SVAR
Answers in Life Science

THE COMPLEMENT SYSTEM
A Specialist Review

An expert overview combining
core biology, recent research, clinical,
and pharmaceutical relevance.

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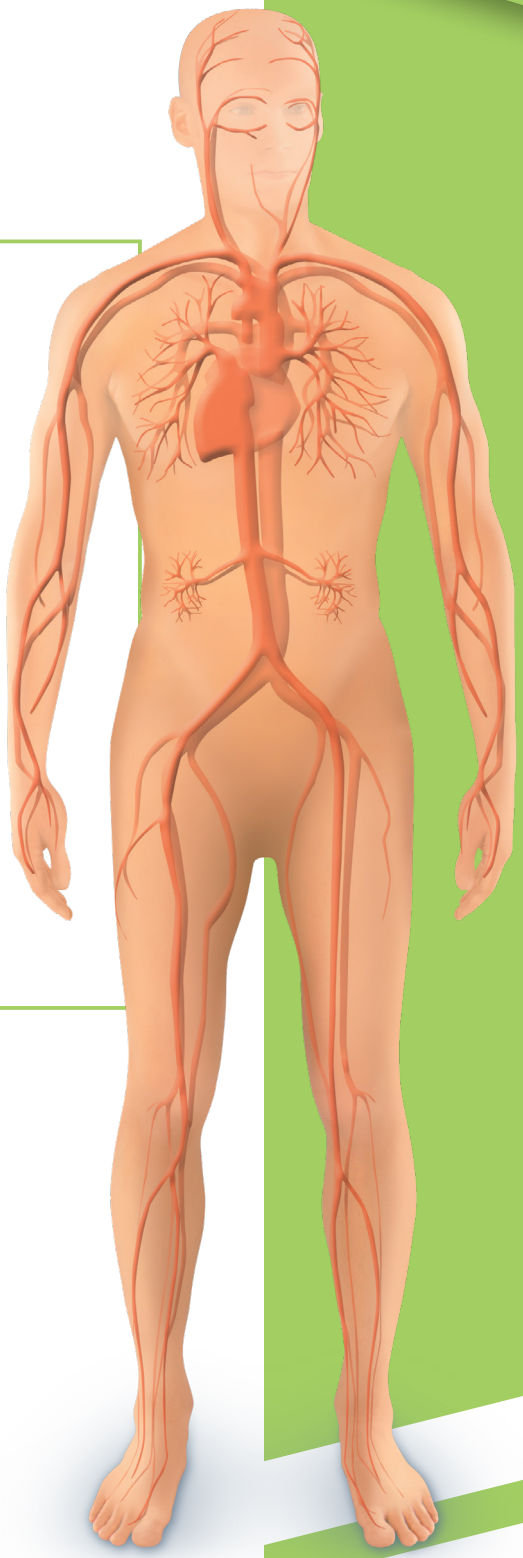

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1. THE COMPLEMENT SYSTEM

1.1 STRUCTURE

Complement is a recognition system and part of the host innate defense with several biological effects, many of which contribute to the inflammatory reaction mainly by activating cells, like leukocytes and endothelial cells. An intact complement system is required to protect against infection and maintain internal tissue homeostasis.

However, the system is a double-edged sword since improper, enhanced or uncontrolled activation is disadvantageous and potentially harmful for the host.

Complement comprises more than 50 proteins acting together in a highly specific manner and is strictly controlled by regulatory proteins. The system can be activated by three initial pathways, converging at C3 which initiates the terminal pathway (Figure 1).

The activation of the different pathways is described in the legend of Figure 1.

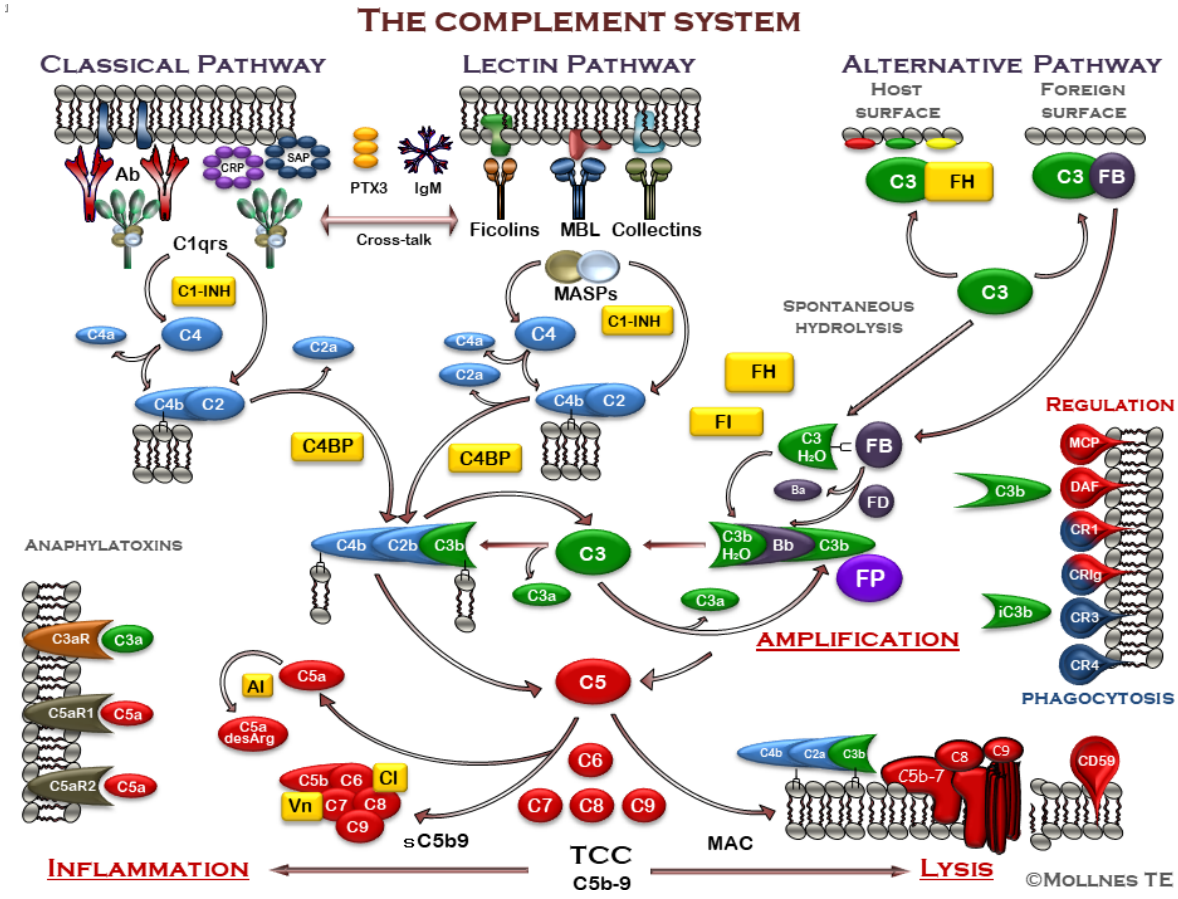


FIGURE 1. The Complement System

The complement system can be activated through three pathways (upper part of figure), all converging to the cleavage of C3 to generate C3a and C3b (middle part of figure). Antibodies typically activate the classical pathway (CP), but also pentraxins including C-reactive Protein (CRP), serum amyloid P component (SAP) and pentraxin 3 (PTX3) can activate C1q. The lectin pathway (LP) is activated through the recognition of carbohydrates by mannose binding lectin (MBL), ficolins and collectins.

