

# Disease-Causing Mutations in Genes of the Complement System

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Recent studies have revealed profound developmental consequences of mutations in genes encoding proteins of the lectin pathway of complement activation, a central component of the innate immune system. Apart from impairment of immunity against microorganisms, it is known that hereditary deficiencies of this system predispose one to autoimmune conditions. Polymorphisms in complement genes are linked to, for example, atypical hemolytic uremia and age-dependent macular degeneration. The complement system comprises three convergent pathways of activation: the classical, the alternative, and the lectin pathway. The recently discovered lectin pathway is less studied, but polymorphisms in the plasma pattern-recognition molecule mannan-binding lectin (MBL) are known to impact its level, and polymorphisms in the MBL-associated serine protease-2 (MASP-2) result in defects of complement activation. Recent studies have described roles outside complement and immunity of another MBL-associated serine protease, MASP-3, in the etiology of 3MC syndrome, an autosomal-recessive disorder involving a spectrum of developmental features, including characteristic facial dysmorphism. Syndrome-causing mutations were identified in *MASP1*, encoding MASP-3 and two additional proteins, MASP-1 and MAP44. Furthermore, an association was discovered between 3MC syndrome and mutations in *COLEC11*, encoding CL-K1, another molecule of the lectin pathway. The findings were confirmed in zebrafish, indicating that MASP-3 and CL-K1 underlie an evolutionarily conserved pathway of embryonic development. Along with the discovery of a role of C1q in pruning synapses in mice, these recent advances point toward a broader role of complement in development. Here, we compare the functional immunologic consequences of “conventional” complement deficiencies with these newly described developmental roles.

## Complement System and Components

As a prelude to discussing the polymorphisms and genetics of the complement system, we wish to set the stage with a brief introduction to the complement pathways and the proteins involved.

The complement system is the humoral backbone of the innate immune defense. It is thus involved in a number of diverse processes involving antimicrobial defense, clearance of immune complexes, and tissue regeneration. The system comprises more than 35 humoral and cell-associated proteins forming three converging enzymatic cascades: the classical pathway, the alternative pathway, and the lectin pathway.<sup>1</sup> An overview is shown in Figure 1, where some of the proteins mentioned in the following are placed in their context.

The classical pathway is especially important for control of infections with pyogenic encapsulated bacteria, such as *Haemophilus influenzae* and *Streptococcus pneumoniae*,<sup>2</sup> although it has also been implicated in control of viral infections, such as influenza.<sup>3</sup> The pathway is initiated when the recognition protein C1q binds to antibodies bound to microbes or in immune complexes.<sup>2,4</sup> Preexisting “natural IgM” produced by naive B cells<sup>5</sup> and IgM or IgG generated through an adaptive response (primary or secondary) will bind to, for example, determinants on the capsule of pathogenic bacteria, leading to agglutination. Binding of IgM exposes cryptic binding sites on IgM, allowing C1q to bind, and binding of arrays of IgG similarly allows high-avidity binding of C1q. C1q is in complex with two serine proteases, C1r and C1s. Upon binding of C1q, conformational changes lead to autoactivation of C1r, which in turn activates C1s. Activated C1s is capable of cleaving C4 and C2. A fragment of C4, C4b, is deposited on the activating surface, where it is covalently attached through a reactive thioester, while a small fragment, C4a, is released. The proenzyme C2 is likewise cleaved by C1s into two fragments, and C2a joins C4b on the surface. C2a is a serine protease, capable of cleaving C3. Cleaved C3 in the form of C3b is covalently bound to the surface-attached C4bC2a complex, while the concomitantly generated small fragment C3a is released to mediate inflammation. C2a in the newly formed complex, C4bC2aC3b, is now able to cleave C5, and two fragments are again generated: C5a is a potent inflammatory mediator, while C5b initiates the formation of a membrane attack complex (MAC), which is expanded through deposition of C6, C7, C8 and C9 (Figure 1), leading to the formation of a hole in the target cell membrane. More importantly, the deposited components C4b and C3b function as molecular tags (opsonins) interacting with complement receptors, hereby facilitating engulfment by phagocytes and activation of B cells.

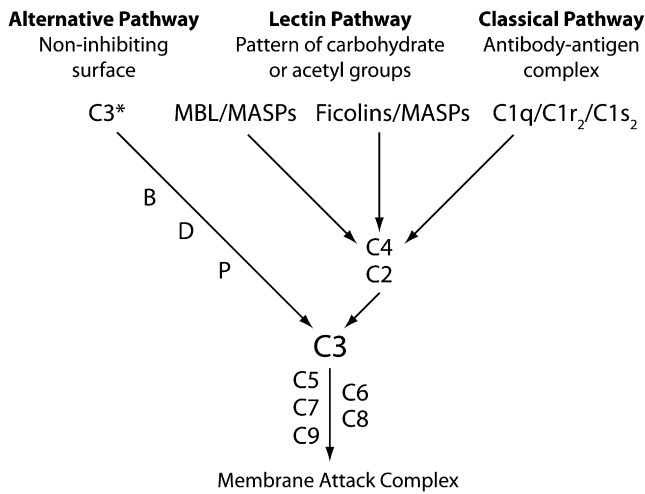
The lectin pathway is quite similar to the classical pathway.<sup>6</sup> An overview of the components of this pathway and the structure of some of the proteins is presented in Figure 2. The lectin pathway is also involved mainly in the control of bacterial infections, as has been observed in the case of children suffering from recurrent pyogenic infections.<sup>2,7</sup> It would appear that the lectin pathway is especially important during the interval between the loss

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## Complement Activation Pathways



**Figure 1. Overview of the Complement Pathways**

The three activating pathways and the terminal pathway are shown. The acronyms of participating proteins are given. The lectin pathway is activated when MBL or ficolins in complex with MASPs recognize foreign patterns of carbohydrate or acetyl groups. The classical pathway is activated by C1q in complex with C1s and C1r upon binding of antibody-antigen complexes. The alternative pathway is constitutively activated and inhibited on self-surfaces, but is allowed to proceed on foreign surfaces. The three pathways converge on C3 and C5 and the common terminal pathway leading to formation of the membrane attack complex.

of passively acquired maternal antibody and the acquisition of a mature immunologic repertoire, as well as in individuals with immunosuppression. The pathway is initiated when one or more recognition molecules bind to patterns of carbohydrates or patterns of acetyl groups on the surface of, for example, bacteria or viruses. Four such recognition molecules are capable of activating the lectin pathway: mannan-binding lectin (MBL), H-ficolin, L-ficolin, and M-ficolin.<sup>8</sup> These pattern-recognition molecules (PRMs) are found in complexes with three serine proteases (termed MBL-associated serine proteases [MASPs]), MASP-1, MASP-2, and MASP-3, as well as two nonenzymatic fragments hereof (termed MBL-associated proteins), MAp19 and MAp44 (Figure 2A). When one of the recognition molecules binds to an adequate pattern, the MASPs are activated. MASP-1 and MASP-2 are responsible for complement activation through cleavage of C2 and C4 (Figure 2B).<sup>9</sup> The cascade then proceeds as described above for the classical pathway.

Recently, a fifth PRM, CL-K1,<sup>10</sup> was reported to associate with MASPs.<sup>11</sup> Although it reportedly binds to various pathogen-associated molecular patterns (PAMPs), it remains to be determined whether CL-K1 can activate complement.

