**Complement as a Diagnostic tool**

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**Abbreviations used:** aHUS: atypical Haemolytic Uraemic Syndrome; AMD: Age-related Macular Degeneration; C3G: C3 Glomerulopathy; HAE: Hereditary Angioedema; MPGN-II: Membranoproliferative Glomerulonephritis Type II; PNH: Paroxismal Nocturnal Hemoglobinuria; RCA: Regulators of Complement Activation gene cluster; SLE: Systemic Lupus Erythemathosus; 3MC: Malpuech-Michels-Mingarelli-Carnevale syndrome.

**Highlights**

Complement system simultaneously senses, discriminates and either attacks or protects cells according to a tightly, sequentially regulated activation process. Complement deficiency or dysfunction may result in infectious disease due to defective elimination of pathogens, but also in excessive inflammation and autologous damage when regulation is defective or surpassed. Complement measurement is informative for the diagnosis of autoimmune, infectious, renal and haemostatic diseases and a wide display of techniques have been developed throughout the years for analyzing complement activation and regulation status. Here, we review the main complement-mediated diseases, standard laboratory techniques and most widely established analytical parameters for up-to-date complement monitoring.

**Abstract**

The complement system is a complex and autoregulated multistep cascade at the interface of innate and adaptive immunity. It is activated by immune complexes or apoptotic cells (classical pathway), pathogen-associated glycoproteins (lectin pathway) or a variety of molecular and cellular surfaces (alternative pathway). Upon activation, complement triggers the generation of proteolytic fragments that allow the elimination of the activating surface by enhancing inflammation, opsonization, phagocytosis, and cellular lysis. Moreover, complement efficiently discriminates self from non-self surfaces by means of soluble and membrane-bound complement regulators which are critical for innate self-tolerance. Complement deficiency or dysfunction disturb complement homeostasis and give rise to diseases as diverse as bacterial infections, autoimmunity, or renal and neurological disorders. Research on complement-targeted therapies is an expanding field that has already improved the prognosis of severe diseases such as atypical Haemolytic Uremic syndrome or Paroxysmal Nocturnal Haemoglobinuria. Therefore, complement analysis and monitoring provides valuable information with deep implications for diagnosis and therapy. In addition to its important role as an extracellular defense system, it has now become evident that complement is also present intracellularly, and its activation has profound implications for leukocyte survival and function. In this review, we summarize the essential, up-to-date information on the use of complement as a diagnostic and therapeutic tool in the clinics.

## 1. Introduction

Complement is an autoregulated complex system that it is normally in stand-by. Discovered in 1894 by Jules Bordet by mixing a normal serum and a heat-inactivated immune serum, complement was named in 1901 by Paul Erlich as a serum activity that *complemented* antibody bactericidal activity. Complement system, however, is far more ancient than antibodies, as it is already present in invertebrates. As a part of the innate immune system, complement destroys pathogens and avoids autologous damage by removing immunocomplexes and eliminating apoptotic and tumoral cells (*1*). It also contributes to adaptive immunity by enhancing antibody generation, immunological memory, and T-cell responses *(2)*. In addition, ongoing research anticipates an unsuspected and important role for intracellular complement (i.e. the “complosome”) in T-cell homeostasis and induction of Th1 responses (*3*).

Complement is integrated by many plasma and membrane proteins that are constitutively synthesized by the liver and other tissues, and that function as activators or as regulators. Complement regulators keep under control the low-intensity, spontaneous complement activation that takes place in plasma, thus preventing

complement damage to autologous cells and tissues. Complement activators take part in the proteolytic cascade initiated when this low-grade complement activity is overcome by pathogens, damaged autologous cells or specific molecular complexes. Depending on the nature of the activating surface, complement activators initiate the classical (CP), lectin (LP) or alternative (AP) pathways. Although the process of complement activation is initiated in plasma, proper complement activity requires the interaction of several complement activation fragments with specific receptors on cells of the innate and adaptive immune system (*4*).

The crucial biochemical event in complement activation is the proteolytic cleavage of the central component C3 into two fragments, C3a and C3b, by the action of enzymatic complexes accordingly know as "C3 convertases". This initial step provokes deposition of clusters of C3b on the activating surface (i.e. a pathogen or a damaged cell), a process know as opsonization. Opsonization enhances phagocytosis of the target surface by polymorphonuclear leukocytes, and decreases the amount of antigen necessary to trigger antibody generation by B lymphocytes. The C3b molecules also take part in the second step of the proteolytic complement cascade, allowing generation of the "C5 convertases", which cleave the C5 molecule into C5a and C5b. C5a is a potent anaphylatoxin (also C3a, but in a lower grade) that recruits inflammatory immune cells (i.e. monocytes/macrophages and neutrophils) to the sites of complement activation and further help destroy the pathogen or damaged cell. The C5b molecules initiate a final series of molecular interactions (referred to as the complement Terminal Pathway, TP) that give rise to the Terminal Complement Complex (TCC)/Membrane Attack Complex (MAC) on the membrane/cellular wall of the activating surface, and provokes its destruction by osmotic lysis. Once activated, the complement cascade is constitutively controlled by the spontaneous inactivation of the C3 and C5 convertases, and by the critical activity of the complement regulators (*5*) which act at all steps of the complement cascade to avoid consumption (i.e. hypocomplementemia), exacerbated inflammation, and autologous damage (*Figure 1*).

While the undesirable effects of deficient complement activation are very intuitive, it is important to underline that an excessive or unspecific activation also has pathological relevance. Thus, primary deficiencies in complement activators generate bacterial infections and autoimmune diseases, while deficiencies in complement regulators give rise to haematological, haemostatic, renal or ocular damage. A prompt screening of complement defects in these patients is critical to implement appropriate complement-targeted therapies currently available. In this review, we summarize how this screening is performed in the clinical setting, within an immunological unit.

## 2. Main complement-mediated diseases

### *2.1. Infectious diseases*

Complement analysis is used in certain clinical settings for monitoring the course of infection and the serum levels of some components correlate with disease severity or mortality. The critical role of complement in the battling against pathogens is best demonstrated in patients who are deficient for a complement component. Complement deficiencies can be inherited or acquired. Inherited deficiencies of complement are rare and occur in about 0.03% of the general population. Acquired deficiencies are more common and can result from impaired synthesis, increased consumption or excretion of complement components.

Homozygous deficiency of any complement component implies an increased risk of infection but, as inheritance of most complement genes is co-dominant (with the exceptions of X-linked properdin and dominant C1-Inhibitor deficiencies),

heterozygous complement deficiency usually do not confers an increased risk of infectious diseases. Inherited deficiencies of CP components are associated with autoimmune diseases, deficiencies in the LP predispose to autoimmunity or infections by a broad range of pathogens, and deficiencies in the AP and TP are strongly linked with increased incidence of invasive infections by encapsulated bacteria (*Table 1*). Up to 20% of patients with disseminated *Neisserial* infections have complement deficiencies (*6*).