The alternative pathway (AP) is activated by foreign surfaces or damaged own cells, facilitated by the continuous spontaneous hydrolysis of C3. AP also has an essential function in the complement system, providing an amplification loop that enhances C3 activation, independent of which pathway is initially activated. This effect is mainly due to properdin (FP), the only positive regulator in the complement system, which stabilizes the C3 convertase.

Activation of C3 leads to the formation of a C5 convertase, cleaving C5 into C5a and C5b. The anaphylatoxins C3a and C5a bind to the receptors C3aR, C5aR1 and C5aR2, leading to the downstream production of inflammatory mediators (lower left part of figure). C5b initiates the formation of the terminal C5b-9 complement complex (TCC), which either forms soluble sC5b-9 in the fluid phase, or the membrane attack complex (MAC), if inserted into a membrane (bottom part of figure). This may lead to lysis of bacteria and cells, or in sub-lytic doses to activation of cells.

The cleavage and inactivation of C3b generates iC3b, which binds to complement receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18), facilitating phagocytosis, oxidative burst and downstream inflammation (right part of figure).

The complement system is tightly regulated by soluble inhibitors (marked in yellow), including C1-inhibitor (C1-INH), factor H (FH), factor I (FI), C4b-binding protein (C4BP), anaphylatoxin inhibitor (AI), vitronectin (Vn) and clusterin (Cl), keeping the continuous low-grade activation in the fluid phase under control. Host cell membranes are equipped with a number of inhibitors to protect them against attack by complement (right part of figure), including membrane co-factor protein (MCP;CD46), decay accelerating factor (DAF;CD55), complement receptor 1 (CR1;CD35) and CRIg, controlling C4 and C3 activation, and CD59 preventing final assembly of the C5b-9 complex.

Complement activation is strictly regulated by inhibitory control proteins (Figure 1). In the fluid-phase C1-inhibitor (C1-INH) controls C1r, C1s and mannan-binding lectin-associated serine proteases (MASPs), whereas Carboxypeptidase N (Anaphylatoxin Inactivator) inactivates the anaphylatoxins C5a and C3a by splitting off the terminal arginine. Factor I cleaves and inactivates C4b and C3b and uses the soluble co-factors C4b-binding protein (C4BP) in the classical/lectin pathway and factor H in the alternative pathway.

The membrane regulators complement receptor 1 (CR1; CD35), the CR1 analog CRIg, membrane co-factor protein (MCP; CD46), and decay accelerating factor (DAF; CD55) regulate complement activation by either acting as co-factors for factor I mediated cleavage of C4b and C3b (CR1 and MCP), or accelerating the decay of the biomolecular C3 and C5 convertases (CR1 and DAF). CD59 prevents the binding of C9 into to the C5b-8 complex.

1.2 FUNCTION

Complement is a biologically highly potent system that is intended to act locally to protect the host against danger. As soon as an infection or other activation of the cascade spreads and induces a systemic activation, the host will be more threatened by a self-destructive uncontrolled action rather than by an attack of the microbe itself. External dangers like pathogenic microbes express pathogen-associated molecular patterns (PAMPs) that are recognized by the host's pattern recognition molecules/receptors (PRM/PRR). The initial complement components are typically PRMs, initiating the response to fight infection.

The complement PRMs also sense endogenous danger from damage-associated molecular patterns (DAMPs), that are exposed upon tissue damage, including ischemia-reperfusion, trauma and other sterile inflammatory conditions. These recognition patterns may be exposed by molecules that are normally hidden, or in molecules changing their structure, and may then be recognized by naturally occurring complement-activating molecules, such as IgM antibodies. In case of systemic activation, like in multi-trauma, a systemic inflammatory response might occur, leading to multi-organ failure and death. Thus, from an inflammatory point of view, complement is a double-edged sword acting as a friend or foe, or both, depending on the actual circumstances.

The main mechanisms of complement in infection defense are opsonization of pathogens by C4 and C3, leading to direct phagocytosis, mainly through CR3, or transport to reticular cells via CR1 binding (figure 2). Rarely, are bacteria lysed by complement since C5b-9 can penetrate only a subgroup of Gram-negative bacteria, especially Neisseria species.

The complement system, however, does not always induce inflammation. It has a number of non-canonical functions including embryotic organ development, like the nervous system and synapse formation. It contributes to tissue renovation, and an intact complement system is required for tissue regeneration and homeostasis. The recent discovery of intracellular functions of the complement system, termed the complosome, is a new era in the complement world, which is still in its infancy. Most importantly, complement is part of an orchestra cross-talking with many biological systems, including other plasma cascades or branches of innate immunity, including toll-like receptors, and metabolic and neuroendocrine systems.

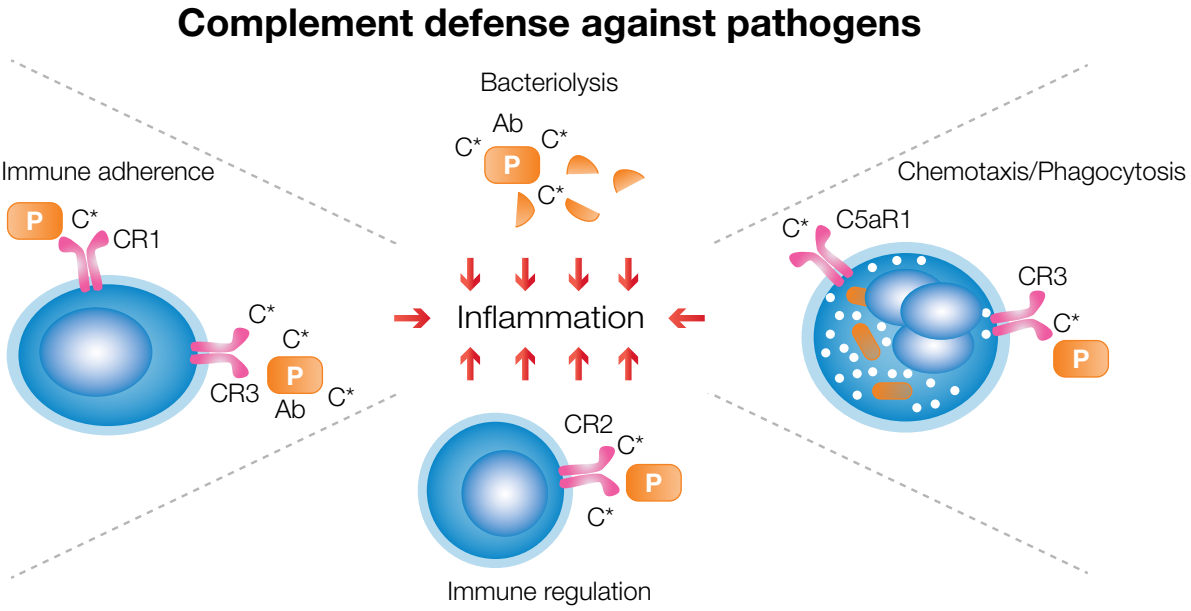


FIGURE 2. Complement in defense against pathogens.