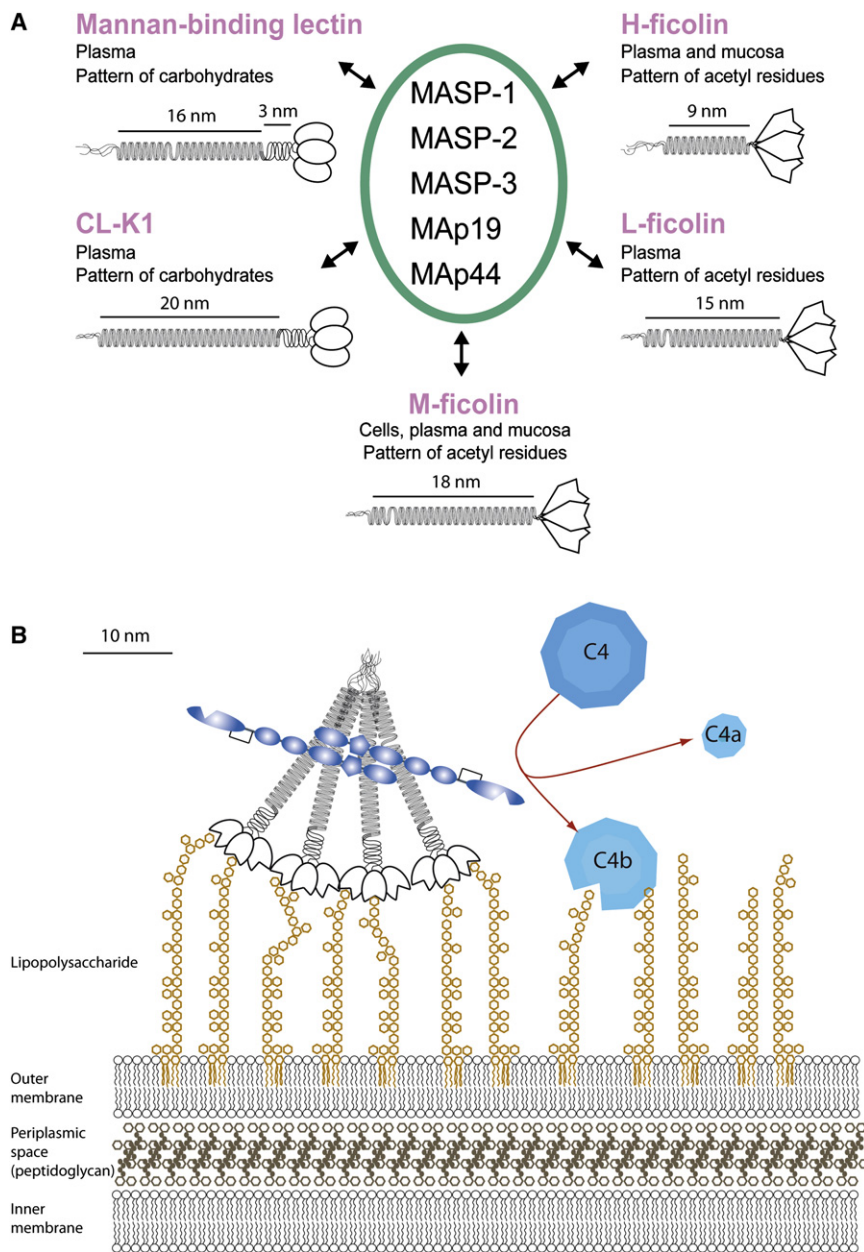
Whereas MASP-1 and MASP-2 have been described to be true enzymes with defined substrates, MASP-3, MAp19, and MAp44 have been suggested to act as regulators of complement activation,<sup>12–14</sup> but the relevance of these

in vitro observations remains unknown. The surprising new findings of potential functions of MASP-3 and CL-K1 are described in detail below.

The third way of activating complement, the alternative pathway, is also presented in Figure 1. In many regards, this pathway can be described as an amplification mechanism, because it is always activated after activation by the classical and the lectin pathways. Thus, the alternative pathway is important for the activity of the classical and lectin pathways. When the C3b fragment is bound to a surface (see above) it forms a complex with factor B. This leads to the cleavage of factor B by factor D, giving rise to an active enzyme complex with the fragment Bb as the enzyme. The substrate for Bb is C3, and efficient deposition of more C3b fragments occurs onto nearby surfaces, again giving rise to even more complex formations. In analogy with C2a, the active Bb enzyme may now also cleave C5 and thus generate all of the functions described above.

In addition to its function as an amplification mechanism of the classical and the lectin pathway, the alternative pathway may be initiated when C3 in plasma is spontaneously activated through hydrolysis of an internal thioester (so-called “tick-over”). This spontaneous event occurs at a constant low rate wherever in the body C3 is present (blood and interstitial fluids) and is an effect of small perturbations of a somewhat unstable structure of C3, exposing the internal thioester to the solvent.<sup>15,16</sup> Thus, generated C3(H<sub>2</sub>O) can form a complex with factor B, leading to cleavage of factor B by factor D and thus the formation of a C3-activating complex, C3(H<sub>2</sub>O)Bb, which upon further cleavage and addition of C3 forms a C5-activating complex, C3(H<sub>2</sub>O)BbC3b. The alternative pathway and the terminal lytic pathway are especially important for control of Neisserial infections, particularly *Neisseria meningitidis*.<sup>2</sup>

Plasma and cell surfaces harbor regulators of complement activation (e.g., factor H and CD59, respectively), immediately causing a local shutdown of the cascade, thus leaving host cells unharmed. On a foreign surface, which does not contain such regulators, the cascade continues undisturbed, ending with opsonization of the target and the formation of membrane-spanning MAC complexes. The regulators of complement activation thus add an extra layer of control to the already specific activation events of the classical and lectin pathways. Notably, in the case of the spontaneous activation of the alternative pathway, the regulators entirely determine the specificity on the basis of the recognition of so-called host-associated molecular patterns (HAMPs), a phenomenon termed “reverse recognition.”<sup>17</sup> Importantly, activation of complement is also tightly regulated in time and space by a very short half-life of activated C3 and C4; i.e., these activated components have a limited diffusion range because of quenching by water, ensuring that only surfaces nearby the site of activation are targeted.<sup>18</sup>



**Figure 2. The Lectin Pathway**  
 (A) The lectin pathway components. Overview of the five different recognition proteins of the lectin pathway, their tissue and humoral localization, and their ligand patterns. They all associate with MASPs and MAPs, except for CL-K1, which has so far only been reported to associate with MASP-1/MASP-3 and is not yet known to activate complement. A schematic drawing of the structural subunit of each protein is shown, composed of three identical polypeptide chains forming a collagen-like helix with the recognition domains extending in the C-terminal end. (B) Figure showing the binding of MBL in complex with MASP-2 to a molecular pattern on the surface of a Gram negative bacterium. MBL (and similarly the ficolins and CL-K1) are multimers (here a tetramer is shown) of the structural subunit drawn in part A of this figure. These molecules thus wield many recognition sites as indicated on the figure. MASP-2 is a homodimer and the domains are indicated with the disulphide bridge connecting the A- and B-chains after activating cleavage in the activation peptide. MASP-2 then activates C4 resulting in deposition of C4b on the surface, which initiates the tagging of the bacterium for phagocytosis. The complement cascade may then progress as indicated in Figure 1.