CP deficiencies are regarded as the major genetic cause for autoimmune diseases, especially systemic lupus erythematosus (SLE), although they also predispose to recurrent, disseminating respiratory infections. Typical pathogens are encapsulated bacteria such as *S. pneumoniae, H. influenzae*, and *N. meningitides*, a similar profile to that seen in hypo- or dysgammaglobulinemias. Complete deficiencies of **C1** (MIM**%**216950, MIM*120550, MIM*120570, MIM*120575) and **C4** (MIM*120810, MIM*120820) are extremely rare, with about 20 cases of C1r/C1s, 40 cases of C1q and 30 cases of C4 deficiencies reported (*7*). Homozygous *C2* deficiency (MIM# 217000) is more common, with an estimated prevalence of 1:10.000 in Caucasians. About 30% of C2-deficient patients develop autoimmunity, and predisposition to infection is lower than in C1 and C4 deficiencies (*6*).

Due to its central role in complement function, inherited homozygous C3 deficiency is uncommon, with no more than 25 cases described to date. Strong predisposition to infection in C3-deficient patients arises from reduced opsonisation capability and secondary impairment in antibody response and dendritic cell maturation (*8*). Pathogens frequently reported in C3-deficient patients include encapsulated bacteria, but *E.coli, S. aureus and S. pyogenes* are not rare (*9*).

Deficiencies of the **AP** regulators Factor H (**FH**) and Factor I (**FI)** present with very low or undetectable C3 and Factor B (FB) levels due to unrestrained C3 convertase formation. FH deficiency (MIM*134370) is mainly associated to type II Membranoproliferative Glomerulonephritis (MPGN II), although half of the patients also have infectious complications. Patients with FI deficiency (MIM#610984) present a similar complement profile that in FH deficiencies, but with greater incidence and a broader spectrum of infections (otitis media, bronchitis, sinusitis, tonsillitis, cutaneous abscesses and vasculitis, reviewed in reference #*9*). **FB** homozygous deficiency (MIM#615561) is extremely rare, and was considered lethal until the recent publication of a complete FB deficient case. The patient presented recurrent bacterial infections from early childhood (pneumococcal peritonitis, meningitis by *Neisseria* and pneumococcal pneumonia) and was compound heterozygote for two loss-of-function FB mutations (*10*). **Properdin** is a trigger and stabilizer factor of AP C3 convertase. Its deficiency is inherited as a recessive X-linked trait. Reduced properdin activity implies an impaired capacity for C3 convertase assembly or stabilization and a consequent reduction in C3 cleavage. Meningitis by *Neisseria*, but also otitis media and pneumonia, are frequent findings in these patients. Complete deficiency of Factor D (**FD)** (MIM#613912), although very uncommon, has been described in humans. As FD cleaves and activates FB, lack of a functional FD leads to reduced AP activation, similarly to observed in properdin deficiency. Patients with FD deficiency suffer from meningitis and gonococcal infections, and their serum lacks bactericidal activity (*11*).

Deficiencies affecting **TP** components (**C5, C6, C7, C8** and **C9**) associate with invasive meningococcal disease, commonly by *Neisseria* species. Except for C9 deficiency, which is relatively common in the Japanese population (1:1000), TP deficiencies are rare. Impaired bacterial lysis in this context can be effectively managed

if good antibody-responses are achieved after vaccination, presumably by increased opsonization (*12*).

Deficiency of any component from the **LP** may be irrelevant in healthy adults due to immune system redundancy. However, LP deficiencies increase susceptibility to secondary infections and autoimmunity in infants and immunocompromised patients (*13*). For example, MBL deficiency increases the risk of invasive infection in C2-deficient patients. **MBL** deficiency (MIM#614372) occurs at high frequency and it is largely explained by three single-point mutations in exon 1 of the MBL gene (*14*). In infants, it is particularly associated with acute respiratory tract infections by extracellular pathogens. MBL is also an important player during intracellular parasitisation, especially by *Leishmania*. Such parasites are known to exploit C3 opsonization and C3 receptors to enter the host cells, so that low MBL levels would reduce C3 activation and opsonization, lowering the chance for parasitisation. Levels of MBL-Associated Serin Proteases (MASPs) and ficolins have been related to a number of viral, bacterial and protozoan infections, and have an impact on development (*15*). **MASP-1** deficiency causes Malpuech-Michels-Mingarelli-Carnevale syndrome (3MC syndrome), a rare disease characterised by mental and growth retardation presenting with several developmental abnormalities. **MASP-2** deficiency (levels below 100ng/ml), occurs in about 4% of Caucasians and up to 18% of some African populations. Some MASP-2-deficient individuals have increased risk of infection or autoimmune disease, but most are asymptomatic. Recently, the first **ficolin-3** deficiency was described in a man who suffered from respiratory infections since childhood, and several episodes of pneumonia and cerebral abscesses during adult life.

### *2.2. Autoimmune diseases*

The complement system is involved in the pathogenesis and clinical manifestations of several autoimmune diseases, such as SLE and rheumatoid arthritis (RA). On one hand, complement is the final effector of tissue injury in autoimmune diseases and on the other, deficiencies of some complement components leads to autoimmunity. For example, 93% of C1r and C1s deficient patients, 75% of C4- and 57% of C1q-deficient patients have SLE. In contrast, only 10% of individuals with C2 deficiency develop SLE (*16*). As a consequence of a defective CP function, apoptotic debris and immune complexes are inefficiently removed in SLE patients, triggering an autoimmune phenomenon, and persistent inflammation and tissue damage. Autoantibodies against C1q, observed in a third of patients with SLE, could result in decreased C1q levels, mimicking a C1q-deficient state with impaired clearance of dying cells (*17*). In patients with active SLE, low levels and systemic complement activation are considered conventional biomarkers for disease activity. Moreover, immunofluorescence studies in SLE patients have shown deposits of immunoglobulins and complement (C3, C4 and his activation fragments) in renal glomeruli and spleen, heart, skin, and liver vessels, suggesting that local complement activation contributes to tissue damage (*18*). In contrast to SLE, complement activation in RA is not fully understood, but evidence of activation in the synovial fluid is abundant. Levels of complement proteins are generally depressed in the synovium of RA patients, reflecting complement consumption. Moreover, elevated levels of cleavage products such as C3a, C3c, C5a, sC5b-9, Bb, and C1-C1INH complexes (*19*), have been observed in the synovial fluid. Interestingly, patients with C1q, C2 or C4B deficiency are prone to develop RA (*18*), but further studies are needed to confirm whether it is a mere association or a casual entity.

