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REVIEW

Complement system network in cell physiology and in human diseases

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ABSTRACT

The complement system is a multi-functional system representing the first line host defense against pathogens in innate immune response, through three different pathways. Impairment of its function, consisting in deficiency or excessive deregulated activation, may lead to severe systemic infections or autoimmune disorders. These diseases may be inherited or acquired. Despite many diagnostic tools are currently available, ranging from traditional, such as hemolytic or ELISA based assays, to innovative ones, like next generation sequencing techniques, these diseases are often not recognized. As for therapeutic aspects, strategies based on the use of targeted drugs are now widespread. The aim of this review is to present an updated overview of complement system pathophysiology, clinical implications of its dysfunction and to summarize diagnostic and therapeutic approaches.

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Introduction

The complement system is a complex multi-functional system, composed of more than 40 plasma and membrane proteins, all concurring to its normal function in either innate and adaptive immunity [1]. It plays a pivotal role in proinflammatory response in which it interacts with inflammasomes and Pathogen Recognition Receptors such as Toll like and Nod like receptors [2], which sense danger signals. The complement network is also involved in cell homeostasis, since it contributes to clearance of apoptotic cells and immune complexes [3], as well as in tissue regeneration.

Complement system impairment, due to dysfunction of its components or regulators, either in congenital or acquired forms, leads to severe and life-threatening bacterial infections but may also be associated with autoimmune, neurodegenerative, and age-related diseases. For instance, atypical Hemolytic Uremic Syndrome and age-related macular degeneration are clinical conditions considered related to complement deficiencies as well [4].

The aim of this review is to summarize the current state of knowledge about complement system pathophysiology, clinical implications of its dysfunction and diagnostic and therapeutic strategies to treat complement related diseases.

Complement system in cell physiology

Complement activation

Three different pathways lead to complement system activation: alternative, classical and **lectin** pathway. All the 3 pathways are activated through the sequential cleavage of inactive precursors, the so-called zymogens and finally converge on the activation of C3, the most abundant member of the complement system. The C3 convertase generates activation products (C3a, C3b) which in turn take part to the formation of the C5 convertase whose products form the Membrane Attack Complex (C5b-9) [5,6]. This process, occurring in both bacterial and human cells, ensues the release of active fragments that enhance host defense [7].

In order to protect host cells from its own attack, the complement activation is tightly and finely regulated. An inefficient stimulation of complement system may lead to increased susceptibility to bacterial infections; on the other hand, an over-stimulation can also be harmful for the host, resulting in chronic inflammation and autoimmune manifestations [8,9].

Classical pathway

The classical pathway is initiated by the interaction of immune complexes, made of the Fc portion of IgM or IgG and antigens, with the first zymogen of the

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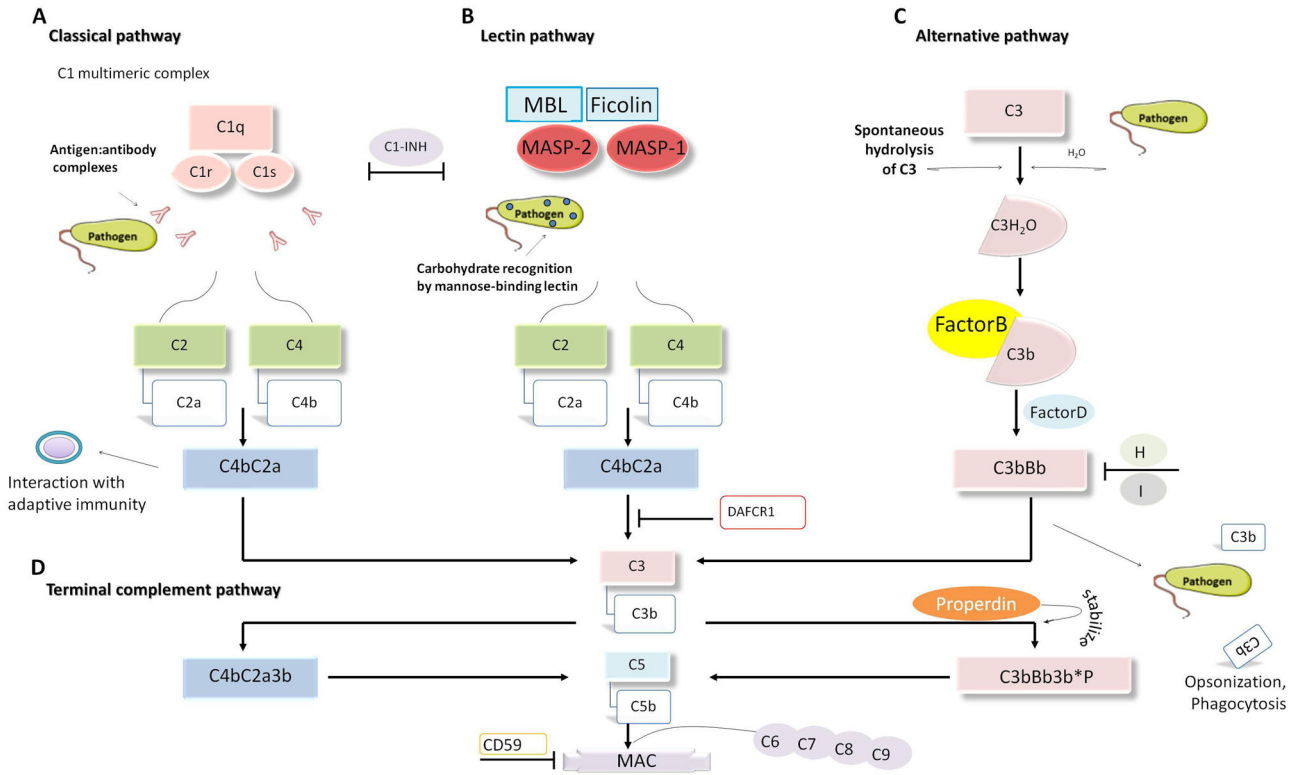