Schematic illustration of four main mechanism by which complement protect the host against pathogens invading a tissue and leads to inflammation.

For details, see the text.

C indicates an activated complement protein or fragment (e.g. C3b, C4b, C5a)*
***P** = pathogen*
***Ab** = antibody*
***CR** = complement receptor*

2. DEFICIENCY AND DYSREGULATION: CLINICAL MANIFESTATION

2.1. REDUCED ACTIVATION POTENTIAL

The traditional complement deficiencies of ordinary components (not the regulators) imply a reduced capacity of complement to be activated when necessary. This mainly implies an increased risk of infections or a reduced capacity to renovate tissue debris, leading to autoimmune-like diseases.

INFECTIONS

Recurrent infections are frequently seen in patients with complement deficiencies. However, given that complement deficiencies are rare (except for MBL), patients with infections would only exceptionally present with a complement defect. In certain cases, it is still important to include complement tests, particularly those with recurrent bacterial infections early in life, where other immune deficiencies cannot explain the condition.

Notably, Neisseria infections are associated with complement deficiencies in the terminal pathway (C5-C9), particularly if there is a family history, if the strain is atypical, or if the infection is systemic, low-grade, and recurrent. The frequency of meningococcal disease reported in C5-C9 deficient individuals reflects a 1000 to 10,000-fold higher risk compared to the general population.

There is also a high prevalence of X-linked properdin deficiency in patients with meningococcal disease. The most common type 1 deficiency is characterized by the absence of properdin in plasma, whereas in type 2 deficiency properdin is low but detectable (<10% of normal). Notably, properdin defects are not detected in conventional hemolytic assay (AH50), but the ELISA-based total complement activity test (Wieslab®) shows a low alternative pathway activity in sera with properdin deficiency (see below for the description of this test).

However, many individuals with complement deficiencies, e.g., of C2, may be found in apparently healthy individuals. In fact, the first two C2 deficiencies were discovered in the early 1960'ies independently by two healthy immunologists who did not get their serum to work in the classical hemolytic assay. However, more than 50% of those with C2 deficiency suffer recurrent infections and require careful follow-up, including vaccination. Thus, this is an example of low penetration, suggesting that additional genetic or environmental factors are necessary to precipitate the phenotype.

Other early deficiencies like C3, however, cause serious infections early in life and kidney diseases. Probably, many complement defects were never diagnosed before death due to severe infections. Therefore, screening for complement deficiencies is critical, especially if other immune defects are excluded.

Mannose-binding lectin (MBL) is a key protein in the complement lectin pathway. MBL variant genes are found in 20-30% of the population, and functional deficiency is found in approximately 5%. Most individuals with a deficiency of MBL are completely healthy, a fact emphasizing the vital redundancy of innate host defense against infection. However, variant MBL genes are somewhat associated with increased infection risk.

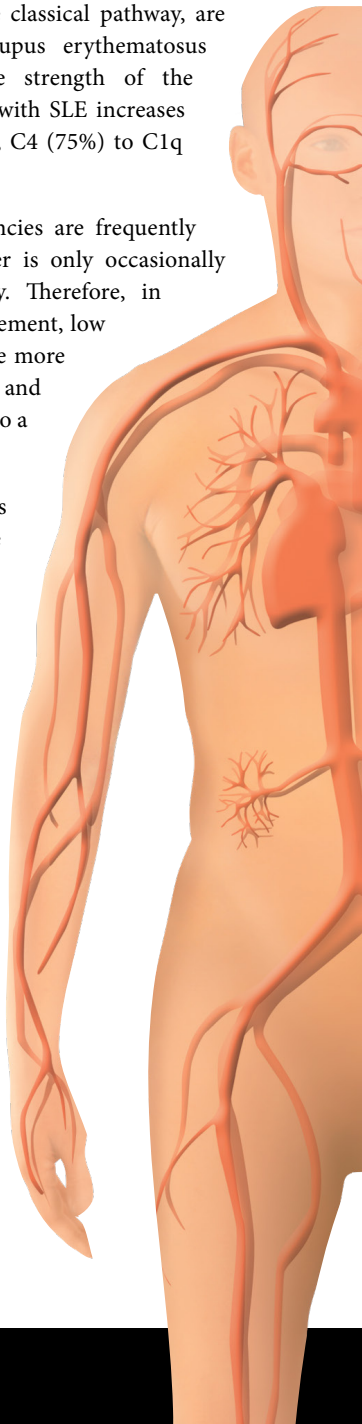
Data indicate that MBL is particularly important in early childhood between the ages of 6 and 18 months, pointing to the important role of innate immunity before establishing and developing the infant's own adaptive immune system. Furthermore, immunocompromised patients, due to hematological disease or immunosuppressive treatment, also suffer infections. Thus, it is recommendable to add MBL to the list of parameters to be tested when immunodeficiency is suspected, although interpretation should be done with caution.

AUTOIMMUNE DISEASES

Complement defects, particularly of the classical pathway, are frequently associated with systemic lupus erythematosus (SLE)- like autoimmune disease. The strength of the association of a complement deficiency with SLE increases from C2 (10% prevalence), C1r/s (57%), C4 (75%) to C1q (95% prevalence).

However, although complement deficiencies are frequently associated with autoimmunity, the latter is only occasionally associated with complement deficiency. Therefore, in active SLE, particularly with renal involvement, low total complement activity and low C4 are more often due to enhanced in vivo activation and thus, an acquired deficiency, rather than to a genetic deficiency.

Neither total complement activity tests nor tests for its activation products are typically included in routine tests for patients with autoimmune conditions, but measuring activation products is highly relevant. Fluctuation of C4 levels may also represent a reasonably good indicator of disease activity, but activation is generally better visible via increased activation markers.



2.2. ENHANCED ACTIVATION POTENTIAL

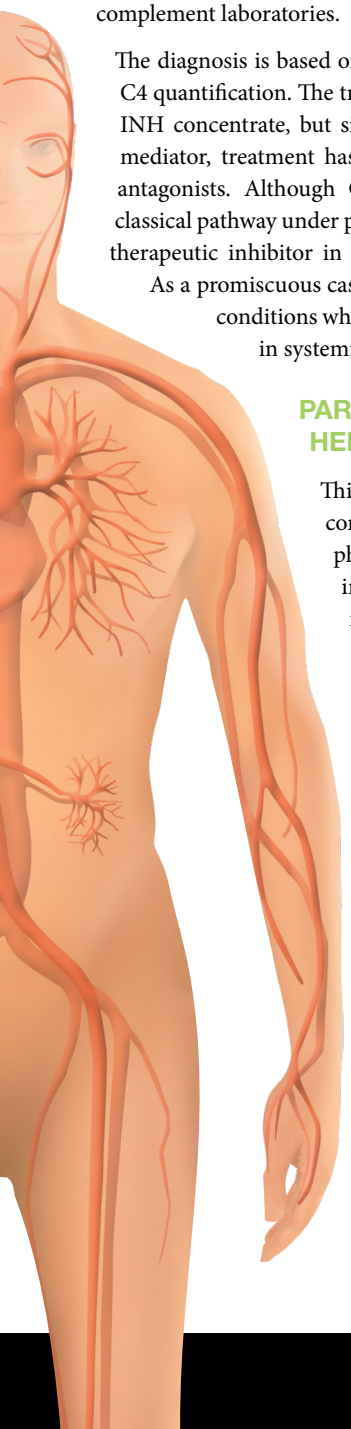
In recent years, the focus on complement in pathophysiology and disease has changed from the traditionally ordinary complement deficiencies leading to infections and autoimmunity, to complement dysregulation due to genetic defects and mutations in the regulatory proteins, e.g. factor H, ("loss of function") or mutations in the ordinary components, e.g. C3 and factor B ("gain of function") – both giving similar phenotypes, leading to diseases due to enhanced and detrimental complement activation.