### *2.3. Coagulation or haematological disorders*

#### *2.3.1 Hereditary or acquired angioedema*

Angioedema due to inherited (HAE) or acquired (AAE) deficiency of C1-Inhibitor (C1INH) is a rare condition characterized by deregulation of the classical pathway of complement and the contact system, triggering spontaneous episodes of edema in the subcutaneous and submucosal layers (*20*). C1INH is a protease inhibitor belonging to the serpin superfamily. It regulates the activation of complement, contact, coagulation and fibrinolytic cascades by irreversibly inhibiting C1r, C1s, C4, C2 and Manose-binding lectin (MBL) (complement system), kallikrein and FXII (contact system), FXI (coagulation), plasmin and Tissue Plasminogen Activator (TPA) (fibrinolysis). In the context of C1INH deficiency, edema in HAE or AAE is due to bradykinin production by the unrestrained contact system and both diseases are consequently considered bradicinergic forms of edema (*21*). Excessive bradykinin production during edematous attacks activates endothelial cells and triggers nitric oxide production, release of endothelial cell markers E-selectin, endothelin 1 and von Willebrand Factor antigen and breakdown of vessel wall integrity (*22*). Thus, HAE and AAE are not primarily complement-driven but haemostatic pathologies, although complement levels are used as gold-standard disease markers.

HAE can present in three subtypes: type I (quantitative serum deficiency of C1INH), type II (with normal levels of a dysfunctional C1INH) and type III (with normal levels and function of C1INH). Types I and II are caused by heterozygous mutations in the *SERPING1* locus encoding C1INH. Type III, which is not due to loss of C1INH function, is a heterogeneous group in which angioedema is estrogen-dependent. It affects predominantly women in high estrogen situations and approximately 50% of patients bear autosomal dominant gain-of-function mutations in the *F12* locus encoding coagulation FXII (*20*).

Biochemically, HAE types I and II are characterized by low C4 levels and C1INH function in serum, furthermore, type I presents distinctively reduced C1INH levels. HAE type III, instead, has no altered complement-profile, with normal C1INH and C4 levels and function.

In AAE, deficiency of C1INH is caused by the presence of monoclonal or oligoclonal anti-C1INH autoantibodies, it is frequently associated to underlying haematological diseases with B cell proliferation and accompanied by autoimmune manifestations (*23*). Accordingly, AAE patients lack family history and the disease is usually of late onset (above 40y.o.). Complement profile in AAE is identical to that seen in HAE types I and II but it usually also features undetectable C1q levels in serum due to over activation of the uninhibited classical complement pathway eventually causing C1q exhaustion.

#### *2.3.2 Paroxismal Noctornal Haemoglobinuria*

PNH is a clonal hematopoietic stem cell disorder that presents with a severe haemolytic anemia resulting from erythrocyte destruction by autologous complement. In PNH, somatic deficiency of the PhosphatidylInositol Glycan A (PIGA) enzyme avoids attachment of the complement regulators DAF/CD55 and CD59 on the erythrocyte membrane through a GPI anchor, provoking an acquired deficiency that render erythrocytes more susceptible to complement-mediated lysis (*24*). The use of Eculizumab, a humanized monoclonal antibody against C5 that inhibits the complement

terminal pathway, prevents complications and improves quality of life and survival in many PNH patients. Monitoring complement activity (CH50 assay; see section 3.2) and free Eculizumab levels could help patient management (*25*). CD55 and CD59 deficiency can be screened by peripheral blood flow cytometry.

### *2.3 Renal and ocular pathologies: aHUS, C3G, IgAN, AMD*

Dysregulation of the complement AP as a consequence of mutations or autoantibodies against specific complement proteins perturbs the activation-regulation equilibrium intrinsic to complement homeostasis, allowing activation of the terminal pathway on different cells and tissues, including the kidney and the eye. Genetic and biochemical studies in different patient cohorts have unveiled the critical role of factor H (FH), the main AP regulator, for efficient protection of autologous cells from complement attack, and the still controversial effects of their homologous factor H-related proteins (FHRs) *(26)*.

The main renal pathologies associated with defects in complement AP are atypical Haemolytic Uraemic Syndrome (aHUS), C3 Glomerulopathies (C3G) and IgA Nephropathy (IgAN). aHUS and C3G are rare diseases that present in children or in adults, while IgAN is the most frequent adult glomerulonephritis. Many C3G and IgAN patients evolve to chronic kidney disease. The prognosis of aHUS, on the contrary, is much worse, but it has drastically improved since the approval of Eculizumab for this disease in 2010.

aHUS is characterized by the clinical triad of Coombs negative-haemolytic anemia, thrombocytopenia and acute kidney damage, and by kidney lesions of Thrombotic Microangiopathy. The pathogenic cascade starts with endothelial damage in the renal microvasculature, which can be idiopathic or secondary to pregnancy/postpartum, infections, autoimmune disorders, or certain pharmacological treatments (e.g. immunosuppressants, antitumorals, and oral contraceptives) *(27)*. The initial endothelial damage is further enhanced by abnormal function of the complement AP, which in about 50% of aHUS patients results from mutations in complement regulators (FH, FI, MCP/CD46), or in complement activators (C3, FB); anti-FH autoantibodies develop in about 10% of pediatric aHUS patients, and they are generally associated with homozygous deficiency of FHR-3 and FHR-1, which are coded in adjacent genes *(28)*. It is important to keep in mind that in aHUS there is a deficient control of complement activation on the surface of autologous cells and tissues (particularly on the kidney microvasculature), but not in plasma; for this reason, the standard assays of complement function in plasma are generally normal, and specific tests to detect this kind of alterations have to be performed (see section 3). In C3G patients, on the contrary, complement defects give rise to a deficient regulation in plasma, therefore generating huge amounts of C3b and its breakdown products (iC3b, C3dg and C3c), which deposit on the glomeruli and are detected by immunofluorescence microscopy; ultrastructural changes in the glomerular basement membrane are also observed by electron microscopy. Mutations in FH and anti-FH autoantibodies are found in some C3G patients, but they did not bind the same functional domains than in aHUS patients (*29*). Other C3G patients present mutations that generate abnormal FHR proteins (*30*) or C3 mutations causing deficiency. Screening of complement defects in aHUS and C3G include mutations causing protein deficiency or dysfunction, and autoantibodies against FH or the C3 convertase *(31)*.

A renal biopsy showing mesangial cell proliferation and hypoglycosylated IgA1 deposits is diagnostic in IgAN. Clinically, IgAN is very heterogeneous, with full recovery of renal function in some patients, and others evolving to chronic kidney

disease in short to long term (*32*). The role of complement defects in IgAN is far from been clear, but it is likely that they help explain the heterogeneous clinical evolution (*33*).

