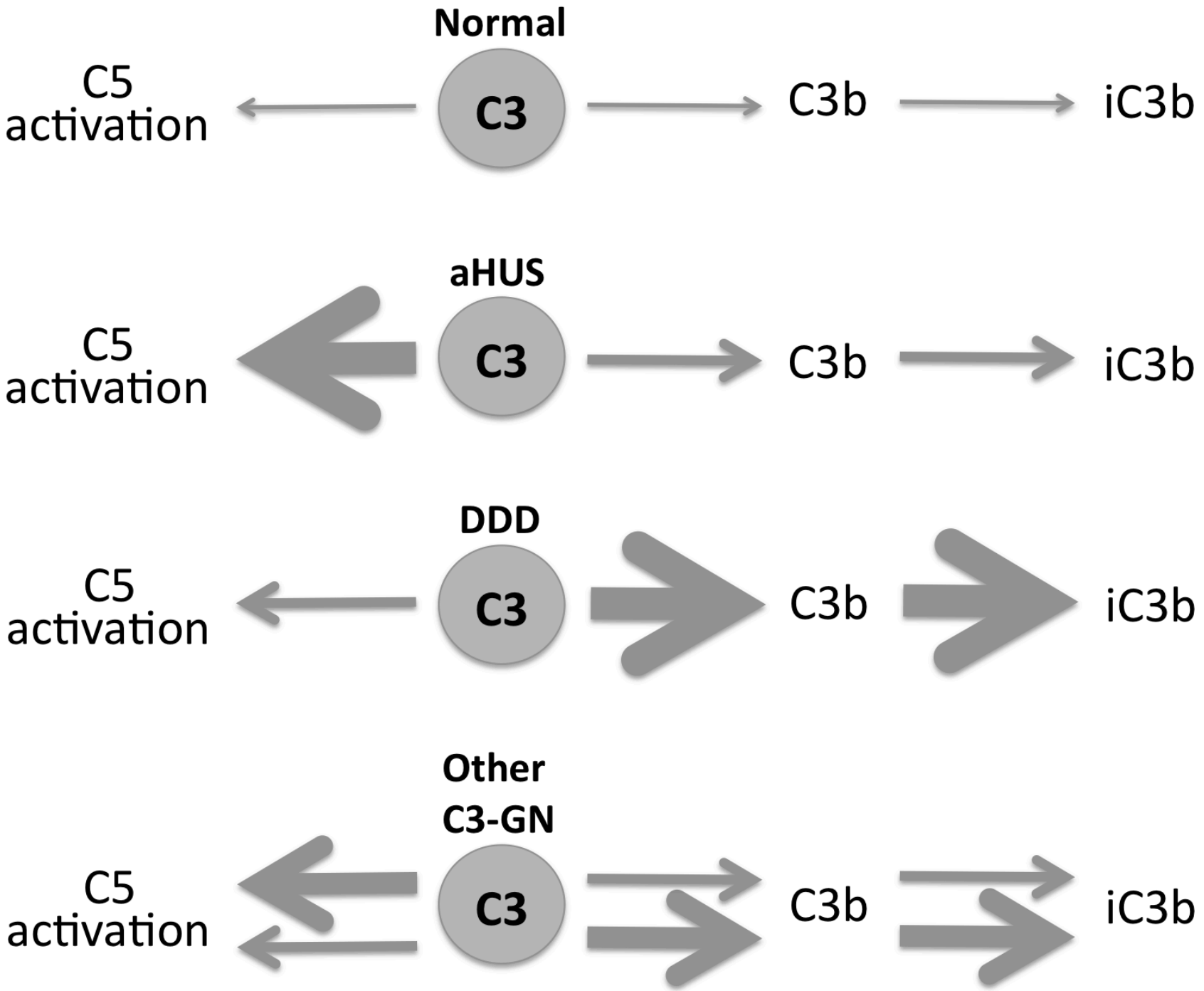
Normal
C5 activation
C3
C3b
iC3b
aHUS
C5 activation
C3
C3b
iC3b
DDD
C5 activation
C3
C3b
iC3b
Other C3-GN
C5 activation
C3
C3b
iC3b