Figure 1. Schematic representation of pathways of activation of complement system (A) Classical pathway is activated by the interaction between immune complexes and C1q leading to the formation of C3 convertase C4bC2a, through sequential cleavage. (B) **MBL and ficolins** recognizing carbohydrate patterns uncommon in the host activate **Lectin** pathway whose final product is the formation the C3 convertase C4bC2a. (C) Constitutively activated, alterative pathway leads to the formation of C3bBb. (D) In terminal complement pathway, the C3 convertases of the three pathways converge on the cleavage of C3 whose fragment C3b takes part in the formation of C5 convertases. C5b, along with terminal components C6, C7, C8, C9, forms the Membrane Attack Complex which lyses complement targets.

cascade, C1q which, in resting conditions, forms an inactive complex with C1r and C1s. Upon interaction with immune complexes, C1q activates C1r, which, in turn, cleaves and activates C1s (Figure 1A). This induces the cleavage of two other components, C2 and C4, with the formation of the complex C4b2a, which hydrolyzes C3 into C3a and C3b that, respectively, recruits inflammatory cells and binds to the C4b2a complex forming C5 convertase [10]. The latter is responsible for the formation of the Membrane Attack Complex that induces lysis of bacterial membranes inserting functional pores in it [1]. Also, viral proteins, apoptotic cells and C-reactive protein can trigger this pathway independently of the presence of antibodies.

Lectin pathway

The Mannose-binding **lectin** (MBL)/MBL-associated serine protease (MASP) pathway is initiated by MBL, **multimeric complexes that recognize specific carbohydrate patterns that are uncommon in the host**. **MBL-associated serine protease**-1 cleaves C3 directly, while **MBL-associated serine protease**-2, activated by **MBL-associated serine protease**-1, cleaves C4 and C2 leading to the formation of a C3 convertase. **MBL-associated serine protease** also interacts with three lectins known as ficolins (ficolin-1, ficolin-2 or ficolin-3), forming active complexes that **also** initiate this pathway [10] (Figure 1B).

Alternative pathway

A so-called "tick over" process, consisting of spontaneous hydrolysis of C3, initiates this pathway, which is permanently active as surveillance system against pathogens [11]. This leads to the formation of C3b, which binds to target structures. Then, Factor B is cleaved by Factor D in order to form the C3 convertase C3bBb, which is stabilized by plasma properdin, a positive regulator secreted by activated neutrophils [6,12] (Figure 1C).

Terminal complement pathway

The C3 convertases produced as a result of the three activation pathways cleave the inactive C3, leading to

the formation of C5 convertase, which, in turn, cleaves C5 releasing C5a that acts as chemoattractant for phagocytes to the site of inflammation. The sequential fusion of the fragments C6, C7, C8, and multiple C9s to C5b finally generates the Membrane Attack Complex [1] (Figure 1D) responsible for the lysis of complement targets mediated by functional pores.

Pathways regulation

Within the classical pathway, C1-inhibitor (C1-INH), secreted by monocytes and liver, directly inhibits the C1r and C1s subunits, establishing a covalent bond with them and disassembling the C1 molecular complex with C1q. Within the alternative pathway, the C3bBb complex is stabilized by properdin, a positive regulator, and dissociated by factor H. C3 and C5 convertases of the classical pathway and alternative pathway are inactivated by Decay acceleration factor. MBL-associated serine protease-1/MBL-associated serine protease-2 of the **lectin** pathway are inhibited by C1-inhibitor [13]. Within the terminal common pathway, CD59 interferes with the formation of Membrane Attack Complex on cell surfaces.

Complement receptors and anaphylatoxins

Four receptors are part of the complement system. Complement Receptor 1 is expressed on macrophages and red blood cells whose role is to promote the clearance of immune-complexes; Complement Receptor 2 is expressed on B cells where, upon binding of the fragment C3d opsonizing antigens, it enhances B cell receptor signaling. Complement Receptor 3 and Complement Receptor 4, belonging to beta-2 (CD18) integrin family [14], are expressed on monocytes, dendritic cells and macrophages and take part to opsonization and phagocytic process.

During the complement activation process, two anaphylatoxins, C3a and C5a, are released. These peptides show pro-inflammatory properties inducing activation of platelets, endothelial cells and recruiting phagocytes.