As opposite to aHUS, homozygous deficiency of FHR-3 and FHR-1 is protective in IgAN and also in Age-related Macular Degeneration (AMD or ARMD), the main cause of blindness in elderly people. Nonetheless, the identification of AMD-associated genetic variants in FH, FHR-1, C3 and FB has provided important insights into disease pathogenesis (*34*). Thus, it is believed that a deficient control of complement activation within the retina enhances the inflammatory response and contributes to the accumulation of cellular debris (drusen) in the macula, and the subsequent loss of central vision characteristic of AMD. A short FH isoform named FHL-1 has emerged as the most relevant regulator of complement activation in the Bruch´s membrane, a major site of AMD pathogenesis (*35*). There are not effective treatments for AMD, but early genetic screening of AMD-risk variants could help acquire prevention habits such as physical exercise or avoid smoking.

## 3. Screening complement defects in the Clinics

Workflow in a complement reference laboratory usually includes three major steps: obtainment of the biological samples and a brief clinical history (i), determination of C3 and C4 levels and quantification of complement function (ii) and immunochemical, functional and genetic screening of individual complement components. The precise order of such steps may vary among laboratories, but C3, C4 and whole complement functional levels are generally of key importance to guide further studies and, as C3 and C4 concentrations are frequently obtained by rapid, high throughput methods (see below), in most cases these are determined first and foremost (*see diagnostic algorithm*). Analytical methods for the complement system can be divided up into (a) assays for the quantification of individual components and its activation fragments and (b) functional tests to evaluate complement activity.

### *3.1 Quantification of individual components and activation fragments*

The concentration of individual protein levels of complement components are usually measured by immunochemical methods, which include immunoprecipitation techniques (double immunodiffusion, radial immunodiffusion, nephelometry or turbidimetry), western blot and Enzyme-Linked Immuno Sorbent Assay (ELISA). These approaches are preferred in most diagnostic and research laboratories due to easy interpretation of the results, straightforward standardization and because they are relatively cheap. Immunoprecipitation techniques usually employ polyclonal antibodies directed against specific complement fragments for measurement of total protein concentration. Working in that manner provides robustness when dealing with complement activation in biological samples (due, for example to inappropriate handling), as polyclonal antibodies against final activation products (for example C4c) will equally recognize its target fragment and also the intact, not-fragmented (i.e. C4) complement component (*36*). Conversely, monoclonal antibodies directed against specific complement fragments have been generated by exploiting the generation of neoepitopes in activation fragments or in non cleaved complement components when these are incorporated into large multiprotein complexes, such as the C3bBbP or the sC5b-9 complexes (*37*).

The most widely used immunochemical methods for the screening of complement defects in the clinical practice are:

**Double immunodiffusion (Outcherlony method)** is a manual method which requires minimal equipment in which wells are manually punched in a properly buffered gel. Antigens and antibodies are placed in separate wells and diffuse towards each other until precipitation occurs at the point of antigen-antibody equivalence. It's a very specific

assay although its sensitivity could be low and long reaction times are necessary (more than 24 hours)

**Radial immunodiffusion (Mancini method)** is another gel method requiring also very few equipment. Similarly to Outcherlony method, it uses a polyclonal antiserum known to precipitate the protein of interest which is added to molten agarose medium. The biological fluid to be tested is pipetted to a punched well and diffuses radially into the agarose, thus forming a ring of antigen-antibody complexes, whose radium is proportional to the initial concentration of antigen and is compared to a standard curve of known antigen concentrations. Although RID is a simple and specific technique, it also has long reaction times (between 24 and 48 hours) which made it not convenient for most clinical routine but it could be available in small laboratories with a few number of samples to be processed.

**Nephelometry and turbidimetry** are based on the scattering of light when it passes through a suspension of particles (e.g. antigen-antibody complexes in a biological fluid). For this, an antibody excess against the protein of interest is added to the sample and the scatter of light is then measured by collecting the dispersed (nephelometry) or transmitted (turbidimetry) light. These techniques have replaced most of the radial immunodifussion applications due to its higher sensitivity, speed and automation. However, the costs of equipment and reagents needed made them more suitable for large number of samples and large volume sample applications.

**ELISA** is nowadays the most widely used immunoprecipitation method in complement analysis because it is more objective and has the capability of easily being high throughput especially when automated. In ELISA techniques, samples containing an unknown concentration of antigen are immobilized on a plate and detected with specific antibodies which may be linked to an enzyme or, alternatively, are used as an antigen for a secondary, enzyme-linked, detection antibody. The reaction is finally developed to produce a coloured product that can be measured by spectrophotometry. To date, the applications of ELISA techniques in complement diagnosis are wide and diverse, ranging from measurement of individual complement components to quantification of clinically relevant autoantibodies (C3NeF and anti-FH in C3G or HUS patients or anti-ADAMTS13 in TTP **(***38,39,40*) to cite just a few examples) to functional ELISA assays.

**Western blot** is another immunochemical method routinely used in some complement laboratories to assess the presence of complement proteins and their activation fragments. It is based on the electrophoretic separation of a protein mixture (e.g. serum, plasma or cell lysates) on a polyacrylamide gel in the presence of SDS. This is followed by transference of the separated proteins to a membrane (typically nitrocellulose or PVDF) were they are blocked and detected with specific antibodies. However, the laboriousness of the technique and the difficulties in automating some steps of the process, make western blot not suitable for protein quantification in the clinical practice. Instead, it is usually employed for qualitative screening of the presence or absence of the antigen of interest, or as a semi-quantitative approximation to its levels. Moreover, as it allows simultaneous visualization of individual proteins, protein complexes and its activation fragments, it is of particular interest for specific complement studies (e.g. FH and FHRs or C3 and its proteolytic species).

*In vitro* measurement of specific complement fragments is used to infer *in vivo* complement activation states. Activation fragments have very different half lives *in vivo* due to receptor binding and clearance from circulation. For example, C3a has a half life of approximately 0.5 hr (*41*) while in the case of C3d,g, it is estimated in 4hr (*42*). Moreover, repeated freeze-thaw cycles or bad sample handling can induce *in vitro* complement activation and artifactually generate detectable amounts of fragments. Because of this, activation end-products like C5b-9 are preferred as disease markers, as they are stable and not easily generated *in vitro* while those produced in the first proteolytic steps (e.g. C3a) are more suitable for *in vitro* induced activation experiments (*43*). In order to minimize

undesired complement activation, quantification of activation fragments is usually performed in EDTA plasma due to its $Mg^{2+}$ and $Ca^{2+}$ chelating properties.

On the other hand, the concentration of a given activation fragment is usually few informative by itself and an accurate assessment of the activation of a single component requires the simultaneous determination of both its native and activated species, which is usually expressed as a ratio. For example, several indicators of disease activity in SLE patients correlate with the C3/C3d ratio in serum (*44*).