but may be of relevance in some situations. With regards to genetic association studies based on polymorphisms, we refer to recent reviews. As a primer to such studies, strong associations between SNPs in the gene encoding factor H (*CFH* [MIM 134370, 609814]) and age-related macular degeneration (AMD [MIM 610698]) have been identified.<sup>20–22</sup>

Polymorphisms in genes encoding other members of the complement system, e.g., factor B (*CFB* [MIM 138470]) and C3 (*C3* [MIM 120700, 613779]), have also been associated with AMD.<sup>23,24</sup> Studies are underway with regards to treatment regimens involving restoration of control mechanisms in this condition. Another example is the finding in atypical hemolytic uremic syndrome (aHUS [MIM 235400]) of a number of “loss-of function” polymorphisms in *CFH* and *CD46* (encoding membrane cofactor protein, MCP or CD46 [MIM 120920]) and “gain-of-function” polymorphisms in *CFB*.<sup>25</sup>

The prevalence of inherited complement deficiencies is difficult to estimate because most deficiencies may be caused by numerous different mutations in the relevant gene. Furthermore, deficiency is only revealed when by chance the physician or clinician decides that the symptoms of the patient warrant examination of the possible

### Conventional Genetic Deficiencies of Complement Components

Experiments with genetically engineered complement-deficient mice have been useful in research on the complement system. However, in general, inbred mouse strains display poor complement activity,<sup>19</sup> and numerous susceptibility differences exist between mouse and man. The study of individuals with increased susceptibility to a variety of clinical symptoms, including suspect immunodeficiency, has proven to be a most important source of information about defects of (and thus function of) the human complement system. In the following, we focus on recessive complement deficiencies based on complete absence of components or functionally defective components. The significance of partial deficiencies is less clear

**Table 1. Conventional Complement Deficiencies**

Protein	Gene symbol	MIM ID	Cases / Homozygosity	Disease Associations	References
<b>Recognition Proteins</b>					
MBL	<i>MBL2</i>	154545	5%	infections in immunocompromised individuals	28,106–108
H-ficolin	<i>FCN3</i>	604973, 613860	3	immune deficiency, necrotizing enterocolitis	40,41
C1q	<i>CIQA</i> , <i>CIQB</i> , <i>CIQC</i>	120550, 120570, 120575, 613652	43	SLE-like syndrome, recurrent bacterial infections	26,33,51
<b>Enzymes</b>					
MASP-2	<i>MASP2</i>	605102	9	immune deficiency	37,38
C1r/s	<i>C1R</i> , <i>C1S</i>	613785, 120580, 613783, 216950	19	SLE-like syndrome, recurrent bacterial infections	26,51
C2	<i>C2</i>	613927, 217000	1:20,000	autoimmune disease	28,32
Factor D	<i>CFD</i>	134350, 613912	<10	meningococcal and encapsulated bacterial infections	26
Factor I	<i>CFI</i>	217030, 610984	31	encapsulated bacterial infections	109,110
<b>Structural Proteins</b>					
C3	<i>C3</i>	120700, 613779	27	bacterial infections, SLE-like syndrome	111,112
C4	<i>C4A</i> , <i>C4B</i>	120810, 120820	26	SLE-like syndrome, encapsulated bacterial infections	33,51
C5	<i>C5</i>	120900, 609536	40	meningococcal infection	113,114
C6	<i>C6</i>	217050, 612446	>50	meningococcal infection	115–117
C7	<i>C7</i>	217070, 610102	>50	meningococcal infection	116,118,119
C8	<i>C8A</i> , <i>C8B</i> , <i>C8G</i>	120950, 120960, 120930, 613790, 613789	>50	meningococcal infection	120
C9	<i>C9</i>	120940, 613825	1:1,000 (Japan)	meningococcal infection	46
<b>Control Proteins</b>					
Properdin	<i>CFP</i>	300383	>50	meningococcal infection	43
Factor H	<i>CFH</i>	134370, 609814	27	hemolytic uremic syndrome (HUS), dense deposit disease	111,121,122
C1-INH	<i>SERPING1</i>	606860	1:50,000	hereditary angioedema (HAE)	33,123
CD11a (LFA-1), CD11b (CR3), CD11c (CR4) /CD18 <sup>1</sup>	<i>ITGAL</i> , <i>ITGAM</i> , <i>ITGAX</i> , <i>ITGB2</i>	153370, 120980, 151510, 600065, 116920	1:1,000,000	leukocyte adhesion deficiency type I (LAD I)	124,125
CD46 (MCP)	<i>CD46</i>	120920		atypical hemolytic uremic syndrome (aHUS)	25,126,127



**Table 1. Continued**

Protein	Gene symbol	MIM ID	Cases / Homozygosity	Disease Associations	References
CD55, CD59 (PIGA) <sup>2</sup>	CD55, CD59, PIGA	125240, 107271, 612300, 311770, 300818	1:1,000,000	paroxysmal nocturnal hemoglobinuria (PNH)	128

Summary of reported cases of homozygosity for complement-system gene mutations leading to functionally deficient proteins. The proteins in question are given on the left, followed by the gene symbol, the MIM ID number (associated with the gene and/or disease), the number of cases or incidence of homozygosity, and associated clinical findings. The numbers in the last column refer to reviews in which most of the original literature is mentioned or to recent case reports where the literature is discussed. Large ethnic differences are seen for C5, C6, C7, C8, and C9 deficiencies; for example, much higher frequencies of C9 deficiency in Japan as compared to people in western Europe. As is evident, most cases of homozygosity are rare; i.e., below 50 cases. Some are marked “>50,” which indicates more than 50 but less than 200 cases.<sup>1</sup> Concomitant loss of LFA-1, CR3, and CR4 is due to loss of the common CD18 chain.<sup>2</sup> Please note that PNH may be caused either directly by a mutation in CD59 or by loss of PIGA function leading to a secondary loss of CD55 and CD59.

involvement of the complement system. As an example, the prevalence of C2 deficiency is fairly well known in southern Sweden because researchers at Lund University have an interest in the complement system. The clinical conditions most frequently associated with complement defects are cases of hereditary angioedema (HAE [MIM 106100]), systemic lupus erythematosus (SLE [MIM 152700]), and recurrent meningococcal infection.<sup>1,26–28</sup> But because it is a multifunctional system and due to redundancies in immune defense mechanisms, the frequency of complement deficiencies in other patient groups is likely underestimated.

Many inherited complement deficiencies have been revealed through association with invasive bacterial infections. This susceptibility appears to be restricted to a limited spectrum of bacteria, mainly encapsulated bacteria. The reason is not clear, but many bacteria (e.g., *Streptococcus pyogenes*, *Staphylococcus aureus*, *Helicobacter pylori*, and *Pseudomonas aeruginosa*) and several other microorganisms<sup>29</sup> have evolved mechanisms to counteract the activities of the complement system and are thus less likely to be associated with complement deficiency.

It is interesting that the strongest disease-susceptibility gene candidates for the development of SLE are found within members of the classical pathway of complement activation. This is speculated to be due to impaired immune complex handling and inefficient clearance of apoptotic cells in such patients.<sup>30</sup> An alternative model has been presented in which complement—together with other components of the innate immune system—participates in the “presentation” of SLE-inducing self-antigens to developing B cells. In this model, the complement system and innate immunity protect against responses to SLE (self) antigens by enhancing the elimination of self-reactive lymphocytes.<sup>31</sup> Homozygous deficiency of C1q is very strongly associated with a lupus-like disease with rash and glomerulonephritis. More than 90% of C1q-deficient individuals were reported to have such symptoms.<sup>30</sup> However, C1q deficiency appears to be very rare (Table 1), and out of all cases of SLE only a tiny fraction are associated with complement deficiency, indicating that SLE is a highly multifactorial disease.