Figure 3

Figure 4

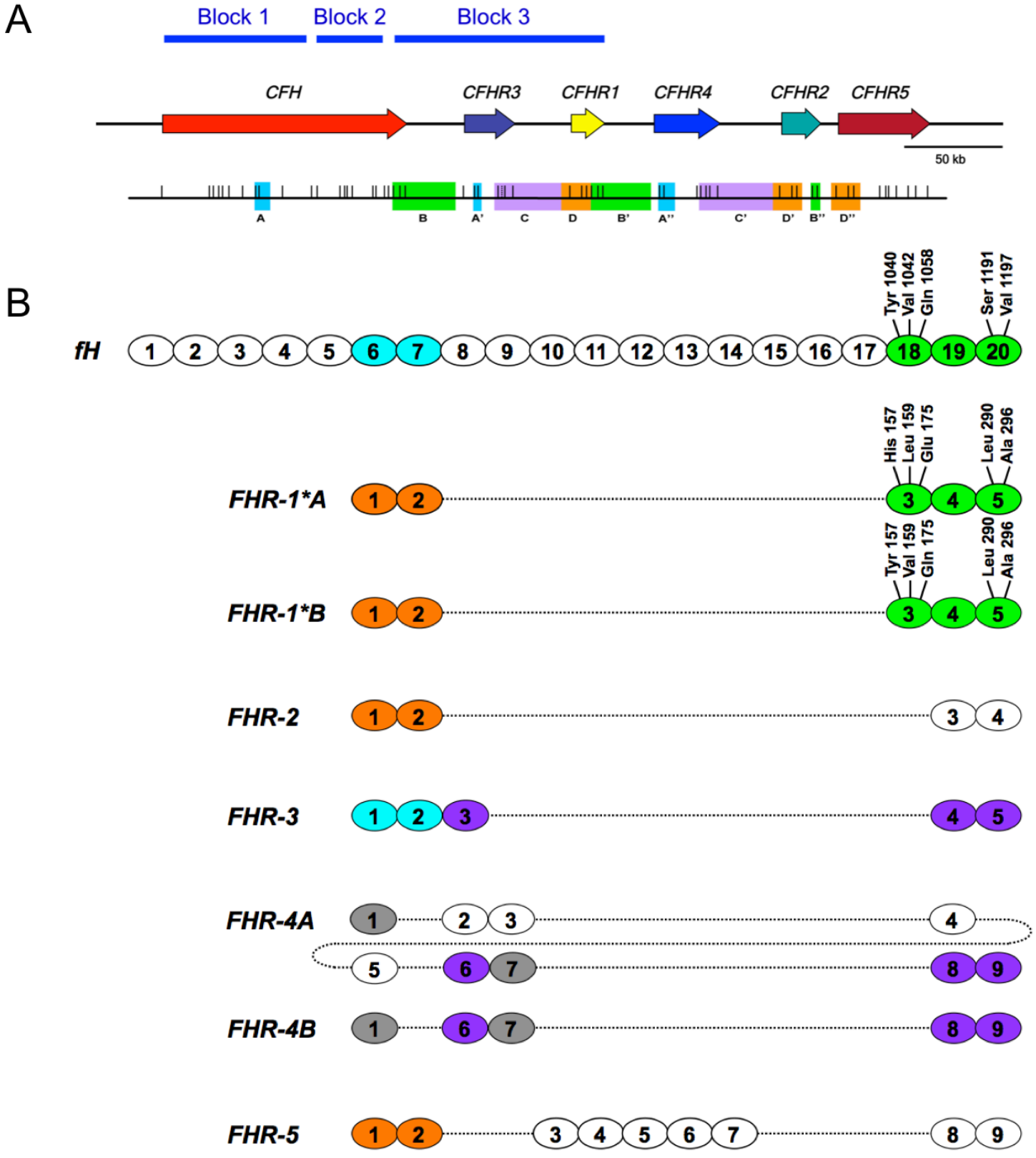
A
Block 1
Block 2
Block 3
CFH
CFHR3
CFHR1
CFHR4
CFHR2
CFHR5
50 kb
A
B
A'
C
D
B'
A''
C'
D'
B''
D''
B
fH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Tyr 1040
Val 1042
Gln 1058
Ser 1191
Val 1197
FHR-1*A
1 2 3 4 5
His 157
Leu 159
Glu 175
Leu 290
Ala 296
FHR-1*B
1 2 3 4 5
Tyr 157
Val 159
Gln 175
Leu 290
Ala 296
FHR-2
1 2 3 4
FHR-3
1 2 3 4 5
FHR-4A
1 2 3 4
5 6 7 8 9
FHR-4B
1 6 7 8 9
FHR-5
1 2 3 4 5 6 7 8 9