Detailed protocols for the most widely used methods can be found in specific reviews (for example, *ref.#45*)

### *3.2 Quantification of complement function*

Diagnosis of a complement defect should also determine the total activity of the classical and alternative pathway either by hemolytic or liposome-based assays or by functional ELISA. Hemolytic assays are the most widely used functional assays for the complement system. The gold standard haemolytic assays are those quantifying the classical and alternative pathway hemolytic activities (termed CH50 and AP50, respectively). Although many variant protocols have been developed throughout the years, the principles of these assays are those originally proposed by Mayer (*46*).

**- Whole complement function**

CH50 exploits the ability of serum to lyse hemolysin-sensitized sheep erythrocytes (*47*) while AP50 is based on the capacity of the alternative pathway regulator FH of discriminating between self and non self cellular surfaces on the basis of their polyanionic composition. Specific self/non-self recognition is achieved by CFH binding only to those cellular surfaces presenting certain sialic acid moieties and thus protecting them from complement attack. Rabbit, guinea pig or chicken erythrocytes lack such polyanionic surfaces and are therefore used in the different AP50 protocols. In AP50, pathway specificity (due for example to the presence of anti-species antibodies in the proband's serum) is ensured by inhibition of the classical and lectin pathways with calcium-chelating EGTA.

Hemolytic assays provide an overall measure of the integrity of the classical and alternative pathways and are expressed as the reciprocal of the dilution at which 50% of the erythrocytes are lysed. A missing or reduced haemolytic activity can indicate primary deficiency (or secondary consumption) of a complement component and guides the specialized physician to further, component-specific investigations.

Liposome chemistry is an alternative to the haemolytic assays. These functional assays are based on the ability of complement to lyse liposomes which carry antibody-antigen complexes on their surface as complement target and encapsulate a reporter enzyme which is released and detected upon lysis (*48*).

In recent years, a functional ELISA assay has been developed which simultaneously analyzes the classical, alternative and lectin-triggered pathways (*49*). This procedure relies on the coating of ELISA plates with a combination of activating molecules for each pathway while specifically inhibiting the remaining two cascades.

**- Component specific assays**

Typically, suspicion of a specific complement component deficiency in a sample with low or undetectable whole complement haemolytic activity is verified by component-specific haemolytic assays in which addition of such complement component to the studied sample recovers normal haemolytic activity and demonstrates specificity.

Several functional tests for the evaluation of complement regulatory components are also available. For example, the capacity of CFH to regulate complement activation on cellular surfaces can be specifically tested with a haemolytic assay in which sheep

erythrocytes, not spontaneously triggering alternative-pathway activation in healthy sera, are used as target in the presence of an EGTA-containing buffer (*50*). This assay, useful for demonstrating the involvement of CFH in a given pathology or to confirm the pathologic nature of an identified CFH sequence variant is nevertheless accessible only for research and specialized complement diagnostic laboratories, as no commercial version is still available. Conversely, chromogenic and complex assays for the measurement of C1-Inhibitor (C1INH) function are commercially accessible. The chromogenic assay measures the inhibition by C1INH of substrate cleavage by C1s, a major target protease of C1INH, and inhibited merely by C1INH. Complex assays infer C1INH function by quantifying the formation of complexes between C1INH and its physiological target proteases C1s, C1r (*51*) or kallikrein (*52*).

### *3.3 Complement Genetics*

As a final step in complement diagnosis, once a quantitative or functional impairment has been already confirmed by other methods and its hereditary nature has been demonstrated or is strongly suspected, characterization of the underlying genetic cause may be determined. Although not available to all laboratories, genetic screening is a powerful diagnostic tool, especially for familial studies of large cohorts, newborns (in whom reference levels may considerably differ from those of adults) or relatives prior to their disease debut in order to provide genetic counselling. The whole process can be straightforward done by conventional polymerase chain reaction followed by Sanger sequencing of the amplified DNA products by standard procedures. Nowadays, disease- or pathway-oriented exome sequencing panels are also commercially available or can be easily designed and obtained. Although still more oriented to research tasks, these panels allow for the rapid identification of genetic variants with clinical significance in cases with very complex or ambiguous presentation.

## 4. Monitorization of complement parameters

Complement analysis is essential for a definitive clinical diagnosis in some entities and for disease follow-up. C3 and C4 levels are widely used for monitoring systemic rheumatic diseases, particularly LES with renal impairment. The recent surge of complement-targeted therapies and their inclusion in clinical trials and practice demands exhaustive complement monitoring to evaluate disease progression and drug effectiveness. A clear example is the use of Eculizumab for aHUS patients.

Eculizumab has changed prognosis of aHUS patients, however an individualized treatment schedule has not yet been stabilised. EMA and FDA guidelines recommend a long-life therapy with standard doses every two weeks in the maintenance phase. These recommendations have been questioned due to the incremented risk of meningococcal infection, possible long-term complications and the extremely high cost of the treatment (*53,54*)

Final drug concentration is modulated by individual pharmacokinetics of Eculizumab, disease condition and individual C5 concentration. Achievement of the dose required for a full blockade of C5 is the goal for individualized treatment, avoiding excessive dosages (*55,56*). Several studies on Eculizumab serum concentration have demonstrated that drug levels in patients receiving treatment every two weeks exceeds the recommended dose for complement inhibition (*55*).

The international consensus of aHUS management recommends assessment of the drug´s activity on the basis of complement inhibition rather than disease activity parameters (*57,58*), but there is yet not consensus on the best analysis options to follow these patients.

Different assays have been proposed for complement monitoring under Eculizumab treatment. These assays should be easy to perform and standardize in clinical laboratories and should have enough sensitivity to detect minimal changes in complement activity.

Total complement blockade can be assessed by haemolytic assays, CH50 or AP50, or ELISA-based assays for complement activity. Eculizumab levels can be easily measured by ELISA. Recommended circulating levels are 50-100 µg/ml and this concentration is sufficient to maintain complement inhibited under normal conditions (*58,59*).

As sC5b-9 plasma levels do not change in aHUS patients during Eculizumab treatment (*53,59*), measuring maximum TCC capacity might be a good parameter to determine complement inhibition in patients experiencing complement activating situations like infections (*53*).

Another proposed assay to follow-up aHUS patients under Eculizumab treatment is determination of drug-C5 complexes. Complexes level inversely correlates with complement inhibition, and they increase with addition of purified C5 or NHS, confirming that Eculizumab is in excess in the majority of patients under standard treatment schedule (*54*).