C4 deficiency is also extremely rare, with only 26 cases having been reported. This could be due to variation in C4 gene copy number. Because two to seven copies are found in the diploid genome, multiple mutations have to occur for deficiency to result.

Deficiency of C2, which is nearly always due to a 28 bp deletion in C2 (MIM 613927, 217000), occurs at higher frequency; for example, there are probably more than 450 in a population of 9 million in Sweden.<sup>32</sup> But whereas the majority of patients with deficiencies of C1 or C4 develop SLE, the association of SLE with C2 deficiency is much weaker.<sup>30</sup> Deficiency of C2 is, on the other hand, generally associated with a slight increase in the prevalence of autoimmune disease. Furthermore, about half of the patients identified as having C2 deficiency had a history of invasive infection with encapsulated bacteria. However, family members lacking C2 but not showing clinical symptoms were often encountered. A recent review<sup>33</sup> provides a detailed picture of the C1q, C2, and C4 deficiencies described above.

With regards to the lectin pathway, MBL deficiency has a surprisingly high prevalence. About 5% of people of European descent show MBL deficiency when it is defined as having a protein level less than 100 ng/ml. The frequency is about twice this in Sub-Saharan Africans. MBL-deficient adults appear healthy, but low levels of MBL are associated with increased risk of infection in toddlers,<sup>7</sup> in cancer patients undergoing chemotherapy, and in organ-transplant patients receiving immunosuppressive drugs, especially recipients of liver transplants.<sup>34,35</sup> The deficiency is due to homozygosity for one of three quite common nonsynonymous SNPs in the coding region of *MBL2* (MIM 154545) (gene frequency in people of European descent: 0.14), as well as a promoter polymorphism (gene frequency: 0.20).<sup>36</sup> A case of MASP-2 deficiency was originally described in an immunodeficient patient with autoimmune manifestations.<sup>37</sup> The deficiency was due to homozygosity of a missense mutation giving rise to a nonfunctional protein and resulting in a very low MASP-2 level. Additional studies revealed that the allele occurs in the general population at a frequency suggesting homozygosity in approximately

6 in 10,000 individuals, but only a low clinical penetrance of MASP-2 deficiency is seen.<sup>38,39</sup> Three cases of H-ficolin deficiency have been described, two in neonates with necrotizing enterocolitis<sup>40</sup> and one in an adult with mixed symptoms of immunodeficiency.<sup>41</sup> All were due to a frameshift mutation occurring at a gene frequency of 0.011 among suspected immunodeficient patients as well as among controls.

With regards to deficiency in proteins of the alternative pathway, factor D deficiency, which is very rare (fewer than 10 cases reported<sup>42</sup>), and properdin deficiency both result in selective impairment of the alternative pathway.<sup>32</sup> In both cases meningococcal disease is the cause for investigating the complement system, and indeed this bacterium is the most prevalent infectious agent in factor-D-deficient individuals. Properdin deficiency is inherited in an X-linked manner and is found as absence of the protein or as a dysfunctional protein.<sup>43</sup> In some families with properdin deficiency, fulminant meningococcal infections, particularly meningococcal meningitis, were the most frequent problem encountered. Considering the complement system as a general antimicrobial defense system, it is surprising that susceptibility to other bacteria is not evident. Associations of factor D or P deficiency with autoimmune diseases have not been reported.

Inherited deficiency of the central component, complement factor C3, is very rare (27 cases reported), but when identified it has been found in patients with life-threatening infections with encapsulated bacteria.<sup>28</sup> C3 deficiency is also seen secondary to genetic deficiency of regulatory proteins of the alternative pathway. Lack of factor I and of factor H thus leads to uncontrolled complement activation and consumption of C3. aHUS has been associated with mutations in the genes encoding factor H and factor I leading to failure of regulation of the alternative pathway.<sup>44</sup> This disorder is characterized by hemolytic anemia, thrombocytopenia, and acute renal failure, which is probably caused by uncontrolled activation of the complement system at the renal endothelium.

Deficiencies in the terminal MAC components are typically identified in patients with recurrent invasive infections caused by *N. meningitidis* and *N. gonorrhoeae*.<sup>45</sup> However, deficiencies of proteins in the MAC may remain undetected until old age, if at all detected. Deficiencies have been observed in all of the MAC components, C5, C6, C7, and C8, at a very low frequency in European populations (Table 1). There are large differences among ethnicities, and the highest frequency is found in Japan, where 1 in 1000 individuals are C9 deficient (due to a nonsense mutation).<sup>46</sup> This high frequency may even indicate an advantage of MAC deficiency under certain conditions. There seems to be no association between MAC deficiency and autoimmune diseases.

Studies on the genes encoding regulatory complement components have revealed a number of interesting findings. HAE, resulting from C1 inhibitor (C1-INH) deficiency, is an autosomal-dominant disorder characterized

by recurrent episodes of angioedema with abdominal attacks. The incidence of HAE is approximately 1:50,000, with no ethnicity-based differences. C1-INH is a serine protease inhibitor of the serpin family, named after its inhibitory activity toward the C1 enzymes, C1r and C1s, but it also controls the MASPs of the lectin pathway. However, the angioedema is due to loss of control of kallikrein of the contact system and is not a consequence of failure of control of the classical or the lectin pathway. The attacks frequently lead to hospitalization and are associated with a substantial mortality. Two forms of hereditary angioedema are found: type I (85% of cases), characterized by low levels and low functional activity of C1-INH, and type II, characterized by normal levels of C1-INH but low functional activity. Both types are due to mutations in *SERPING1* (MIM 606860), encoding C1-INH. The genetics and therapy of C1-INH deficiency (using C1-INH purified from donor plasma) was recently reviewed by Cugno et al.<sup>47</sup>

Two of the important membrane-bound inhibitors of the complement system, decay-accelerating factor (DAF, CD55) and membrane inhibitor of reactive lysis (MIRL, CD59), are tethered to the cell surface through a glycosyl phosphatidylinositol (GPI) anchor. Rare mutations (1 in 1,000,000) in *PIGA* (MIM 311770), encoding phosphatidylinositol glycan class A, which is involved in GPI production, thus lead to loss of these regulators. This results in paroxysmal nocturnal hemoglobinuria (PNH [MIM 300818]) because the complement system autoattacks the erythrocytes lacking the control proteins. Such patients can be treated with compounds inhibiting the complement system, such as anti-C5 antibody.<sup>48</sup> Direct mutations in *CD59* (MIM 107271) can similarly result in CD59 deficiency (MIM 612300), causing PNH.