Additionally, as part of the intracellular store of complement proteins, known as “complosome”, they act as regulators of the adaptive immune response as they promote Antigen Presenting Cells maturation and T-cell activation through an autocrine and paracrine C5a/C3a/C3b signaling at the Antigen Presenting Cell-T cell interface [15].

Role in innate immunity

During an infectious process, the recognition of foreign antigens, different for each pathway, triggers complement system activation that, in turn, initiates an inflammatory response, consisting of opsonization, phagocytosis and clearance of the pathogen, eventually resulting in the activation of adaptive immunity [11].

Pathogens that are susceptible to complement system are promptly identified by C3b that is constantly produced as a result of the constitutive activation of the alternative pathway. C3 cleavage is accelerated by an amplification loop whose final step is the covalent binding of C3b to target cells that, in this way, are opsonized. Components of the classical and lectin pathway, namely C1q and Mannose Binding Lectins, recognize immune-complexes and danger associated signals, respectively; likewise, the cleavage of C3 is followed by the production of C3b, the opsonization of pathogens and their phagocytosis. Through subsequent events of the complement cascades, the formation of Membrane Attack Complex ensues, contributing to pathogen elimination [8,11].

Role in adaptive immunity

The complement system is implicated in both B- and T-cell responses. As for the B-cell compartment, antigen-antibody complexes, opsonized by C3 component, are presented by follicular dendritic cells to B cells in germinal centers, thus inducing memory and effector B cells.

In addition, C4, another early component of the complement cascade, is supposed to be a key regulator of B-cell autoreactivity. In fact, its deficiency has been associated to an impaired antigen presentation by Antigen Presenting Cell essential to establish the peripheral B-cell tolerance [8]. C4 has been shown to take part in the clearance of apoptotic cells whose impairment interferes with host antigen presentation.

As for the T-cell compartment, complement anaphylatoxins C3a and C5a, are able to bind to specific receptors on T cells, promoting their activation and facilitating T-helper response. Thus, the complement is involved both in induction and effector phases of immune response [16–18].

Some complement molecules are key players as a bridge between innate and adaptive immunity: namely, C1q, C3b and the above mentioned anaphylatoxins. In particular, C1q-opsonized antigens upregulate the production of IL-12 and TNFα in dendritic cells, thus stimulating Th1 polarization [19]. Moreover, the phagocytosis of antigens opsonized by

Table 1. Classification of the main complement components defects with phenotypic and laboratory hallmarks. SLE: systemic lupus erythematosus; aHUS: atypical **Hemolytic**-uremic; PNH: Paroxysmal Nocturnal Hemoglobinuria.

Functional defect	Gene	Inheritance	Clinical phenotype	Laboratory
Classical pathway deficiencies				
C1q	*C1QA, C1QB, C1QC*	AR	Infections with encapsulated bacteria, SLE	Absent CH50
C1r	*C1R*	AR	Infections with encapsulated bacteria, SLE, immune thrombocytopenia	Absent CH50
C1s	*C1S*	AR	Infections with encapsulated bacteria, SLE	Absent CH50
C4	*C4A + C4B*	AR	Infections with encapsulated bacteria, SLE	Absent CH50
C2	*C2*	AR	Infections with encapsulated bacteria, SLE	Absent CH50
C3	*C3*	AR	Infections with encapsulated bacteria, glomerulonephritis	Absent CH50 and AH50
Lectin pathway deficiencies				
MASP2	*MASP2*	AR	Infections with encapsulated bacteria, chronic lung disease	Decreased lectin pathway activation
Ficolin 3	*FCN3*	AR	Abscesses, lower respiratory tract infections	Decreased lectin pathway activation
Factor B GOF	*CFB*	AD	aHUS	Increased AH50
Factor B LOF	*CFB*	AR	Infections with encapsulated bacteria	Decreased AH50
Factor D	*CFD*	AR	*Neisseria* infection	Absent AH50
Terminal component deficiencies				
C6	*C6*	AR	Disseminated infections with encapsulated bacteria	Absent CH50 and AH50
C7	*C7*	AR	Disseminated infections with encapsulated bacteria	Absent CH50 and AH50
C8a	*C8A*	AR	Disseminated infections with encapsulated bacteria	Absent CH50 and AH50
C8b	*C8B*	AR	Disseminated infections with encapsulated bacteria	Absent CH50 and AH50
C8g	*C8G*	AR	Disseminated infections with encapsulated bacteria	Absent CH50 and AH50
C9	*C9*	AR	Disseminated infections with encapsulated bacteria	Decreased CH50 and AH50
Pathway regulators deficiencies				
C1-Inhibitor	*SERPING1*	AD	Hereditary angioedema	Decreased C4 and C2
CD59	*CD59*	AR	PNH	Increased erythrocyte susceptibility to lysis
CD55	*CD55*	AR	Protein-losing enteropathy	Complement hyperactivation
Properdin	*CFP*	XL	*Neisseria* infection	Absent AH50
Factor H	*CFH*	AD or AR	aHUS, invasive *Neisseria* infection	Decreased C3

this molecule in macrophages stimulates the production of IL-27, a cytokine that inhibits Th1/Th17 polarization, thus halting inflammatory process during apoptosis [20].