Checking complement blockade on long-term maintenance therapy is not necessary once it is achieved unless ongoing known complement activation situations, such as infection, immunization, surgery, pregnancy or changes of previously normalized clinical parameters (haptoglobin, LDH, platelets counts, proteinuria….) (*58*). Conversely, if an individualized therapy is intended (by adjusting the dose or administration intervals), complement blockade should be closely monitored (*54,57*). Together with complement activity, Eculizumab levels, TCC capacity or Eculizumab-C5 complexes can be used to adjust administration intervals or dosage in the absence of active disease.

To date, larger studies are required to provide detailed evidence on which complement test should be routinely used to monitor and design individualized Eculizumab treatment schedules.

## 5. Complement in other clinical settings

### *5.1. Complement in neurological/neurodegenerative diseases*

Complement plays an important role in normal homeostasis and brain functioning. Local synthesis of complement components by glia and nerve cells in the brain is crucial for proper functioning of the local defense system (*60*). Moreover, complement is also involved in neurological disorders such as Alzheimer´s disease (AD), Parkinson's disease, Pick's disease, multiple sclerosis, Huntington's disease and amyotrophic lateral sclerosis (*61*).

Amyloid β peptide (Aβ), the major component of senile plaques, serves as a focus of complement activation and amplification during pathogenesis of AD. Predominantly CP activation products (C1q, C4d and C3d) were found to co-localize with Aβ deposits, although AP components have also been found associated with amyloid plaques in both human AD (*60*) and in murine models of AD (*62*). In later AD stages, complement deposits are more prominent and terminal components (C5b-9) appear (*63*). Several studies reported that brain cells express complement regulatory proteins (*61*) in response to complement attack. However, regulation of complement activation in AD brain is not sufficient or is impaired. For example, CD59 expression was found to be downregulated in the affected tissue of AD patients. Furthermore,

amyloid aggregates bind C3b covalently, which promotes phagocytosis of Aβ through CR1 binding by glial cells, although risk polymorphisms exist in CR1, which are related with a poor phagocytic activity.

### *5.2. Complement in dermatological diseases*

The synthesis of certain complement proteins by human skin keratinocytes and fibroblasts plays an important role in skin defense against microbial infection but also mediate inflammation and tissue injury (*64*). The presence of autoantibodies against skin antigens induces complement overactivation causing direct tissue damage and inflammation due a cytotoxic effect of the MAC on epidermal or vascular cells (*65*). In autoimmune bullous diseases, as pemphigus and bullous pemphigoid (BP), pathogenesis has been demonstrated to be complement-dependent. Direct immunofluorescence of perilesional skin from BP patients shows linear deposits of C3 and, in most cases, IgG (*66*). Activation of complement has been demonstrated locally in blisters and systemically in blood by low concentrations of CP and AP components in patients diagnosed with pemphigus (*67*). Another example of complement involvement in dermatological diseases has been reported in anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV). Recent results showed that plasma levels of C3a, C5a, soluble C5b-9 and Bb were elevated in patients with active AAV, whereas properdin was reduced, as compared with patients in the remission phase (*68*).

### *5.3. Complement in trauma, shock and sepsis*

Complement activation after polytrauma substantially contributes to the development of systemic inflammatory response syndrome (SIRS) and multiple organ failure and is a critical event in the pathogenesis of sepsis and septic shock (*69*). There are evidences that suggest that changes in the levels of certain complement components or their activated products reflect the course of sepsis and may thus serve as potential staging markers. Numerous studies have shown that patients in septic shock have markedly decreased levels of C4 and reduced total complement activity (*70,71,72*). In other studies, while AP activation fragments (C3a and Bb) were elevated in septic shock patients and correlated with mortality (*73*) serum properdin was significantly low (*74*). There are controversial results regarding MBL's involvement in sepsis, but an early clinical study suggested that deficiency of MBL function was associated with bloodstream infection and the development of septic shock (*75*).

### *5.4. Complement in acquired lipodystrophies*

The connection between complement and adipose tissue has a long history. In 1989, Rosen et al. reported that adipsin, a major protein of mature adipocytes, was highly homologous to FD (*76*); subsequently it was shown that both proteins were identical and that adipose tissue synthesized more than 95% of FD *in vivo* (*77*). Adipocytes also express C3, FB (*78*) and complement regulators like properdin (*79*) and FH (*80*).

Local activation of AP in the vicinity of the adipocyte stimulates pre-adipocyte differentiation (*78*). However, although the AP activation has an important physiological role for adipose tissue, AP dysregulation has been related with fat mass-distribution diseases. Lipodystrophies are clinically heterogeneous acquired or inherited disorders characterized by the selective loss of adipose tissue in the absence of nutritional deprivation or catabolic state (*81*). Acquired partial lipodystrophy (APL) presents with low C3 levels and C3NeF in more than 90% of cases (*82*). As consequence of AP dysregulation, approximately 25% of APL patients develop C3G

about eight years after onset (*83*). In addition, Savage et al. (*84*) have reported three patients with acquired generalized lipodystrophy, decreased serum C4 levels, and autoimmune hepatitis. Although the connection between complement abnormalities and renal disease has been established, the exactly mechanism of fat loss remain unclear. Mathieson and collaborators demonstrated that C3NeF could induce complement-mediated lysis of adipocytes *in vitro* (*85*). However, the fact that only a small proportion of patients with C3NeF develop lipodystrophy remains unexplained

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## Figure legends

**Figure 1. Activation of the complement system**.
**Step 1**. Complement activation through the classical and lectin pathways generates the same CP C3 convertase. Activation of the alternative pathway generates the AP C3 convertase, which further amplifies complement activation through a feedback mechanism. The C3 convertases generate C3a and C3b, which enhances phagocytosis and favours antibody generation. **Step 2**. The CP C5 convertase and the AP C5 convertase generates C5a, which potentiates inflammation, and C5b. **Step 3**. C5b initiates the TP and MAC assembly, which results in pathogen lysis. Soluble (C1INH, C4BP, FH, FI, Vn, Cn) and membrane-bound complement regulators (CD35, CD46, CD55, CD59) avoid autologous damage. (For complement nomenclature see ref#*86*).