A number of diseases are related to dysfunctional complement regulation, many are candidates for complement therapy (see below).

C1-INHIBITOR (C1-INH) AND HEREDITARY ANGIOEDEMA (HAE)

HAE is an autosomal dominant condition with reduced concentration (type 1) or function (type 2) of the complement regulator C1-INH. Considering the life-threatening consequences of edema formation, early diagnosis of the C1-inhibitor deficiency in these patients is extremely important. The pathophysiology of HAE is complex, but it is now generally accepted that the formation of bradykinin through activation of the kallikrein-kinin system, which C1-INH also controls, is the major inductor of the edema. Thus, HAE may not be regarded among the complement dysfunction diseases, but since the diagnosis is based on complement analyses, it has a natural place on this list in complement laboratories.

The diagnosis is based on C1-INH concentration and function and C4 quantification. The treatment of attacks can still be made by C1-INH concentrate, but since it is now clear that bradykinin is the mediator, treatment has gradually shifted to bradykinin receptor antagonists. Although C1-INH is important for control of the classical pathway under physiological conditions, it is not an efficient therapeutic inhibitor in specifically complement-induced diseases. As a promiscuous cascade inhibitor, it might still have a place in conditions where several of the cascades are activated, like in systemic inflammatory response conditions.



PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH)

This is a prototype of a disease where complement activation is the central patho-physiologic mediator. It is not genetically inherited, but due to a somatic mutation in a bone marrow cell gene coding for the phosphatidylinositol (PI) anchor (PIG-A) results in the development of a clone of cells with decreased expression of membrane proteins linked to this structure, including the complement regulators CD55 and CD59. This renders the red blood cells (RBCs) susceptible to complement-mediated lysis, which is the hallmark of this condition.

Diagnosis of PNH was traditionally made using Ham's test (acid lysis test) but is now specifically assessed by flow cytometric analysis of the respective cell surface proteins. The lysis is mainly intravascular and completely complement-dependent. The patients suffer from anemia and thromboses, and treatment consisted, for a long time, of blood transfusions only.

PNH was the first disease approved by the FDA for treatment with a complement inhibitor, eculizumab , a monoclonal antibody blocking the cleavage of C5. The treatment is effective and expensive. Most of the patients do not need transfusions and do not suffer from thromboses when treated with this drug. Still, some patients have "breakthrough" lysis due to extravascular red blood cells (RBCs) lysis. These patients may benefit from inhibiting C3, which prevents complement opsonization.

ATYPICAL HEMOLYTIC UREMIC SYNDROME (aHUS)

Complement mediated thrombotic microangiopathy (TMA), also known as atypical Hemolytic Uremic Syndrome (aHUS), is a severe disease often occurring in children and, in many cases, leads to kidney failure and the need for transplantation. Approximately 2/3 of the patients have dysregulation of the complement system with increased activation due to "loss of function" mutations in a regulatory protein (e.g., factor H, factor I, membrane co-factor protein (CD46)) or to "gain of function" mutations in the ordinary components (e.g. C3, factor B). It is important to note that the concentration of the actual component in serum may be normal even if the function is impaired.

aHUS is also FDA-approved for treatment with C5 inhibitory complement drugs. The treatment is generally highly effective, prevents progression to kidney failure, and most patients now have a good prognosis. In contrast to PNH, which needs life-long treatment, many aHUS patients remain in remission after discontinuing the treatment. They are closely followed, and in case of exacerbation, treatment is re-started, and kidney function, in most cases, is preserved.

The presence of autoantibodies to factor H, or to the C3-, C4- or C5 convertases ("nephritic factors") present with a similar phenotype as patients with genetic defects, and it is thus important to test for these to decide for the correct diagnosis and treatment.

RENAL DISEASES

Dysregulation of the alternative pathway with particular involvement of C3 may lead to a group of kidney diseases classified as C3 glomerulopathies (C3G). Among these is the dense deposit disease, previously termed membranoproliferative glomerulonephritis type II, and if the main feature is glomerulonephritis, the condition is termed C3GN. Many of these patients have low levels of serum C3 due to consumption and consequently display low activity in all pathways in the total complement activity tests.

IgA nephropathy has been approved for complement inhibition. In other renal diseases, the pathophysiology is more complex. Still, a role for complement has been suggested in nephropathy, lupus nephritis, other forms of glomerulonephritis, and antibody-mediated kidney transplant rejection.

Case reports and early clinical trials indicate that some of these conditions may benefit from complement inhibitory treatment, but larger controlled trials are needed to confirm complement inhibition as a future clinical option for different kidney diseases.

NEUROLOGICAL AND EYE DISEASES

A role for complement in the pathogenesis of several neurodegenerative diseases has been suggested, including Guillain-Barré syndrome, Alzheimer's disease, multiple sclerosis, and amyotrophic lateral sclerosis. Myasthenia gravis presents with autoantibodies to the acetylcholine receptor and impairs neuromuscular transmission signals and is currently under routine treatment with complement inhibitors. Neuromyelitis optica spectrum disorder, previously regarded as a subgroup of multiple sclerosis, is a separate entity where the pathogenic factor are autoantibodies to aquaporin 4. It might lead to blindness and is now approved for complement inhibitory treatment.

The eye disease age-related macular degeneration (AMD) is the most common cause of blindness in industrialized countries. It is closely related to mutation in the same genes as described for aHUS above, indicating a dysregulation of the alternative pathway. Clinical trials with complement inhibitors are underway. Evidence also indicates a role for complement in chorioretinitis and possibly also in glaucoma.

... AND MANY OTHER DISEASES

The above-mentioned are diseases where a role for complement has been described, and in some of them, complement therapy is already in clinical use. However, the list is much longer, including diseases like ANCA-associated vasculitis (approved for treatment with the C5aR antagonist avacopan), cold agglutinin syndrome (approved for treatment with the C1s inhibitor subtimlimab), HELLP syndrome, catastrophic anti-phospholipid syndrome, lung diseases, atherosclerosis, ischemia-reperfusion injury like myocardial infarction and stroke, trauma and sepsis leading to the systemic inflammatory response syndrome, and many more. Among these are endemic virus diseases, since increased complement activation correlated with lung function and death during Covid-19, and inhibition with a complement inhibitor increased the survival rate. Moreover the role for complement in cancer remains an area of active research.