New emerging roles in pathophysiology

Apart from its ancient and well-known functions in cell homeostasis and immune system, recent findings helped expand our understanding of non-canonical activities of complement system and of uncommon locations. The intracellular complement system, referred as complosome as opposed to extracellular active one, has been attributed plenty of new functions. Firstly, C3a generated in inactive CD4+ and CD8+ T cells provides homeostatic survival of T cells through a constitutive mTOR activation via C3a Receptor on lysosomes. In addition, during T Cell Receptor activation, intracellular C3b translocates to the cell surface contributing to overall T cell homeostasis [9]. Secondly, C3 helps in protection from intracellular pathogen invasion by "labelling" pathogens, thus acting as a Damage Associated Molecular Pattern, that activates innate immunity [3]. The production of C3 occurring in various cell types as B cells, endothelial and epithelial cells as well as fibroblasts, has been hypothesized to contribute to cell survival and intracellular host immunity as well [13].

Factor H prevents excessive complement activation and related autoimmune phenomena and inflammation [21], by ensuring an efficient opsonization and disposal of coated cellular debris.

Of note, West and colleagues recently focused on distinct location-driven activities of complement system, speculating that the intracellular location may be focused on safeguarding normal cell physiology; the extracellular expression may be mainly deputed to pathogen clearance exerting a pro-inflammatory role. Eventually, the surface expression may promote the crosstalk between innate and adaptive immune

responses and allow environment signals sensing (Pathogen Associated Molecular Patterns and Damage Associated Molecular Patterns) driving pathogen associated or sterile inflammation [9]. Indeed, complement components act as amplifier of sterile inflammation triggered by various danger signals. Thus, it is involved in the pathogenesis of widespread non-communicable diseases, as cancer, hypertension and multiple sclerosis. In tumorigenesis, C5a serves as a chemokine for myeloid-derived suppressor cells which differentiate into tumor-associated macrophages that induce immunosuppressive and Treg polarizing cytokines thus enhancing neoplastic proliferation. In multiple sclerosis, Complement Receptor 3 and Complement Receptor 4 have been shown to influence T-cell activation and induce autoimmune attack, impairing T-reg function and IL-10 production [15].

A growing body of evidence suggests a role of complement system components in neurobiological processes such as neurogenesis, migration of neurons, brain connectivity and response to insults. In particular, the anaphylatoxins C3a and C5a regulate cell proliferation and migration while C1q, C3 and C4 shape brain architecture and wiring "labelling" weaker synapses that will be removed by microglia. Abnormal complement signaling due to genetic mutations or to inflammatory damage in neurodevelopment have been implicated in the pathogenesis of autism spectrum disorders, Rett syndrome and in schizophrenia [22]. For instance, in 22q11.2 deletion syndrome, the patients who displayed increased complement activation were more prone to develop psychiatric disorders [23]. Complement system has been also linked to the maintenance of the integrity of intestinal barrier function: in intestinal epithelial cells, locally expressed complement components, such as C1q, C4 and C3 and regulatory proteins like CD46 and CD59, contribute to intestinal epithelial cell proliferation and to mucosal response against pathogens [24]. As a consequence, an imbalance in intestinal complement system has been deemed responsible for the development of intestinal inflammation of inflammatory bowel diseases: in particular an increased activation of the classical pathway has been identified in ulcerative colitis as opposed to an over-activation of alternative pathway in Crohn's disease [25]. Finally, complement abnormalities have also been associated to certain skin diseases. Activation of both classical and alternative pathways is involved in psoriasis, whereas the interplay between complement system and the endothelial cells is involved in the pro-inflammatory response seen in Cutaneous Small Vessels Vasculitis, where C5a and Membrane Attack Complex may determine structural and functional damage of the endothelium [26].

Epidemiology and genetics of complement deficiencies

Complement deficiencies can be hereditary (Table 1) or acquired. The inherited forms are all transmitted in an autosomal recessive manner, with the exception of properdin deficiency, which is X-linked recessive and C1-esterase inhibitor, that is autosomal dominant [1]. Complement deficiencies account for approximately 1–6% of all inborn errors of immunity and are generally caused by null alleles [1,27,28]. In Caucasian population, C2 deficiency occurs in 1:20,000 individuals. A prevalence of 5% has been reported for Mannose Binding Lectin deficiency, while deficiencies of C4A and C4B show prevalence rates of 11–22% and 30–45%, [7,29]. Overall, these defects have been observed in up to 20% of patients with disseminated *Neisseria* infections [30].

Clinical phenotypes

Classical pathway defects

Deficiency of the early proteins of the classical pathway is strongly associated with the development of a lupus-like disease characterized by a younger age onset, prominent skin involvement, sometimes with low anti-DNA antibodies and elevated anti-Ro (SSA) antibodies [31–33]. Hereditary defects in classical components such as C1q, C1r, C1s, and C4 are associated with the most severe forms [1]: on the contrary, C3 deficiency is rarely associated with systemic lupus erythematosus.