Deficiency of complement receptors may also cause pathology, as seen in the case of leukocyte adhesion deficiency (LAD) type I (MIM 116920), an autosomal-recessive disorder of leukocyte function resulting from a deficiency of the beta-2 integrin subunit (CD18, encoded by *ITGB2* [MIM 600065]) of the CD11/CD18 complex.<sup>49,50</sup> The CD11/CD18 complex is important in adhesion and phagocytosis. Three CD11 alpha chains, CD11a (integrin alpha-L, encoded by *ITGAL* [MIM 153370]), CD11b (integrin alpha-M, encoded by *ITGAM* [MIM 120980]), and CD11c (integrin alpha-X, encoded by *ITGAX* [MIM 151510]), form heterodimer transmembrane complexes with the CD18 beta chain: CD11a/CD18 (lymphocyte function-associated antigen 1, LFA-1), CD11b/CD18 (complement receptor 3, CR3), and CD11c/CD18 (CR4). Thus, CD18 deficiency results in a combined loss of expression of LFA-1, CR3, and CR4, causing defects in neutrophil, macrophage, T cell, and B cell adhesion to the vascular endothelium and inefficient migration into infectious sites, as well as impairment of normal T cell and B cell functions.<sup>51</sup> Clinically, LAD is characterized by recurrent bacterial infections and impaired pus formation and wound healing.<sup>52</sup>

We have summarized these conventional complement deficiencies in Table 1. Investigations of more and larger groups of patients are expanding our understanding of the role of complement in disease. Young patients with invasive infections caused by encapsulated bacteria should be screened for complement deficiency. As evident from recent findings in studies on gene associations with AMD, surprising angles on the clinical importance of the complement system may be found through the analysis of other patient groups. Although the association of, for example, factor H deficiency with AMD fits its role in complement-mediated reactions, genetic analyses have now revealed completely unexpected roles of complement components beyond the hitherto known functions of the complement system.

### Unexpected Roles of Proteins of the Lectin Pathway of Complement

In a recent study in this journal, Sirmaci and colleagues harnessed the power of homozygosity mapping and next-generation exome sequencing to identify a novel missense mutation in the exon encoding the serine protease domain of the protein MASP-3 (mannan-binding lectin-associated serine protease 3) in a family afflicted by the autosomal-recessive 3MC syndrome.<sup>53</sup> The 3MC syndrome is a term encompassing the overlapping Mingarelli (MIM 265050),<sup>54</sup> Malpuech (MIM 248340),<sup>55</sup> Michels (MIM 257920),<sup>56</sup> and Carnevale (also MIM 265050)<sup>57</sup> syndromes, which were independently described but appear clinically to be unified<sup>58</sup> and, as will be clear, have underlying mutations and etiology that now appear to be unified as well. Patients with 3MC syndrome (see Figure 1 in <sup>53</sup>) may exhibit a spectrum of developmental features, including developmental delay, growth and mental retardation, and characteristic facial dysmorphism, such as hypertelorism, telecanthus, blepharophimosis, blepharoptosis, and epicanthus inversus. They may also suffer from anterior-chamber eye anomalies, mixed hearing loss, cleft lip and/or palate, periumbilical defects, and skeletal anomalies with radioulnar synostosis and caudal appendage.

MASP-3, MASP-1, and MAP44 are all encoded by *MASPI*. Sirmaci and colleagues proceeded to identify a nonsense mutation in a part of *MASPI* (MIM 600521) common to all three splice variants in an unrelated family with 3MC syndrome. Thus, a novel and unexpected role was discovered of what was so far regarded “only” as a complement component. A more recent paper by Rooryck and colleagues presented an independent confirmation of this finding and also demonstrated 3MC-causing mutations in another gene, *COLEC11* (MIM 612502), encoding a protein, collectin kidney 1 (CL-K1 or collectin-11), of the same innate immune defense system, the lectin pathway of complement activation.<sup>59</sup> Their studies on zebrafish further support the developmental influence of mutations in these two genes, as morphant fish showed severe craniofacial abnormalities. Importantly, this finding also indi-

cates the presence of an evolutionarily conserved pathway of embryogenesis. In the following, we will give a more detailed description of the proteins encoded by *COLEC11* and *MASPI* and their suggested function.

### CL-K1

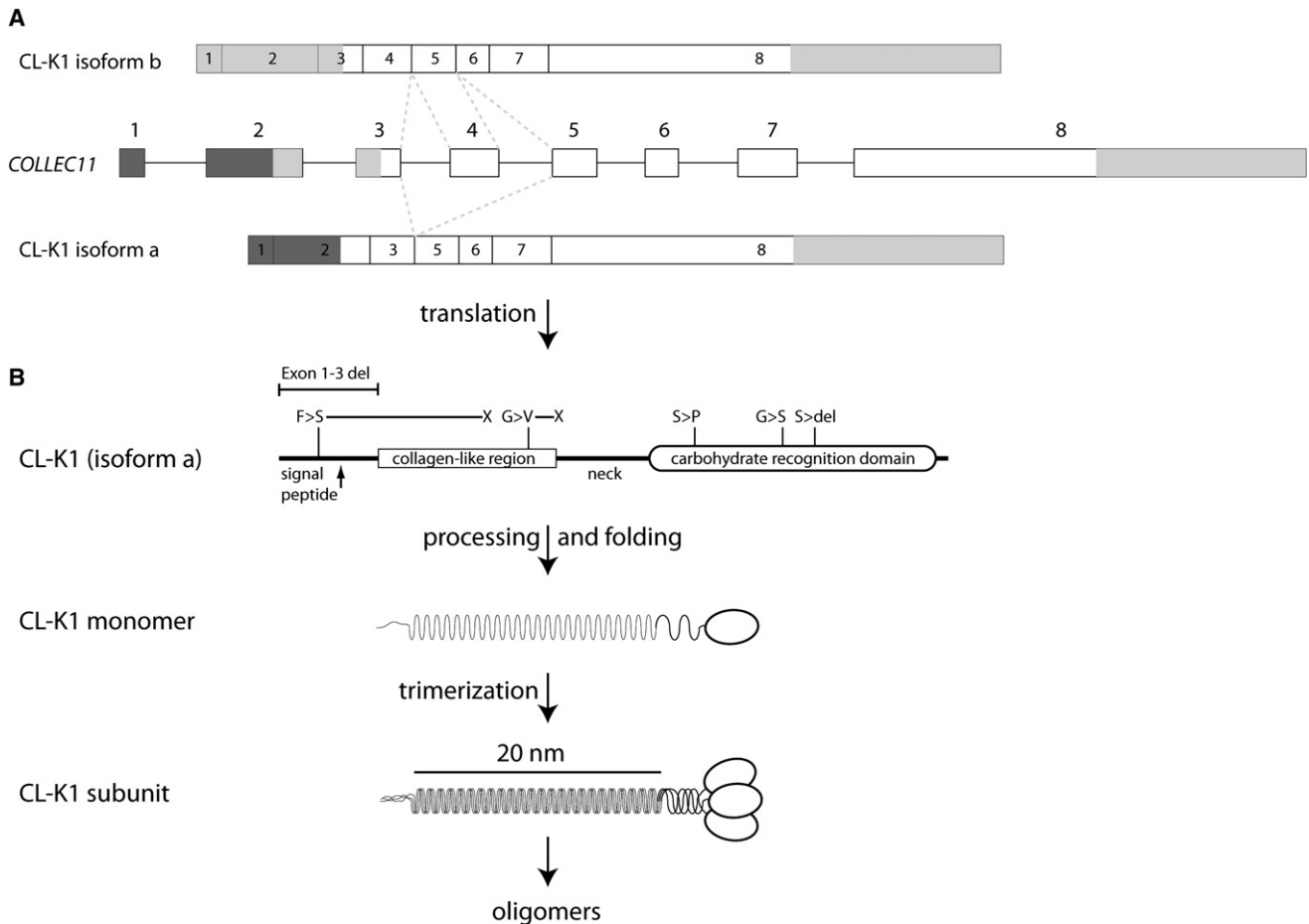
As mentioned above, CL-K1 is a recently described member of the collectin family of humoral pattern-recognition molecules.<sup>10,11</sup> So far, only two papers have been directed at describing this protein. The evidence for its function in the innate immune defense and in the complement system is only fragmentary and is largely deduced from structural similarities with MBL and ficolins (Figure 2A). These molecules are composed of subunits consisting of three identical polypeptides with a collagen region and a C-terminal carbohydrate-recognition domain (CRD). Three or more subunits are joined into oligomers forming collagen-like coiled regions. Dimers of MASPs associate with the collagen region and mediate complement activation when MBL or ficolins bind to their targets (Figure 2B). It appears that CL-K1, composed of 34 kDa polypeptides with a collagen region and a C-type CRD, must present a similar overall structure (Figure 3), and it seems also to associate with MASPs<sup>11</sup> and to bind to a number of microbial polysaccharides.<sup>10</sup> The synthesis of CL-K1 is widely distributed in the body, and, like MBL and ficolins, it is secreted and circulates in the blood.