Historically, the cell lytic potential of complement was the earliest function described, but for immune defense against pathogens opsonophagocytosis appears to be the dominant mechanism. However, the lytic activity of complement is undeniably crucial in the context of cancer.

An often overlooked molecule is hereby C7, the only terminal complement component not predominantly synthesized in the liver and postulated to be the rate-limiting factor in the terminal pathway. In line with this, recent studies could show that increasing C7 expression clearly suppressed tumor growth in vitro.

Immunotherapy using monoclonal antibodies is escalating in cancer treatment. From a complement point of view, there are two potential strategies to increase the success of this therapy; the first is to design the antibodies to activate complement effectively, and the second is to inhibit membrane complement regulators at the cancer cell surface (e.g. DAF and CD59), which can be done by bi-specific/tri-specific antibody technology.

Thus, the question that we should pose is perhaps not how many diseases complement is involved in; that list is already very long and it will likely increase. Rather, the questions is: In how many diseases is complement not involved in pathogenesis in some way or another? We suggest that such a list is unlikely to appear.

3. COMPLEMENT EVASION

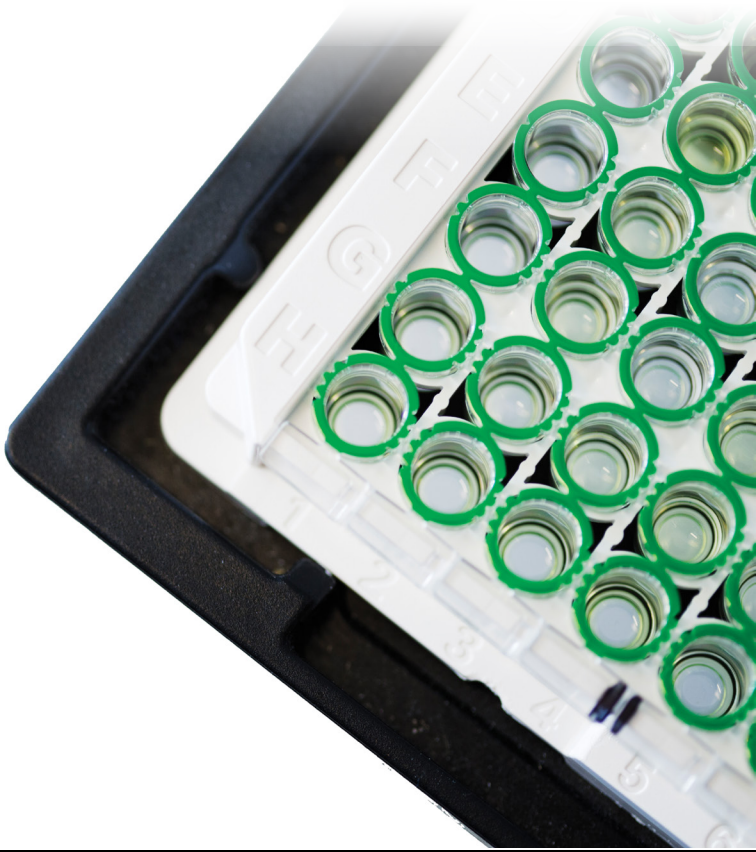
Almost all pathogens systemically invading the human host have adopted numerous mechanisms to evade complement action.

A very effective measure is simply to evade the recognition by shedding complement activation moieties, displaying poorly activating molecules, and if immunoglobulins have bound, then shedding these either alone or by sacrificing their own surface material together with the bound antibodies.

Similarly, the pathogen can shed deposited, activated complement components, avoiding further activation. Moreover, some pathogens can inhibit complement by cleaving or modifying the components, rendering them inactive.

A very clever way of evading complement is by employing host molecules, either for defense, such as recruiting factor H by displaying factor H binding molecules, or for entry into cells, such as displaying C3-like moieties, which then bind to complement receptors on the target cells.

This is in many ways facilitated by molecular mimicry, i.e., the pathogens mimic a target molecule, which then attracts the ligand host molecule for their advantage. Successful therapy must then target these pathogen molecules.



4. COMPLEMENT TESTS

4.1. SCREENING FOR DEFICIENCY

The traditional complement deficiencies of ordinary components (not the regulators) imply a reduced capacity of complement to be activated when necessary. This mainly implies an increased risk of infections or a reduced capacity to renovate tissue debris, leading to autoimmune-like diseases.

Screening of the functional activity of the complement system is first of all indicated when a deficiency of complement cannot be excluded as a cause of immune disturbances, primarily in recurrent infections and occasionally in autoimmune diseases. These tests have traditionally been performed using hemolytic assays. The principles of complement hemolytic assays are illustrated in Figure 3. They provide insight into the integrity of the entire cascade reaction. For the classical pathway (CP), serial dilutions of the sample to be analyzed are incubated with antibody-sensitized sheep erythrocytes. The results are usually expressed as a reciprocal dilution of the sample required to produce 50% lysis, the CH50 test. Tests evaluating the functional activity of the alternative pathway (AH50) also work with lysis as the readout, but with non-treated rabbit erythrocytes (alternatively chicken or guinea pig) as target cells.

A novel methodological approach, a total complement screen enzyme immunoassay (Wieslab®), has been developed to separately detect the complement activity of the different pathways, CP, LP, and AP (Figure 4). The activity of the three pathways is tested in microtiter wells coated with IgM, mannan, or lipopolysaccharide, respectively. When adding serum, the activation will continue until the assembly of C5b-C9, which can then be quantified by an antibody specific to activated C9 (Figure 4). Thus, the principle is very similar to the hemolytic assays, but this assay is more robust since it is not dependent on the quality of living cells, and it is suitable for large-scale complement deficiency screening.

The following defects should be considered depending on the pattern observed in the screening:

- Abolished CP, normal LP, and AP: C1q, C1r, C1s.
- Abolished LP, normal CP, and AP: MBL, MASPs.
- Abolished AP, normal CP, and LP: properdin, factor D and B.
- Abolished CP and LP, normal AP: C2, C4.
- Abolished CP, LP, AP: C3, C5, C6, C7, C8, C9 (or by *in vitro* inactivated serum! – always new test).

CP	MP	AP	Possible deficiency/blockade
			NONE
↓			C1q, C1r, Cls
		↓	Properdin, Factor B, Factor D
	↓		MBL, MASP2
↓	↓	↓	C3, C5, C6, C7, C8, C9, Factor H*, Factor I*
↓	↓		C4, C2

**Deficiency of Factor H and/or Factor I cause consumption of C3, i.e. secondary C3-deficiency and thereby reduced pathway activity.*

CH50 - COMPLEMENT HAEMOLYTIC ACTIVITY

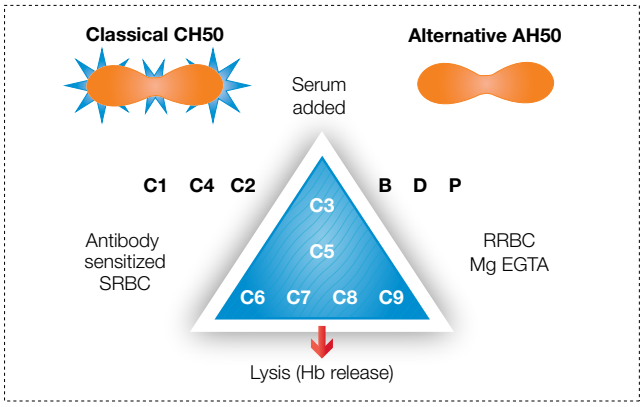


FIGURE 3. Total complement haemolytic activity.