A small percentage of patients with C2 deficiency (10 to 20%) develop lupus. The most common complement deficiency, C2 defect, may be due to nonsense mutation abolishing protein synthesis (type I) or to defective protein secretion (type II, less frequent). Clinical phenotype includes muskolo-skeletal and muco-cutaneous lupus manifestations, mainly occurring in adult females [34]. In patients with lupus nephritis, autoantibodies against classical complement component such as C1q have been identified [35].

Deficiencies of complement components C4A and C4B may be considered genetic risk factors in that they increase the susceptibility of developing systemic lupus erythematosus or a lupus-like disease [36]. Concerning the role of complement deficiencies in systemic lupus erythematosus pathogenesis, several hypotheses have emerged: according to the "waste-

disposal' hypothesis", apoptotic debris, accumulated due to defective clearance, act as a trigger for autoantibodies production, thus eliciting an overt autoimmune response [32,37,38]. The lupus-like phenotype may also develop as immune complexes escape effective clearance and deposit in peripheral tissues, causing inflammation and tissue damage [36].

Deficiencies in components involved in the initial activation, including C1s, C1r and C2, also lead to increased susceptibility to severe infections, usually occurring in early childhood, and generally caused by both gram positive and negative encapsulated bacteria, such as *Streptococcus pneumoniae* and *Haemophilus influenzae* [39,40]

Lectin pathway deficiencies

Deficiencies of **lectin** pathway components may be clinically irrelevant in healthy individuals due to redundancy of the immune system [41]. However, due to the ability of Mannose Binding Lectin to bind to a broad spectrum of pathogens including bacteria, fungi and viruses, the **lectin** pathway appears to be implicated in innate response to several infections in humans. In particular, complement proteins are also synthesized by cells of Central Nervous System, which is separated from circulating plasma complements. Thus, local complement proteins seem to play a pivotal role in immunity against viruses, as Herpes Simplex Virus in Central Nervous System. In support of this, *MASP-2* gene mutations associated to Herpes Simplex Virus encephalitis have been reported in two adults [42].

An increased risk of meningococcal infection has been observed in children with decreased levels of Mannose Binding **Lectin**, while an increased susceptibility to a broader spectrum of microorganisms including bacteria but also fungi, protozoa and viruses has been documented in adults. The incidence of this condition in the general Caucasian population ranges between 5 and 10% [43]. However, it should be noted that the vast majority of Mannose Binding Lectin deficient subjects are healthy.

Alternative pathway and terminal complement component deficiencies

Deficiencies of Factor B, D, H, I and properdin have been described in association with increased risk of severe pyogenic infections. This is presumably caused by an uncontrolled activation of the alternative pathway, resulting in consumption of complement components [44]. For these defects no relation to autoimmunity has been thus far observed [4].

Excessive activation of the alternative complement pathway may result in C3 glomerulopathy, formerly known as membrano-proliferative glomerulonephritis, a rare clinical entity presenting with proteinuria, hematuria, hypertension and renal failure that may evolve in end-stage kidney disease. Laboratory testing usually show low C3 with normal C4. Nonetheless, low levels of C4 or normal level of C3 do not exclude the C3 glomerulopahty. Considering its nonspecific clinical picture, often resembling that of post-infectious glomerulonephritis, the definitive diagnosis is histological, showing C3 deposits in the glomeruli. This may be due to gain of function mutations in C3 gene or loss of function mutations in *CFH* gene coding for Factor H which exerts a negative regulatory function on this cascade whereas, in acquired forms, the so-called nephritic factors, auto-antibodies stabilizing the C3 or C5 convertases, are involved. Therefore, C3 active fragments deposit in glomerular membrane where inflammation leads to glomerular damage.

Patients deficient in terminal complement components exhibit an extremely high risk (7000- to 10,000-fold higher than general population) of developing infections with *Neisseria meningitidis* [44], which tend to be recurrent, and frequently caused by uncommon serogroups such as Y, W135 and X [45]. Overall, conjugate vaccines, not only against *Neisseria meningitidis* but also against *Haemophilus influenzae* and *Streptococcus pneumoniae* are strongly recommended for long term individual protection, in both early and late components deficiencies [46,47]. These should be followed by booster dose of tetravalent conjugate meningococcal vaccine every 3 years for children aged 2 months to 6 year or every 5 years for older patients.

The dysfunctional regulation of alternative complement pathway due to Factor H or I deficiency or C3 unresponsiveness to inhibition leads to the development of the atypical form of Hemolytic Uremic Syndrome. Usually caused, in its typical form, by Shiga-toxin producing *Escherichia coli,* atypical Hemolytic Uremic Syndrome is a thrombotic microangiopathy disease affecting mainly kidneys, in which deposits of C5b-C9 damage glomerular endothelium. Thereafter, intravascular hemolysis and platelets activation result in the formation of microthrombi [48]. Atypical forms account for 5 to 20% of cases of Hemolytic Uremic Syndrome and may be either due to mutations in one of more genes coding for regulatory or cascade proteins (loss of function mutations in *CFH, CFI* or gain of function mutations in *CFB* and

C3) [49,50] or due to autoantibodies to factor H, detected in 5-13% of European atypical Hemolytic Uremic Syndrome patients. In such patients, genetic and autoantibodies testing are essential: the detection of anti-factor H autoantibodies may suggest the use of immunosuppressive drugs combined with plasma exchange, as therapeutic approach [51] as opposed to sero-negative atypical Hemolytic Uremic Syndrome forms in which the standard treatment choice is a humanized monoclonal antibody, Eculizumab, that binds the terminal complement component C5, as below described [51].