### The CL-K1-Encoding Gene, *COLEC11*

*COLEC11*, encoding CL-K1, is located on chromosome 2, at 2p25.3. It spans about 50 kb and encodes two splice forms (Figure 3). Transcript variant 1 comprises 7 exons (1–3, 5–8) and 1320 nt. Transcript variant 2 is 8 exons (1–8) and 1399 nt long. The resulting isoforms a and b are 271 aa and 268 aa long, respectively. Isoform a is predicted by SignalP<sup>60</sup> to contain a signal peptide (aa 1–25), whereas isoform b does not harbor a signal peptide and cleavage motif. The existence of both isoforms at the mRNA level is well supported by expressed sequence tags (ESTs). At the protein level, the secreted isoform a of CL-K1 is the form described in the literature. It has 24 Gly-Xaa-Yaa repeats, starting at aa position 41, constituting a collagen-like stalk, and a C-terminal CRD (aa 151–266), thus identifying it as a member of the collectins (Figure 3).

### The MASPs

The MASPs are modular proteases composed of well-described domains in the order CUB-EGF-CUB-CCP-CCP-SP (Figure 2B and Figure 4). The CUB (C<sub>1</sub>r/C1s, embryonic sea urchin protein [Uefg], and Bone-morphogenetic protein 1 [Bmp1]) domain is around 110 aa and is found in a diverse set of proteins involved mainly in developmental processes.<sup>61</sup> The EGF (epidermal growth factor) domain of approximately 50 aa is also found in many proteins and is known to mediate protein-protein interactions. The CUB and EGF domains of the MASPs are responsible for dimerization and for the calcium-dependent



**Figure 3. Gene Structure, Alternative Splicing, and Protein Products of *COLEC11***

(A) Overview of the gene/primary transcript and the two suggested alternative splice variants. Mutually exclusive splice events are indicated by the dotted lines. Greyed areas indicate the 5' and 3' UTRs. Notice the predicted use of alternative translation initiation sites in isoforms a and b.

(B) The protein structure of the secreted isoform is illustrated and polymorphisms are indicated, along with the mutations reported by Rooryck et al. The exact locations of the mutations are: c.496T>C (p.Ser169Pro), c.610G>A (p.Gly204Ser), c.45delC (p.Phe16SerfsX85), c.648\_650delCTC (p.Ser217del), and c.300delT (p.Gly101ValfsX113), as well as the 27 kb exon 1–3 deletion. The formation of the structural subunit composed by three identical polypeptide chains and the ensuing oligomerization is illustrated.

binding to MBL and ficolins.<sup>62–64</sup> The CCP (complement control protein) domains of around 60 aa are found in a number of complement factors and other proteins.<sup>65</sup> The contiguous CCPs of MASP-1 and MASP-2, especially the second CCP domain, have been implicated in the binding of macromolecular substrates.<sup>66–71</sup> Finally, the SP (serine protease) domain is the catalytically active unit of the proteases and defines them as part of the S1A family of chymotrypsin-like proteases. The second CCP domain and the SP domain are connected by a somewhat flexible activation peptide, which lies just upstream of a site, which is cleaved upon activation of the MASP (Figure 4).

#### *MASP1*

*MASP1* is located on chromosome 3q27-28 and gives rise to three alternative splice products: MASP-1, MASP-3, and MASP-4 (Figure 4).<sup>12,13,72,73</sup> The gene encompasses 18 exons, of which the first 8 exons plus exons 10 and 11 encode the five N-terminal domains shared by MASP-1

and MASP-3, CUB-EGF-CUB-CCP-CCP. The serine protease domain of MASP-1 is encoded by six separate exons (13–18), whereas for MASP-3 it is encoded by a single exon (12).<sup>12,74,75</sup> (Figure 4). Thus MASP-1 and MASP-3 share their first five domains but have distinct serine protease domains (Figure 4). The third splice product, encoding MASP-4, is composed of nine exons: the first eight exons are shared with MASP-1 and MASP-3, and they encode the first four domains, CUB-EGF-CUB-CCP, whereas the ninth exon is unique to MASP-4 (Figure 4). An additional adenosine nucleotide from exon 8 combined with the first 50 nt of exon 9 encodes the unique 17 C-terminal aa of MASP-4.

In addition to the three expressed splice products, a fourth splice variant has been recorded in the NCBI database. However, the RNA arising from this splice pattern does not constitute mRNA, but is predicted to be degraded by the nonsense-mediated mRNA decay (NMD) pathway.<sup>76</sup> The fourth splice variant is identical to MASP-3 mRNA, save for the exclusion of exon 2, giving rise to





acid substitutions all occur in the CRD of CL-K1. These are otherwise well-conserved residues, and none of the mutations were found in 368 ethnically matched controls or in the dbSNP or 1000 Genomes databases. Indeed, no CL-K1 was found in the serum of two patients with the p.Gly204Ser mutation.<sup>59</sup>

Analysis of the localization of the protein in the mouse revealed broad tissue distribution during embryogenesis. This was also found by *in situ* expression analyses in zebrafish. The effect of loss of function of this protein during zebrafish embryogenesis was examined by the use of morpholinos directed against the initiation site and the exon 2-intron 2 splice site. Notably, these morpholinos target both isoforms. There were evident differences in the craniofacial skeleton compared with uninjected or control morpholino-injected embryos. Importantly, it was found that the phenotype of *COLEC11* morphant fish could be rescued by coinjecting embryos with the full-length human CL-K1 mRNA.

#### *MASP1 Mutations and 3MC Syndrome*

A large number of SNPs have been reported for *MASP1*, but until now, no mutations with impact on the function of either of the three protein products have been reported. An overview of the nonsilent polymorphisms from dbSNP as well as those reported by Sirmaci and colleagues and Rooryck and colleagues is given in Table 2. The fact that two completely different mutations were found by Sirmaci and colleagues in two independent families, and that three additional mutations were found independently by Rooryck and colleagues in another four families, lends credibility to the conclusion that *MASP1* dysfunctionality is causative of the phenotype. One of the mutations identified by Sirmaci and colleagues is a nonsense mutation in the common part of the three alternative splice products (Figure 4). However, it would appear that MASP-3 is the central product, as this is the only component affected in the other family as well as the four families reported by Rooryck and colleagues. The affected residue in the MASP-3-specific mutation of Sirmaci et al., G687, is located within the active site close to the junction of two  $\beta$ -barrels and in close proximity to D658 (Figure 4). On the basis of structural modeling, Sirmaci and colleagues present the elegant hypothesis that given the close proximity of D658 to G687, the G687R variant would likely result in the formation of an intermolecular salt bridge, thereby inhibiting catalytic activity.<sup>53</sup> Indeed, Asp658 in MASP-3 corresponds to Asp189 in chymotrypsin (c189, chymotrypsin numbering used in the following for reference), and this residue is responsible for primary substrate specificity in the chymotrypsin family. In MASP-1, the side chain of Arg677 (c224) is rotated toward the Asp640 (c189) and forms a salt bridge, which competes with the S1-P1 interaction upon substrate binding.<sup>78</sup> This is envisioned to modulate the activity of MASP-1. Thus, it seems plausible that the reported mutation in the MASP-3-specific exon could similarly disrupt or suppress the protease activity.