Table 1

TABLE 1. Risk factors associated with aHUS, C3-Glomerulopathies and AMD

	aHUS	C3 Glomerulopathy		AMD
		MPGN1, C3GN,fMPGN3 CFHR5 nephropathy	DDD	
Genetic factors				
- Mutations	*CFH* *MCP* *CFI* *CFB* *C3* *CFHR1* *THBD*	*CFH* *CFI* *MCP* *CFHR5* *CFHR1*	*CFH* *C3*	*CFH*
- Polymorphisms	*CFH* *MCP* *CFHR1* *CFHR3*	*CFH* *CFHR1* *CFHR3* *CFHR5*	*CFH* *CFHR1* *CFHR3* *CFHR5*	*CFH* *CFI* *CFB* *C2* *C3* *C7* *CFHR1* *CFHR3* *AMRS2 (LOC387715)*
Autoantibodies	**Factor H**	**C3Nef**	**C3Nef, Factor H**	
Environmental factors	**Infection** **Immunosupp. drugs** **Cancer therapies** **Oral contraceptives** **Pregnancy** **Childbirth, etc.**	**Infection**	**Infection**	**Smoking** **Diet** **Exercise, etc.**

TABLE 2. CFHRs gene rearrangements associated with disease.

Disease		Genetic findings	Outcome	Significance	Risk/ Protection	Prevalence	Ref.
aHUS		CFH::CFHR1 hybrid genes	Substitution of the C-terminal SCRs of fH for those in FHR-1.	Loss of complement regulation at cell surfaces.	R	Several unrelated cases described	(Venables et al., 2006)
		CFH::CFHR3 hybrid gene	Substitution of the last C-terminal SCR20 of fH for the whole FHR-3.	Loss of complement regulation at cell surfaces	R	Very rare	(Francis et al., 2012)
		DelCFHR3-CFHR1 DelCFHR1-CFHR4	Loss of FHR-3 and FHR-1. Loss of FHR-1 and FHR-4.	Associated with auto anti fH antibodies impairing cell surface regulation.	R	Common	(Abarrategui-Garrido et al., 2009; Moore et al., 2010; Zipfel et al., 2007)
		CFHR*B	Allelic variant in which FHR-1 SCR3 is identical to fH SCR18.	Unknown	R	Common	(Abarrategui-Garrido et al., 2009)
C3 Glomerulopathy	**DDD**	DupCFHR1	Mutant FHR-1 with SCR123412345	Unknown	R	Very rare	(Abarrategui-Garrido et al., 2010)
	C3-GN	CFHR3::CFHR1 hybrid gene	Hybrid protein containing SCR1-2 of FHR-3 followed by the whole FHR-1 molecule.	Unknown	R	Very rare	(Malik et al., 2012)
	FHR5 Nephropathy	DupCFHR5	Mutant FHR-5 with SCR12123456789	Unknown	R	Several related cases described	(Gale et al., 2010)
AMD		DelCFHR3-CFHR1	Loss of FHR-3 and FHR-1.	Unknown	P	Common	(Hughes et al., 2006)
		CFHR*A	Allelic variant in which FHR-1 SCR3 differs from fH SCR18 in three amino acids.	Unknown	R	Common	(Martinez-Barricarte et al., 2012)
SLE		DelCFHR3-CFHR1	Loss of FHR-3 and FHR-1.	Unknown	R	Common	(Zhao et al., 2011)

***Detailed Response to Reviewers**

We have revised the manuscript in response to the helpful comments of the reviewers. We have updated figure 2 to include recently described mutations. We have replaced Figure 4 with a modified, clearer version, we have added text in several places (highlighted in submitted Ms) to address omissions noted in review. We have added four additional references in response to reviewer comments.