Schematic illustration of haemolytic screening tests for the activity of classical (CH50) and alternative (AH50) pathway. Serum is added to sensitized sheep red blood cells (SRBC) for the CH50 test.

If all components of the classical and terminal pathway are present, the red cells will lyse and the readout haemoglobin (Hb) is released - "a chain is not stronger than the weakest link". Similarly, AH50 is measured using rabbit red blood cells (RRBC).

Unfortunately, this assay does not detect properdin deficiency, which is of clinical importance, whereas factor D and B deficiencies are extremely rare.

TOTAL COMPLEMENT SYSTEM ACTIVITY

Serum added to microtiter wells

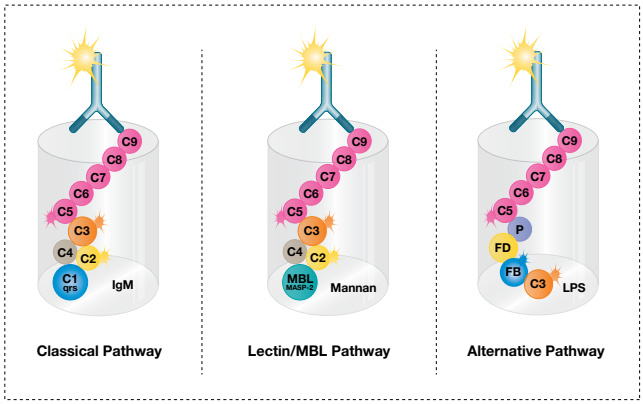


FIGURE 4. Total complement activity test (Wieslab®).

Schematic illustration of an enzyme-immuno assay screening test for separate detection of the CP, LP and AP activity. Serum is added to the wells and allowed to react with the specific activators. If all components are present in the serum, the cascade will continue until activated C9 is bound.

The principle is thus similar to the haemolytic tests, but the readout is not dependent on red cell lysis. Detection is facilitated by a monoclonal antibody reacting with a neoepitope in activated C9. Reduced activity will be seen if one or more components are low or missing.

Properdin deficiencies are detected in the AP assay, whereas AH50 is unable to do so.

4.2. INDIVIDUAL COMPONENTS

When defects are detected in the screening assay, specific assays for single components must follow. These are mostly performed by immunochemical quantification.

The initial screening will exclude many components as candidates and will likely already focus the search on components of only one pathway, which should then be verified by quantifying the individual components in question. Some homozygous deficiencies, such as factor B, factor D, or MASPs deficiencies are extremely rare. In contrast, the lack of LP activity is in >99% due to an MBL defect.

Total complement activity is sensitive to in vitro activation of complement in serum. If serum is heat-inactivated, has been stored for a long time at room temperature (see "treatment of samples" below), or contains complement activating agents (e.g. immune complexes, cold agglutinins), the activity is reduced and may even be zero. Thus, if the activity of all pathways is abolished, a new sample with fresh serum should always be tested before further investigation is done.

When immunochemical assays do not reveal any deficiency, the actual component may be functionally inactive, and a functional or genetic assay can then verify the diagnosis.

Direct identification of single components without total complement activity screening is indicated in certain cases. Thus, in routine diagnosis, C3 and C4 are most frequently measured, particularly associated with autoimmune vasculitis and glomerulonephritis. These two molecules are those that clinicians have traditionally used for disease activity monitoring for decades.

The results should, however, be interpreted with caution since several factors influence the serum C3 and C4 levels – these components:

- are produced by the liver, thus liver failure will reduce the level
- are acute phase reactants and will increase during inflammation
- can be decreased due to in vivo activation and consumption
- can be decreased, e.g., in ICU units where a lot of infusions are given, diluting all plasma proteins

The sum of the individual contributions of these factors will define the level of C3 and C4; this variability, exemplified in table 1, illustrates the advantage of measuring both native components and activation products.

	Patient 1 (F24)	Patient 2 (F35)	Reference range
C3	0.20	0.30	0.50 - 1.00 g/L
C4	0.09	0.06	0.10 - 0.50 g/L
C3dg	25	126	20 - 45 AU/mL
sC5b-9	3.9	15	2.2 - 6.6 AU/mL
Diagnosis:	**Liver failure**	**Chronic active hepatitis**	

TABLE 1. Quantification of total C3 and C4 compared with complement activation products C3dg and sC5b-9.

Two female patients aged 24 and 35 years presented with low C3 and C4 levels. One had liver failure with reduced synthesis of C3 and C4 and low activation products (C3dg, sC5b-9), the other had hepatitis without liver failure, but with substantially increased activation products consistent with in vivo complement activation leading to C3 and C4 consumption.

Thus, two completely different pathophysiological states with similar C3 and C4 profiles.

4.3. ACTIVATION PRODUCTS

A low concentration of a complement component may be due to genetic deficiency, decreased synthesis (mostly liver failure), or increased consumption due to in vivo activation, whereas acute phase reactions may increase the concentrations of a number of the components. Thus, the level of a single component may be difficult to interpret. In order to evaluate the degree of complement activation in vivo, specific activation products need to be quantified.

Novel sensitive, specific, and reliable enzyme immunoassays to detect complement activation products are based on monoclonal antibodies directed against neoepitopes, i.e. antigenic determinants exposed in the activation products but not accessible to the native components. Only the activation product will bind when a neoepitope specific antibody is used as the capture antibody in the assay. After washing, there is no native component left in the well that can compete with the activation product, and a second antibody, not necessarily neoepitope specific but detecting the activation product, can then be used for detection. By this approach, the exact amount of an activation product can be quantified directly in a sample, as illustrated for the terminal complement complex (TCC/sC5b-9; Figure 5).

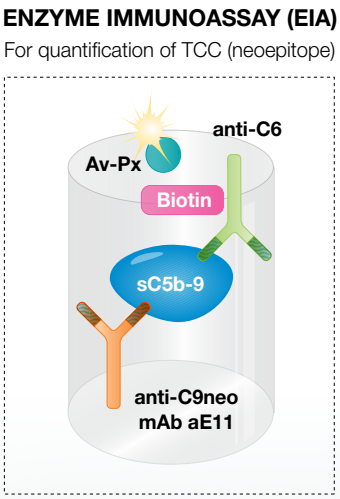


FIGURE 5. Detection of complement activation product.

Schematic illustration of an ELISA quantifying the final activation product, the soluble TCC, sC5b-9, measured by a capture monoclonal antibody (aE11) reacting with a neoepitope in activated C9 and detected with an anti-C6 antibody.