Deficiencies of the pathways regulators

The absence of the CD18, a subunit shared by two complement receptors, CR3 (CD11b/CD18) and CR4 (CD11c/CD18) is the cause of Leukocyte Adhesion Deficiency I, a well-known inborn error of immunity, characterized by severe bacterial infections [52].

Hereditary deficiency of C1-esterase inhibitor is a rare disorder, affecting approximately 1 in 50–100,000 individuals [53]. Three types have been described so far: type I is caused by inadequate production of the protein, while type II is due to dysfunctional production of the enzyme (C1-INH), both caused by mutations in the *SERPING1* gene, located on the chromosome 11. They are both inherited in an autosomal dominant manner, but *de novo* mutations have also been described. In type III, currently regarded as "unknown" hereditary angioedema, no genetic alteration has been identified [54]. This form is characterized by a later age of onset, between the second and the third decade of life and it appears to be more frequent in females, usually worsening during pregnancy. The consumption of C1-esterase inhibitor may lead to an acquired form of angioedema in the context of lymphoproliferative or autoimmune diseases [55].

The clinical picture is dominated by recurrent episodes of non pruriginous submucosal and subcutaneous edema, usually lasting 24–72 h, that may occur spontaneously or may be triggered by infections, drugs, trauma, or even stress. The target tissues are the skin, the upper respiratory tract, sometimes with laryngeal edema and obstruction, which may be fatal, and gastrointestinal system, with vomiting and diarrhea due to mucosal swelling [1,5]. As for the pathogenesis, the deficiency of C1-esterase inhibitor results in the formation of bradykinin, a vasoactive peptide, that increases vascular permeability and triggers pro-inflammatory pathways.

Another major clinical disorder related to abnormalities of the regulatory network of the complement cascade is the Paroxysmal Nocturnal Hemoglobinuria. Paroxysmal Nocturnal Hemoglobinuria is a rare hematopoietic stem cell disease due to the clonal expansion of hematopoietic stem cells carrying a somatic mutation in the gene phosphatidylinositol glycan anchor biosynthesis, class A (*PIG-A)* located on X chromosome. This gene encodes for an enzyme involved in the biosynthesis of phosphatidylinositol glycan that serves as anchor for membrane proteins such as Decay Accelerating Factor and CD59, whose physiological function is to inhibit complement system activation. DAF inhibits C3 convertase while CD59 competes with the C9 for the binding to the C5b-C8 complex thus interfering with Membrane Attack Complex formation. When their regulatory effect on complement system is impaired, complement mediated lysis of cells occurs leading to profound intravascular hemolysis and hemoglobinuria, hallmarks of the disease. Patients affected with Paroxysmal Nocturnal Hemoglobinuria show moderate to severe anemia and are prone to thromboembolic events usually affecting hepatic or cerebral veins and representing the main cause of morbidity and mortality associated with the disease [56]. Intravascular hemolysis releases free hemoglobin, which causes typical symptoms like dysphagia and abdominal pain, due to smooth muscles dystonia consequent to nitric oxide (NO) depletion.

Diagnostic approach

A stepwise approach (on clinical, functional, protein and molecular level) should start from the warning signs suggestive of complement deficiencies (Figure 2) [30]. These include:

- recurrent and severe bacterial infections, especially those caused by *Neisseria meningitidis*;
- angioedema without urticaria;
- severe autoimmune diseases and lupus like manifestations;
- inflammatory disease involving kidney

To assess the integrity of the whole complement cascade, hemolytic techniques have traditionally been used. Since in vitro activation can occur when evaluating complement cascade, sample collection and storage should be accurate: plasma should be collected in ethylene-diamine-tetra-acetic acid (EDTA*)* that chelates Ca^{2+} and Mg^{2+}, thus blocking the function of the C1 complex and of C3 convertases [57], and