The three mutations identified by Rooryck and colleagues are all located in the MASP-3-specific exon (Figure 4). They find two homozygous missense mutations in two new 3MC families (c.1489C>T and c.1888T>C, at the protein level resulting in p.His497Tyr and p.Cys630Arg, respectively) and a third homozygous missense mutation in two previously described 3MC families (c.1997G>A, encoding p.Gly666Glu). All of these mutations are found in well-conserved positions, are predicted to be damaging (Polyphen), and are not found in 286 ethnically matched controls.<sup>59</sup>

Rooryck et al. targeted *MASP1* expression in developing zebrafish embryos by use of morpholinos specific for the initiation site and the exon 3-intron 3 splice site.<sup>59</sup> Such targeting should disrupt expression of the two splice forms (MAP44 and MASP-3), which are predicted to be found in zebrafish.<sup>13</sup> Pigment and craniofacial cartilage defects similar to those seen in CL-K1 morphants resulted.

#### *MASP-3 and CL-K1 Likely Act in the Same Pathway, Possibly as Chemoattractants*

Rooryck and colleagues stab at the mechanistic link by examining the effects of *COLEC11* and *MASP1* mutations on the migration of cranial neural crest cells during zebrafish development. It appears that they may be early guidance cues directing the migration of neural crest cells, and this is followed up by the demonstration that recombinant CL-K1 attracts neural crest cells. The effect of CL-K1 was confirmed in a quail neural tube explant assay.<sup>59</sup> Other proteins with collagen-like regions linked to a CRD domain (surfactant protein D<sup>79-81</sup> and surfactant protein A<sup>82</sup>) have previously been noted to act as chemoattractants. Whereas the possibility remains that several members of this family have dual roles in immunity and development, the observed directional cell movement may be caused by a "matrix-like" effect of the collagen helix in the proteins, or, indeed, these molecules may activate cells to secrete other chemoattractants.

Notwithstanding the zeal of the investigators, the mechanism behind the link between *MASP1* and *COLEC11* mutations and 3MC syndrome remains obscure. Sirmaci and colleagues speculate that the effect may be mediated through an activity of MASP-3 toward insulin-like growth factor-binding protein 5 (IGFBP-5).<sup>53</sup> This is based on one study demonstrating cleavage of IGFBP-5 by MASP-3.<sup>83</sup> However, such an activity has not been firmly established, but is based on the observation of a weak activity of recombinant serine protease domain of MASP-3 toward IGFBP-5. IGFBP-5-cleaving activity has also been reported for a number of other enzymes; for example, thrombin,<sup>84</sup> C1s,<sup>85</sup> and the inflammation-related neutrophil proteases cathepsin G and elastase,<sup>86</sup> all of which are serine proteases involved in or related to host defense. It is tempting to suggest a function in the release of sequestered IGF for promotion of wound healing and repair. A more cynical explanation, based on the large number of reported IGFBP-5 proteases,<sup>87</sup> is that IGFBP-5 is a quite promiscuous

**Table 2. Overview of the Nonsilent Polymorphisms in the Coding Parts of *MASP1* and the Resulting Amino Acid Substitutions**

Chr. Position	Coding Pos.	dbSNP rs #/Ref.	Heterozygosity	Contig Ref.	Missense	Amino Acid	Contig Ref.	Substitution
<b>Common</b>								
187003844	6	rs116384821	N.D.	G	T	2	Arg [R]	Ser [S]
187003788	62	rs1062049	0.005	C	T	21	Thr [T]	Ile [I]
187003786	64	rs77189011	0.022	G	A	22	Val [V]	Met [M]
186980367	379	rs115647447	N.D.	C	T	127	Arg [R]	Cys [C]
186978610	466	rs78137557	0.236	T	C	156	Tyr [Y]	His [H]
186978589	487	rs115241263	N.D.	G	A	163	Gly [G]	Ser [S]
186974493	703	rs3203210	N.D.	G	C	235	Glu [E]	Gln [Q]
186974465	731	rs28945071	N.D.	A	G	244	Tyr [Y]	Cys [C]
186971076	772	rs866085	N.D.	C	G	258	Pro [P]	Ala [A]
186970985	863	rs116001173	N.D.	G	A	288	Arg [R]	Gln [Q]
<b>186970978</b>	<b>870</b>	<b>Sirmaci et al., 2010<sup>1</sup></b>	<b>N.D.</b>	<b>G</b>	<b>A</b>	<b>290</b>	<b>Trp[W]</b>	<b>Stop[X]</b>
186970964	884	rs73886128	N.D.	G	A	295	Arg [R]	Lys [K]
<b>MAP44</b>								
186965162	1102	rs113938200	N.D.	G	A	368	Asp [D]	Asn [N]
<b>MASP-1/3</b>								
186959343	1229	rs114365879	N.D.	G	A	410	Gly [G]	Asp [D]
186959295	1277	rs28945068	0.034	G	A	426	Gly [G]	Glu [E]
<b>MASP-3</b>								
<b>186954170</b>	<b>1489</b>	<b>Rooryck et al., 2011<sup>7</sup></b>	<b>N.D.</b>	<b>C</b>	<b>T</b>	<b>497</b>	<b>His [H]</b>	<b>Tyr [Y]</b>
186954107	1552	rs73068950	N.D.	G	A	518	Val [V]	Ile [I]
186953975	1684	rs72549155	0.010	C	G	562	Pro [P]	Ala [A]
186953932	1727	rs72549154	0.058	G	T	576	Arg [R]	Met [M]
186953906	1753	rs111908734	N.D.	C	A	585	His [H]	Asn [N]
186953812	1847	rs2461280	N.D.	T	C	616	Val [V]	Ala [A]
<b>186953771</b>	<b>1888</b>	<b>Rooryck et al., 2011<sup>7</sup></b>	<b>N.D.</b>	<b>T</b>	<b>C</b>	<b>630</b>	<b>Cys [C]</b>	<b>Arg [R]</b>
186953749	1910	rs115022399	N.D.	G	T	637	Arg [R]	Leu [L]
<b>186953662</b>	<b>1997</b>	<b>Rooryck et al., 2011<sup>7</sup></b>	<b>N.D.</b>	<b>G</b>	<b>A</b>	<b>666</b>	<b>Gly [G]</b>	<b>Glu [E]</b>
<b>186953600</b>	<b>2059</b>	<b>Sirmaci et al., 2010<sup>1</sup></b>	<b>N.D.</b>	<b>G</b>	<b>A</b>	<b>687</b>	<b>Gly [G]</b>	<b>Arg [R]</b>
186953491	2168	rs115897757	N.D.	A	G	723	Glu [E]	Gly [G]
<b>MASP-1</b>								
186944222	1528	rs28945070	0.010	A	G	510	Ser [S]	Gly [G]
186943280	1573	rs28945073	N.D.	C	T	525	Arg [R]	Trp [W]
186943150	1703	rs13322090	0.041	T	C	568	Val [V]	Ala [A]
186937924	2035	rs3774266	0.006	G	A	679	Gly [G]	Arg [R]