The principle is the same as for the functional screening assay – both are detecting the TCC – the functional ELISA detects in vitro formation of C5b-9 on a surface, whereas the biomarker ELISA detects sC5b-9 in solution.

A number of assays have been designed to detect complement activation products from different parts of the cascade. sC5b-9 (soluble TCC) indicates that the terminal pathway has been activated to its end, irrespective of which initial pathway was activated. TCC is less prone to in vitro activation than most of the other activation products. Together with a C3 activation product (e.g. C3a, C3bc, C3dg) it serves as a good general "screening" for the activation of the entire system.

However, to evaluate which of the initial pathway(s) are activated, other assays have to be used. C4 activation products (e.g., C4a, C4bc, C4d) indicate classical and/or lectin pathway activation, C1rs/C1-INH complexes reflect classical pathway activation and MASP-2/C1-INH complexes reflect lectin pathway activation, whereas alternative pathway activation is reflected by factor B activation (Ba, Bb) or convertase formation (C3bBbP). C5a can detect terminal pathway activation in addition to sC5b-9.

Complement activation products are usually present only in trace amounts in vivo but they are rapidly generated in vitro. Therefore, it is crucial that the samples are collected and stored properly in order to avoid in vitro activation (see below).

Furthermore, the various different in vivo half-lives of complement activation products have to be taken into account for a proper interpretation of the data. Due to rapid receptor binding, the biologically highly active and important C5a fragment has a half-life of approximately one minute. Thus, detecting this split product in samples obtained in vivo is challenging. In contrast, the various C3 activation products are readily detectable due to half-lives of a few hours, sC5b-9 has a half-life of 50-60 minutes. Thawing and freezing also increase many of the activation products, particularly those in the early part of the cascade at the C3 and alternative pathway level, whereas sC5b-9 is relatively resistant.

It has been postulated that the ratio between an activation product and the native component (e.g. C3d/C3) is a more sensitive indicator of in vivo activation. In our experience, the activation product alone is the most sensitive activation indicator. Routine use of activation products is still limited, partly due to the strict guidelines for sample handling and partly due to lack of clinical experience. However, these tests are widely used to study complement activation and to monitor complement inhibition. Since complement inhibition therapy is now increasing in the clinics, the need for these tests will likely increase.

4.4. DYSREGULATION AND GENETICS

Some disease conditions are due to complement dysregulation, most frequently due to a regulator malfunction. In the case of PNH, diagnosis is rapidly done using flow cytometry. If the clinical feature suggests aHUS or a form of C3GN, rapid immunological analysis of autoantibodies can be performed (anti-factor H, nephritic factors). Except for these analyses, few complement tests can immediately help the diagnosis. C3, C4, total functional complement screen test, and an activation product may be valuable as supplementary tests, but these might be normal despite a functional defect in, e.g., factor H.

If the mutation involves binding to endothelial cells in glomeruli, it will lead to complement attack on these cells because factor H acts as an important surface protection on endothelial cells against self-attack, but this might not affect any of the serum and plasma complement analyses mentioned. Thus, genetic tests are required.

A problem with the genetic tests has been the long response time, but with the new generation of genetic analysis, it should be possible to get the results after a few days. "Disease packages" have been set up by many laboratories, implying that when the clinical diagnosis is, e.g., aHUS, the laboratory performs genetics first on the proteins already known to be associated with the disease (e.g., Factor H, Factor I, MCP, C3, factor B).

The importance of such tests will increase in the future, both because new mutations are continuously found in the already known diseases related to complement, and new diseases will be added to the list associated with complement dysfunction.

5. HANDLING OF SAMPLES

Correct handling and treatment of samples to be analyzed for complement are critical to obtaining reliable results.

The following guidelines are recommended:

- **Total complement activity** (hemolytic assays like CH50/AH50 are now mainly replaced by total complement activity in ELISA-based assays, e.g. Wieslab® assays or similar): fresh normal serum prepared from whole blood without anticoagulants allowed to clot for 1 hr; alternatively with clot activator for 30 min; avoid gel in the tube. Storage at 4°C until frozen at -80°C, preferentially within the same day (2-4 hours). If transport to hospital is needed, 24 hours is acceptable; if the result is normal, it is fully reliable.
- **Functional assays of single proteins:** fresh normal serum as described for total complement activity above (e.g., C1-INH function) or as indicated by the instructons for use (e.g., functional Properdin assay with Serum or Plasma)
- **Protein quantification of single components** (e.g., C1-INH, C1q, C3, C4, MBL) **and autoantibodies** (e.g. anti-factor H, nephritic factors): normal serum. If only protein concentration is to be measured, the length of storage is not critical, since the assays used normally are not sensitive for in vitro activation.
- **Complement activation products:** Sampling and handling are critically important to avoid activation in vitro. Blood is drawn directly into EDTA-containing tubes, turned gently 4-5 times, and place on slushed ice until centrifuged (preferentially cooling centrifuge) for 15 min at 1,500 x g. Plasma should be removed carefully (avoid cellular elements), and stored immediately in aliquots at -70°C, or below.
- **Genetic testing:** Fresh or frozen EDTA whole blood (avoid thawing of frozen samples by shipping on dry ice).

TO BE NOTED:

- You can generally trust a normal complement result (exception: the sample has been mixed and is from another person). A pathological in vivo complement condition cannot change to normal in vitro (in contrast to the opposite).
- If a pathological result is obtained for a functional test (including total functional complement screening) or for an activation product, repeat the test on a new sample documented to be obtained and stored according to the guidelines before proceeding with more extensive analyses.

6. COMPLEMENT THERAPEUTICS

6.1. THERAPEUTIC APPROACHES

Treating patients with complement inhibitors present challenges for the complement field in developing and validating methods to monitor the efficacy of the drug. Observations made in the 1980s in Göttingen showed that a monoclonal antibody could almost completely inhibit the generation of TCC even after activation. This led to the development of eculizumab, which inhibits activation of C5. Currently many other complement inhibitors are either in clinical use, undergoing clinical trials, or continuously being developed.

The last three decades have witnessed a "complement revolution" that we could not have imagined 50 years ago. Complement inhibition is now in clinical use not only for the "prototype" condition of paroxysmal nocturnal hemoglobinuria (PNH) but is also approved for a variety of other conditions including atypical hemolytic uremic syndrome (aHUS), myasthenia gravis, neuromyelitis optica spectrum disorder (NMOSD), ANCA-associated vasculitis, IgA nephritis, age-related macular degeneration (AMD), Chaple syndrome and cold agglutinin syndrome, as well as in severe cases of COVID-19.

Numerous diseases are being considered for potential complement treatments, and many have been successfully treated off-label.

The critical question when deciding for a drug to treat complement-mediated diseases is not which drug is available but rather what the pathophysiology of the disease is. Consider the following:

- **Is there a need for substitution of a deficient component?** This is hardly the case; while MBL and FH have been suggested, there is currently no indication for their substitution.
- **Is there a need for developing complement inhibitors?** Yes, definitely. This is summarized below in the text and in Figure 6.