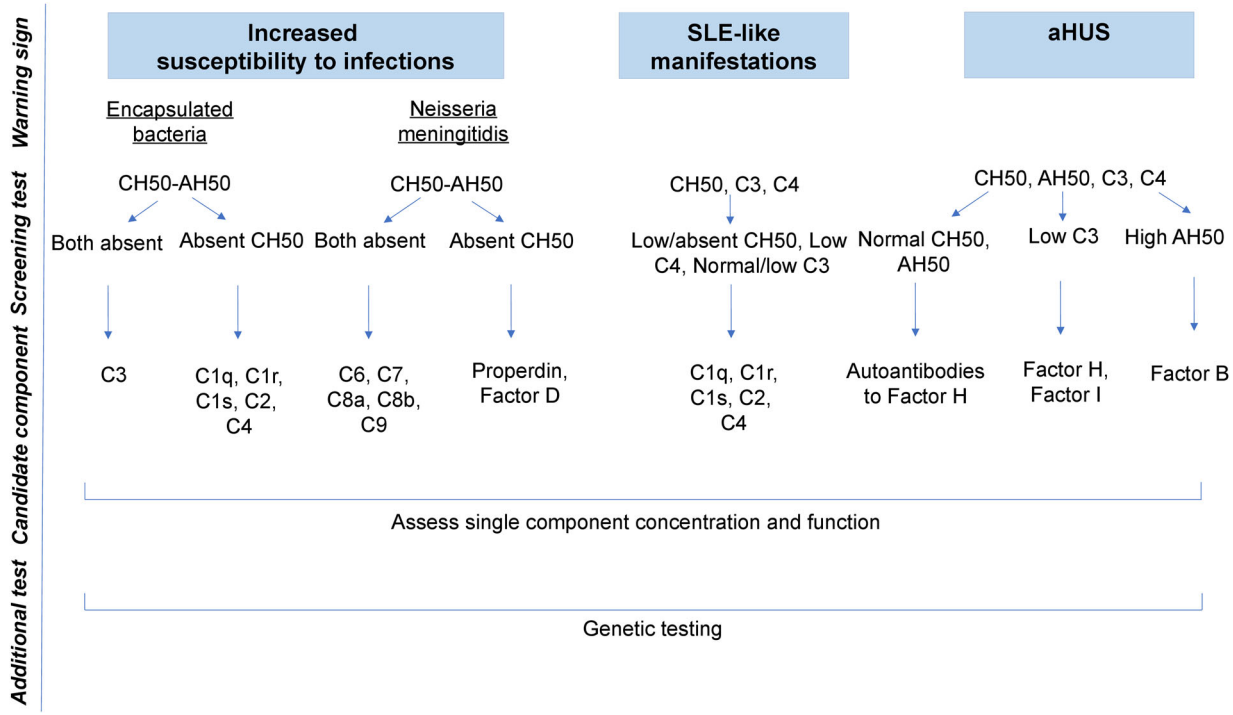

Figure 2. A stepwise approach to complement system deficiencies. Starting from predominant clinical warning signs, preliminary laboratory tests to perform and their interpretations are shown: single complement components likely to be deficient, according to clinical phenotype and laboratory assays results, should be tested in terms of concentration and function and, afterwards, genetic testing should be performed.

should be promptly separated and drawn to the laboratory on dry ice or frozen −70 °C for a proper analysis [58]. The dilution of patient's serum capable to lyse 50% of erythrocytes is defined CH_{50} and can be easily measured [59]. The CH_{50} tests serum complement components of the classical and terminal pathway for their capability to lyse sheep red blood cells, pre-coated with anti-sheep red blood cells antibodies. Once coated, the sheep red blood cells are incubated with test serum and hemolysis is initiated, as a result of complement system activation.

Complement abnormalities in the alternative pathway are screened through a similar test, the AH50 assay: a buffer is used to block the activation of the classical pathway and, afterwards, rabbit erythrocytes are used to activate the alternative pathway.

In the absence of a complement system component, the CH_{50} level will be null since no lysis will be elicited while the decrease in one or more components will result in reduced CH_{50} level. The presence of all functionally active proteins of both the classical pathway and the terminal pathway is required for a normal result. An undetectable CH_{50} may be due to both congenital or acquired complement deficiencies. As afore-mentioned, alterations of CH_{50} may also be due to increased consumption of complement components due to autoimmune diseases. For instance, absent CH_{50} together with low C4 may be observed in autoimmune conditions such as SLE, Sjögren's syndrome, rheumatoid arthritis [60]. Under certain circumstances, as severe liver disease, reduced synthesis of the components may be implicated.

A far less time-consuming test using parallel Enzyme-Immuno-Assays to quantify the function of the three activation pathways is now available, showing an excellent concordance with hemolytic assay [57]. As for the interpretation of screening test results, some preliminary considerations should be done: a very low or non-existing function in one or all the pathways may reflect a deficiency but also a consumption due to overactivation or dysregulation. For this reason, measurement of activation products such as C3a, Bb, C5b, should be performed to sort this out. Finally, since cryoglobulins can inactivate both classical pathway and terminal pathway in serum, cryoglobulinemia should be excluded [57,61].

Overall, a normal AH50 with absent CH50 suggests an early classical pathway deficiency, involving a complement protein or a regulatory factor, while undetectable AH50 and normal CH50 activity imply a deficiency in one of the alternative pathway components; absent AH50 and CH50 suggest a late component deficiency. If clinical suspicion for complement deficiency is high, in spite of normal CH50 and

AH50, a defect in **lectin** pathway may be ruled out measuring MBL. Once candidate proteins have been identified (Figure 2), single components should be tested in terms of both concentration and function since normal expression does not distinguish between a deficit of a protein due to an underlying gene mutation or to the presence of autoantibodies. The presence of autoantibodies appears to be likely when low levels of multiple components are detected [62]. Enzyme-Immuno-Assays, western blot, immunoprecipitation and nephelometry are techniques used to measure single component concentration [4,63]. The latter is routinely used to measure C3 and C4 plasma levels. Thereafter, serum tests may be used to assess the function: patient's serum is mixed with complement depleted serum in order to evaluate hemolysis [63].