The polymorphisms are divided into those from the common part, the MAP44-specific part, the part common for MASP-1 and MASP-3, the MASP-3-specific part, and the MASP-1-specific part (see Figure 4). The mutations reported by Sirmaci et al.<sup>1</sup> and by Rooryck et al.<sup>7</sup> are indicated in bold.

substrate. The weak activity of MASP-3 could also be explained as an evolutionary remnant on the basis of homology with the proteases known to cleave IGFBP-5. It would be worthwhile to examine whether mutated MASP-3 is produced and whether it is indeed catalytically

active or not. Unfortunately, the latter is difficult because a convincing substrate for MASP-3 remains to be identified.

Notably, there exists a mouse that was targeted in exon two of the *MASP1* ortholog (*masp1*) and is hence (presumably) deficient in MASP-1, MASP-3, and MAP44.<sup>88</sup> It has

been reported to be more susceptible to influenza virus infection, and more recently it was found that the mouse is deficient in alternative pathway function, with MASP-1 reportedly being the enzyme responsible for activation of murine factor D by cleavage of pro-factor D.<sup>89</sup> Apart from small size and low weight, no phenotypic characterization of the mouse has been presented. One would have expected a murine “3MC” phenotype to be immediately apparent.

#### *Evolutionary Aspects*

MASPs are found all the way back to cephalochordates and urochordates. MASP-2 and MASP-3 are believed to have evolved from an ancestral MASP-1 by gene duplication and divergence.<sup>72,90</sup> In this connection, it is peculiar that birds<sup>91</sup> and fish seem to have no MASP-1, whereas they have MASP-2 and MASP-3, but this could represent a loss or inactivation of the MASP-1 exon(s) in each of their ancestors subsequent to the duplication of MASP-1.<sup>92</sup> Interestingly, the chicken also lacks the related haptoglobin, which is believed to have evolved from an ancestral MASP.<sup>93</sup>

Krem and colleagues created a large phylogenetic tree of the protease domains of all the then-known members of the S1 family.<sup>94</sup> The tree was rooted with the use of marine arthropod enzymes, chosen for the primordially of the species and certain molecular markers. This phylogenetic tree suggested two origins of proteases that participate in the innate immune system. Proteases of mast cells, neutrophils, and other immune cells are most closely related to kallikrein enzymes, which probably evolved from an ancestral gene distinct from degradative, clotting, and complement lineages.<sup>95,96</sup> Complement proteases, with the exceptions of factors D and I, share ancestry with clotting proteases,<sup>97</sup> most likely having evolved from clotting-immune systems of the type found in the horseshoe crab.<sup>98,99</sup> Interestingly, complement and coagulation proteases diverge in close relation to embryonic developmental proteases. There are some functional overlaps between arthropod developmental and hemolymph proteases, and these evolutionary connections between development, clotting, and immunity extend beyond the proteases to their substrates.<sup>95</sup> In the complement system there is a clear distinction of origin between enzymes of the classical/lectin and alternative pathways. Factor D diverges early with enzymes of cell-mediated immunity and shares common ancestry with arthropod degradative enzymes. Factor I also groups with degradative enzymes, but of later origin, whereas factors B and C2 diverge together and share common ancestry with fibrinolytic proteases. Finally, the MASPs, C1r, and C1s emerge in close connection with enzymes of blood coagulation.<sup>95</sup>

#### **Extending the Scope**

Notably, the 3MC patients suffer moderate mental retardation. In recent years, evidence has been mounting of unrelated or alternative roles of complement outside immunity.

In the mouse, Stevens and colleagues reported that the classical complement cascade mediates CNS synapse elimination<sup>100</sup> and that consequently C1q knockout mice display enhanced synaptic connectivity and suffer from epilepsy.<sup>101</sup> No neural defects have been reported in the identified human C1q-deficiency cases. In contrast, the finding of elevated levels of MBL/MASP-2 complexes within the schizophrenic brain<sup>102</sup> may implicate the lectin pathway in neural functions in humans. A model describing a possible mechanism for activity-dependent synapse elimination was suggested nearly two decades ago.<sup>103</sup> According to this model, strong synapses facilitate elimination of weaker synapses by sending both a protective signal for the stronger synapse, resulting in stabilization and strengthening, and a punishment signal for weaker synapses, resulting in their elimination. It has recently been suggested that immune molecules may mediate the punishment signals.<sup>104</sup> Synapse elimination is a prominent process both during development and during neurodegenerative diseases and aging, in the former as a requirement for correct formation of synaptic networks and in the latter as a detrimental process destroying such synaptic networks. An intriguing hypothesis has been proposed: that a common mechanism may underlie this elimination of synapses during development and disease.<sup>104</sup> Indeed, a recent review has highlighted the currently known roles, some detrimental and some protective, of complement in neurodegenerative disorders.<sup>105</sup> The new results on MASP-3 and CL-K1 may spur renewed interest in studies on intellect and the immune system.

#### **Conclusion**

A number of conventional complement deficiencies with immunological consequences have been characterized. Here, we have focused on recessive complement deficiencies based on complete absence of components or functionally defective components. Partial deficiencies and polymorphisms also account for numerous immunological defects. Commonly, these conventional complement deficiencies have not been very severe since the advent of antibiotics and modern treatment modalities, and hence such genetic deficiencies are quite widespread in various populations.

In contrast, newly discovered defects of components of the lectin pathway have implications outside innate immunity, namely in development. These appear to be more severe and are caused by *de novo* mutations, which have arisen in a handful of families and have been fixed by consanguinity. A role for MASP-3 in developmental processes is supported by several lines of circumstantial evidence: (1) a general link between immunity and development, (2) a specific evolutionary relationship between complement and coagulation proteases and embryonic developmental proteases, (3) broad expression of MASP-3 in developing chick and rat embryos,<sup>91</sup> (4) enzymatic activity toward IGFBP-5 and similar broad developmental expression of this purported substrate, itself suggested to



be involved in developmental processes, (5) homology with classical pathway proteases involved in developmental processes in the mouse, (6) CUB-domain-containing proteins often being involved in developmental processes,<sup>61</sup> and (7) a very high degree of phylogenetic conservation of MASP-3. The report by Sirmaci and colleagues has provided the first solid evidence for an important function of MASP-3. This finding is confirmed and substantiated by the report of Rooryck and colleagues, and it is extended with the discovery of a similar role for CL-K1, as well as by the evidence that the two proteins collaborate in the same pathway. Future studies should elucidate the mechanism behind these fascinating observations.

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## Web Resources

The URLs for data presented herein are as follows:

1000 Genomes, <http://www.1000genomes.org/>  
 dbSNP, <http://www.ncbi.nlm.nih.gov/projects/SNP/>  
 Online Mendelian Inheritance in Man (OMIM), <http://www.omim.org>  
 PolyPhen-2, <http://genetics.bwh.harvard.edu/pph2/>  
 SignalP, <http://www.cbs.dtu.dk/services/SignalP/>

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