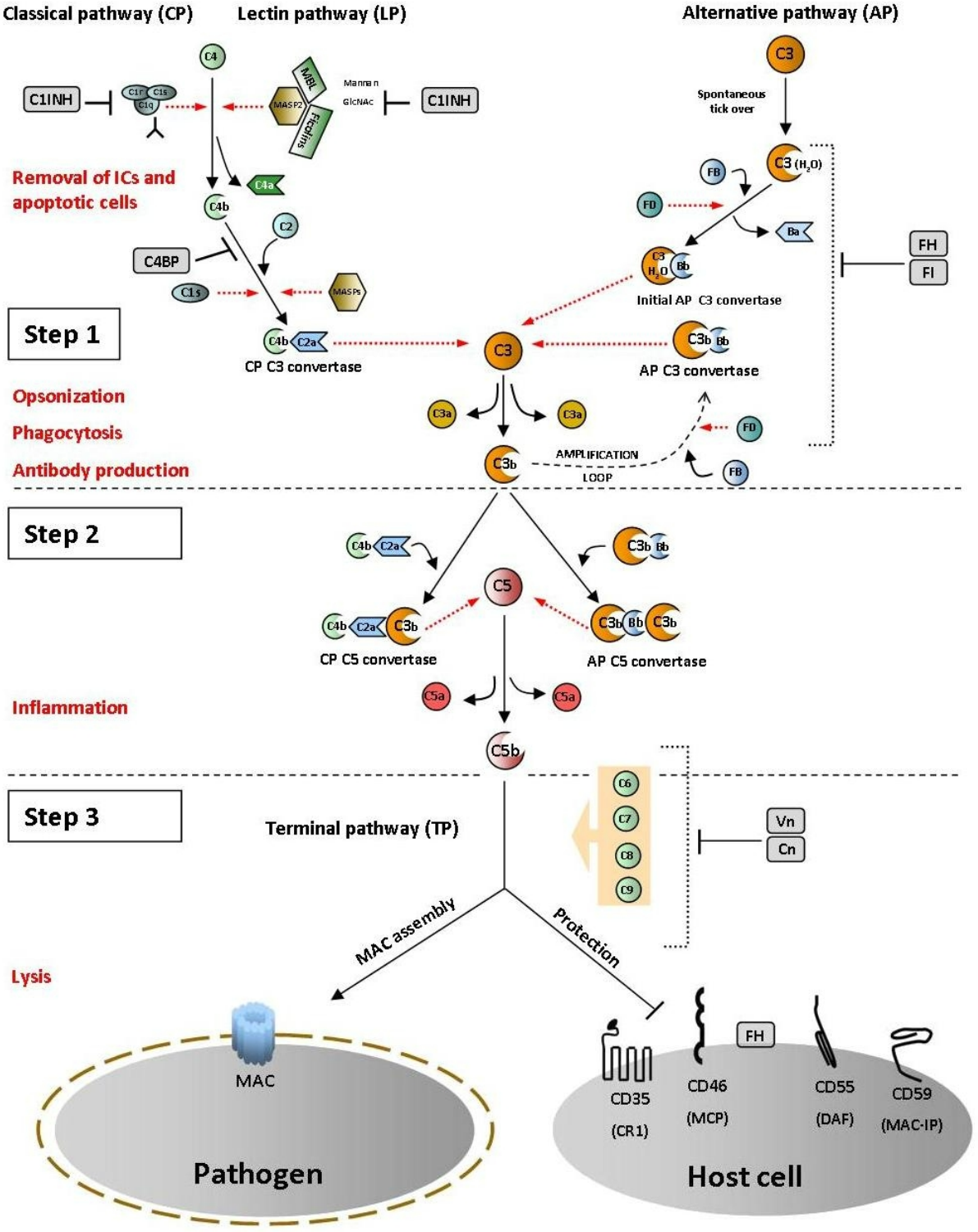
**Figure 2- Flowchart of tests for the characterization of Complement abnormalities.**
**Situation#1**. Normal C3 and C4 levels in the presence of recurrent infections should prompt the physician to analyse the functional integrity of the complement cascade. In this situation, very reduced or undetectable CH-50 levels indicate deficiency of a complement component. **Situation#2**. Normal C3 but reduced C4 levels are indicative of CP activation. Functional abnormalities in C1INH and the presence of crioglobulins should first be discarded; if these two assays are negative and C4 levels are repetitively low, partial or total C4 deficiency should be suspected. **Situation#3**. Low C3 but normal C4 levels suggest AP activation. If autoimmunity due to C3NeF autoantibodies is ruled out, partial or complete deficiency of an AP component is to be suspected. **Situation #4**. A profile with simultaneous low C3 and C4 levels indicates significant complement consumption by immunocomplexes or autoantibodies due to infection or autoimmune disease.

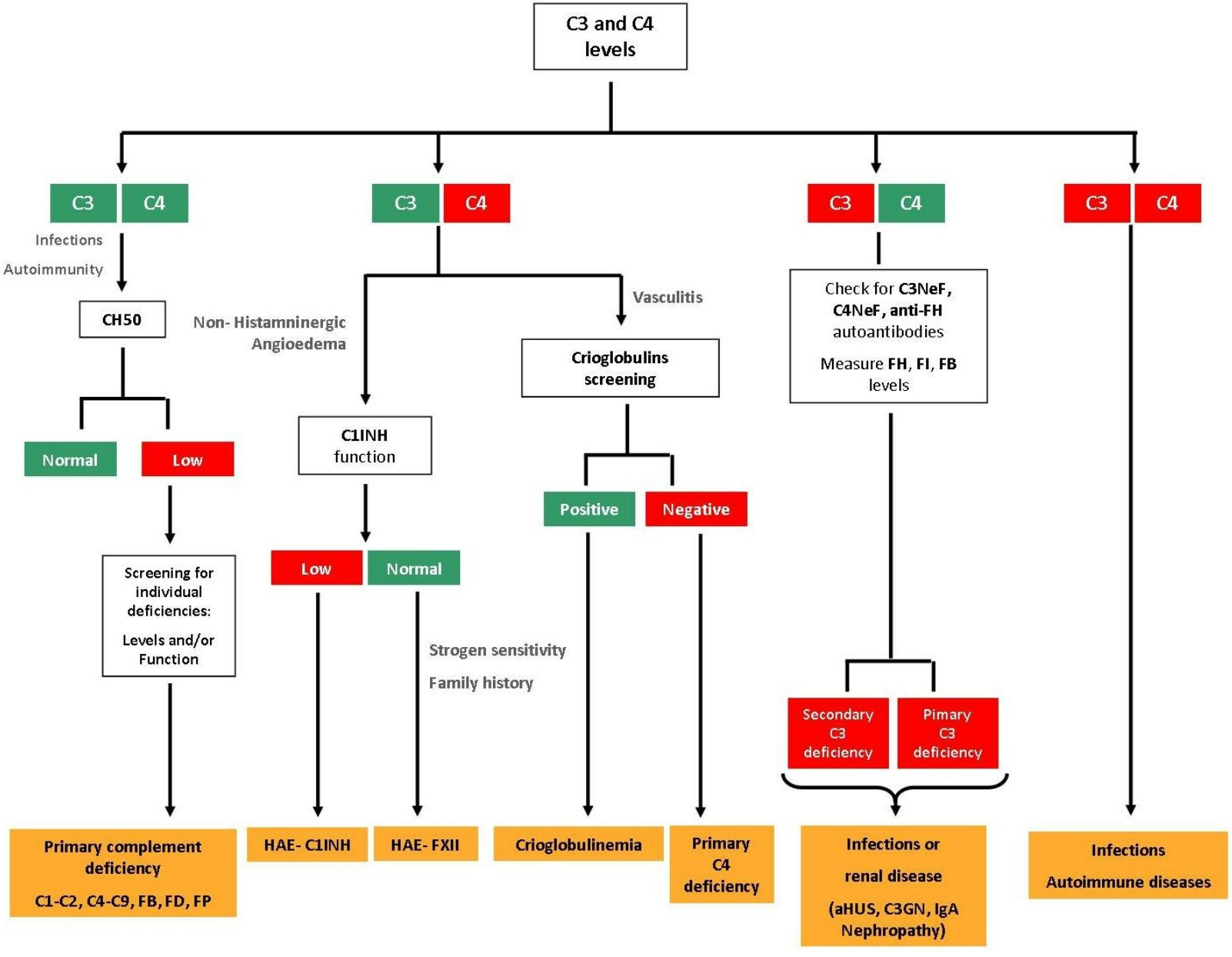
**Table I. Clinical conditions associated with complement deficiencies**
Main pathological situations associated with genetic deficiencies of complement proteins from the classical, alternative, or terminal pathway. Disease prevalence, chromosomal location, and genetic mode of inheritance are also indicated.
AD: Autosomal Dominant. aHUS: atypical Haemolytic Uraemic Syndrome. AMD: Age-related Macular Degeneration. AR: Autosomal Recessive. C3G: C3 Glomerulopathies; HAE: Hereditary Angioedema. HLA: Human Leukocyte Antigen. MPGN-II: Membranoproliferative Glomerulonephritis Type II. PNH: Paroxismal Nocturnal Hemoglobinúria. RCA: Regulators of Complement Activation gene cluster. SLE: Systemic Lupus Erythemathosus. XL: X-linked. 3MC: Malpuech-Michels-Mingarelli-Carnevale syndrome.