Flow cytometry is the tool for the diagnosis of Leukocyte Adhesion Deficiency I and Paroxysmal Nocturnal Hemoglobinuria, in which CD18 or CD59 are, respectively, absent.

As for the diagnosis of C1-esterase inhibitor deficiency, C1-esterase inhibitor antigenic and functionality levels should be assessed, preferentially by gel precipitation or Enzyme-Immuno-Assays, as well as C4 levels, as screening tests. These should be confirmed after at least 3 months and, in case of a second positive result, genetic testing should be considered.

Eventually, after having ruled out acquired etiologies, molecular analysis may be helpful to confirm the diagnosis, especially in the presence of a single component deficiency [64]. Genetic newborn screening may be useful for genetic counseling [65]. Direct Sanger sequencing, once the diagnostic hypotheses have been narrowed, or Next generation sequencing followed by Sanger confirmation and functional assessment of genetic variants are useful diagnostic tool.

Therapeutic strategies

In general, patients affected with complement deficiencies presenting with severe and recurrent infections, despite adequate vaccinations, may benefit from antibiotic prophylactic regimens [4]. Complement targeted therapies have been proven useful in several clinical conditions. A humanized monoclonal antibody, Eculizumab, binding the terminal complement component C5 [51] has found widespread use in complement-associated diseases. In particular, it represents the gold standard treatment for Paroxysmal Nocturnal Hemoglobinura thanks to its ability to block C5 cleavage in C5a and C5b and, subsequently, the formation of Membrane Attack Complex. This compensates the absence of CD59 [66]. In atypical Hemolytic Uremic Syndrome with complement involvement, Eculizumab is used to determine a sufficient inhibition of Membrane Attack Complex to eventually prevent cell damage [51]. The effect of the therapy may be monitored through hemolytic or Enzyme-Immuno-Assays classical pathway and alternative pathway functional tests [57]. It must be mentioned that the inhibition of the terminal pathway may lead to a 1000-fold increase in meningococcal infections [50].

Recently, a novel anti-C5 monoclonal antibody, Ravulizumab, has been approved by Food Drug Administration for Paroxysmal Nocturnal Hemoglobinuria [67]. It is a long-acting inhibitor, engineered from Eculizumab, with increased elimination half-life: in a recent phase 3 single arm study it has been shown to be effective in solving thrombotic microangiopathy in adults affected with Hemolytic Uremic syndrome and also safe, with no major adverse reactions described [68].

As for C3 glomerulopathy, to date, no consensus has been reached for its treatment. Aside from renoprotective and immunosuppressive regimens, complement cascade is a potential therapeutic target. In small cohorts of patients, Eculizumab was used as therapeutic option, especially in those showing high level of C5-9, even though the response was variable [69,70]. **Phase 2 and 3 studies to evaluate the safety and efficacy of oral C5a receptor antagonist, Avacopan, are now ongoing in patients with C3 glomerulopathy and ANCA-associated vasculitis** [71,72].

Lastly, the treatment of hereditary angioedema encompasses prophylactic therapies and management of attacks. As for the acute attacks, first line therapies include two intravenous agents, human plasma derived (pd) C1-esterase inhibitor (Berinert) and recombinant human C1-esterase inhibitor (Ruconest), and two subcutaneous drugs, synthetic bradykinin B2-receptor antagonist (Icatibant) and kallikrein inhibitor (Ecallantide) [64]. If the above-mentioned drugs are not available, acute attacks may be managed with the use of solvent detergent-treated or fresh frozen plasma [73].

The aim of long-term prophylaxis regimen is to reduce the burden of the disease decreasing the number, duration and severity of attacks. First line treatment is based on pd C1-esterase inhibitor, administered via intravenous or subcutaneous route and second line treatment on oral androgens, limiting

the role of anti-fibrinolytic agents, e.g. tranexamic acid, to last resort choice [74].

Recently, a new therapeutic agent has been approved for long term prophylaxis in patients aged ≥12 years, Lanadelumab, which is a subcutaneous, fully human monoclonal antibody that inhibits plasma kallikrein, involved in bradykinin formation [75]. Current data suggests that it is effective and well tolerated [76].

Another field of current investigation is gene therapy: the use of a gene transfer vector expressing normal C1-esterase inhibitor in a hereditary angioedema mouse model has shown positive results, even though further studies in this area are needed [77].

Conclusions

Although complement system has been traditionally considered mainly involved in innate immunity, emerging studies are expanding the spectrum of its functions in cell physiology and in adaptive immunity. Moreover, it is also implicated in tissue regeneration and tumor growth. Inherited disorders of its components are associated to well defined clinical entities. Simple diagnostic tests are easily available. However, a diagnosis is frequently missed due to insufficient awareness of the potential warning signs for these rare disorders. The increased knowledge of the biological role of the complement components and their involvement in the pathogenesis of diseases may help disclose new windows of opportunity in the therapeutic approach of these so far underestimated disorders. Thus, our aim is to alert physicians to promptly recognize such disorders for a better management.

Disclosure of conflicts of interest

The Authors declare that they have no competing interests.

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