Specific inhibition of the initial pathways can be obtained, e.g. C1s (classical pathway), MASP-2 (lectin pathway), or factor D or factor B (alternative pathway). Targeting C3 is particularly potent as it blocks activation of all initial pathways at an early stage of the final common pathway. C3aR may also be a potential target in certain conditions, although this is more intricate as it acts both as a pro- and an anti-inflammatory receptor.

C5 inhibition has been the most attractive candidate to inhibit, for the last 15 years, primarily for treating paroxymal nocturnal hemoglubinuria (PNH) and more recently for conditions such as aHUS, myasthenia gravis, and neuromyelitis optica spectrum disorders (NMOSD). This comprise agents that either block C5 cleavage, the C5a molecule, or target the main proinflammatory molecule C5aR1.

6.2. THERAPEUTIC MONITORING

Complement tests will be increasingly important as complement inhibition expands in clinics and novel inhibitors are developed, as illustrated in Figure 6.

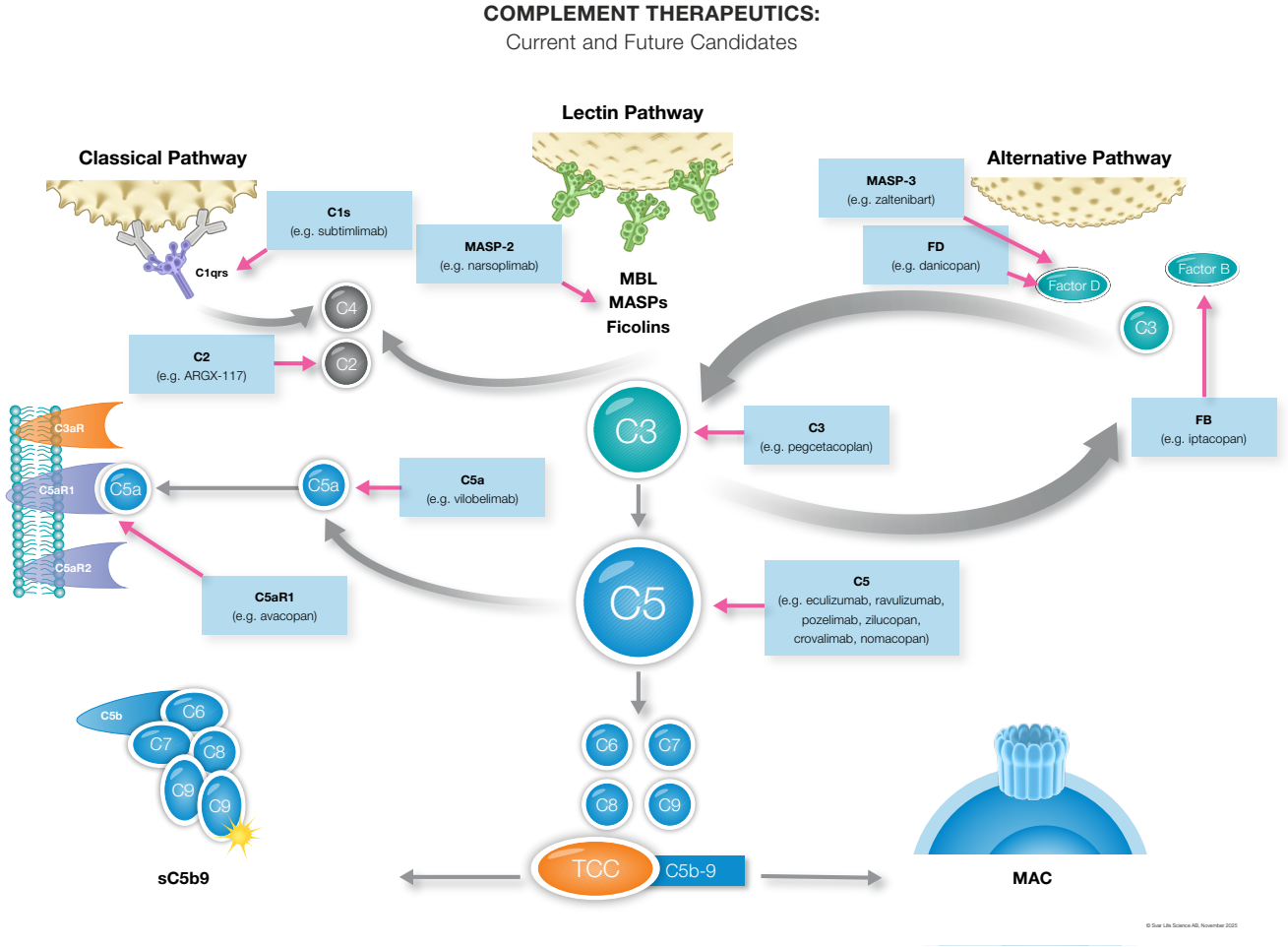


FIGURE 6. Therapeutic inhibition.

The figure illustrates in blue boxes examples of complement inhibitors acting at specific levels of the cascade. Several are FDA approved and already in clinical use whereas others are in phase II or III trials). Inhibition of C5 cleavage by eculizumab has been used since 2007 with PNH and later aHUS as the main indications. The treatment has been found efficient and safe, although an increase in Neisseria infection has been observed. A series of new inhibitors at the C5 level have been developed, as well as specific inhibitors of C3 and of all three initial pathways.

Tests capable of assessing therapeutic complement modulation are already available and used to study the efficacy of potential inhibitors both in vitro and in vivo. The functional activity measurements, from the C5b-9 read-out, in ELISA-based methods like the Wieslab® Screen assay (formerly known as Wielisa®), has the potential to be particularly useful; by individually assessing the activity of the three initial pathways, the whole complement cascade is covered. Thus, intentional blocking of the function of any of the ordinary native components can be detected; the effect of blocking C1s function would be quenched signal for classical pathway activity, MASP-2 inhibition should quench lectin pathway, and FB- or FD-targeting would lead to loss of functional read-out for the alternative pathway.

When blocking the "bottleneck" components, C3 and C5, inhibition can be evaluated using the ELISA-based functional assays, such as the Wieslab® assays, reducing or quenching activity for all three pathways. In clinical trials, this approach has demonstrated an ability to monitor pharmacodynamics, post administration, and it can be hypothesised that this kind of approach could be used to optimise and personalize dosage of complement therapeutics in the future. Complement targeting therapies are currently very costly; it is expected that competition along with development of low-cost modalities, such as small molecule drugs, will reduce the expense of complement modulating therapies.

Treatment with most complement inhibitors can easily be studied with functional complement assays in clinical trial and research settings, utilising their downstream TCC based readout. However, for certain inhibitors, monitoring may be challenging since their end-products diverge from the core complement path; for instance, new assays will likely be required to actively monitor C5a inhibitors, successfully used in treating Covid19 patients, and C5aR1 antagonists, which have seen use in ANCA Associated Vasculitis treatment.

Future pharmacological treatment will likely focus on personalized therapy, i.e. giving optimal doses to obtain effective treatment, avoiding adverse effects and reducing costs. This will be a challenge for complement inhibition therapy in the future. Thus, it will be important to design and validate complement assays which can guide clinicians in follow-up of these patients, giving the right dose at the right time.

(All interpretations and conclusions are drawn from information current as of August 2025)

Answers in Life Science

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