| Protein | Pathology | Prevalence | Gene (location) | Inheritance |
|---|---|---|---|---|
| **Classical pathway (CP)** | | | | |
| C1q | SLE<br>Bacterial infections | ~70 cases | 1p36 | AR |
| C1r | | < 20 cases | 12p13 | AR |
| C1s | | < 20 cases | 12p13 | AR |
| C4 | | ~30 cases | 6p21 (HLA class III) | AR |
| C2 | SLE<br>Bacterial infections<br>Asymptomatic | ~1:10,000 | 6p21 (HLA class III) | AR |
| C1INH | HAE | 1/10,000 – 1:50,000 | 11q12 | AD |
| C4BP | Atypical Morbus Behçet<br>Angioedema | Extremely rare | 1q32 | AR |
| **Alternative pathway (AP)** | | | | |
| Factor D | Bacterial infections | 3 cases | 19p13 | AR |
| Factor B | *Neisseria meningitidis* infections | 2 cases | 6p21 (HLA class III) | AR |
| Properdin | Severe *Neisserial* infections | Extremely rare | Xp11 | XL |
| Factor H | Renal diseases (C3G and aHUS)<br>Bacterial infections<br>AMD<br>SLE-like | <200 cases | 1q32 (RCA) | AR* |
| Factor I | Bacterial infections<br>Renal diseases (MPGN-II and aHUS) | <100 cases | 4q25 | AR* |
| C3 | Bacterial infections<br>MPGN | <50 cases | 19p13 | AR |
| MBL | Bacterial infections in vulnerable populations | 5% Caucasians | 10q11 | AR |
| MASP1 | 3MC syndrome | <1/1,000,000 | 3q27 | AR |
| MASP2 | Bacterial infections<br>Autoimmunity | 4% Caucasians | 1p36 | AR |
| Ficolin 3 | Bacterial infections<br>Necrotizing enterocolitis | Extremely rare | 1p36 | AR |
| **Terminal pathway (TP)** | | | | |
| C5 | *Neisserial* infections | ~50 cases | 9q33 | AR |
| C6 | | 1/2,000 in Afro-American population. Extremely rare elsewhere | 5p13 | AR |
| C7 | | <100 cases | 5p13 | AR |

| | | | | |
|---|---|---|---|---|
| C8 | | <100 cases | 1p32 (α,β chains) 9q34 (ɤ chain) | AR |
| C9 | | 1/1,000 in Japanese population**. Extremely rare elsewhere | 5p14 | AR |
| CD35 (CR1) | SLE Resistance to Malaria | Extremely rare | 1q32 (RCA) | AR |
| CD46 (MCP) | aHUS, C3G | >50 | 1q32 (RCA) | AR |
| CD55 (DAF) | PNH | Extremely rare | 1q32 (RCA) | AR |
| CD59 (MAC-IP) | PNH | Extremely rare | 11p13 | AR |

*Heterozygous mutations in FH or FI predisposes to aHUS. **The R95X mutation is frequent in Japan.


Classical pathway (CP)
Lectin pathway (LP)
Alternative pathway (AP)
C1INH
C1r
C1s
C1q
C4
MBL
MASP2
Ficolins
Mannan
GlcNAc
C1INH
Spontaneous tick over
C3
C3 (H2O)
FB
FD
Ba
FH
FI
Removal of ICs and apoptotic cells
C4a
C4b
C2
C4BP
C1s
MASPs
C3 H2O Bb
Initial AP C3 convertase
Step 1
C4b C2a
CP C3 convertase
C3
C3b Bb
AP C3 convertase
Opsonization
Phagocytosis
Antibody production
C3a
C3a
C3b
AMPLIFICATION LOOP
FD
FB
Step 2
C4b C2a
C3b Bb
C5
C4b C2a C3b
CP C5 convertase
C3b Bb C3b
AP C5 convertase
Inflammation
C5a
C5a
C5b
Step 3
Terminal pathway (TP)
C6
C7
C8
C9
Vn
Cn
MAC assembly
Protection
Lysis
MAC
Pathogen
CD35
(CR1)
CD46
(MCP)
FH
CD55
(DAF)
CD59
(MAC-IP)
Host cell


C3 and C4 levels
C3
C4
Infections
Autoimmunity
CH50
Normal
Low
Screening for individual deficiencies:
Levels and/or Function
Primary complement deficiency
C1-C2, C4-C9, FB, FD, FP
C3
C4
Non- Histamninergic Angioedema
C1INH function
Low
Normal
Strogen sensitivity
Family history
HAE- C1INH
HAE- FXII
Vasculitis
Crioglobulins screening
Positive
Negative
Crioglobulinemia
Primary C4 deficiency
C3
C4
Check for C3NeF, C4NeF, anti-FH autoantibodies
Measure FH, FI, FB levels
Secondary C3 deficiency
Pimary C3 deficiency
Infections or renal disease
(aHUS, C3GN, IgA Nephropathy)
C3
C4
Infections
Autoimmune diseases