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Advances in analytical methods for regulatory complement proteins in research and clinical applications

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ABSTRACT

The canonical complement system and its dysregulation are increasingly realized to be associated with pathologies like age-related macular degeneration, atypical hemolytic uremic syndrome, or dense deposit disease. Resulting drug development, clinical studies, and expanding fundamental research into the complement system and its regulatory proteins all require appropriate analytical methods with which the function of the complement system can be quantified. In clinical settings, two types of routine diagnostics are mostly used. Functional assays such as the hemolytic CH50 and AH50 tests are based on erythrocytes and typically monitor complete activation pathways. Assays that quantify certain complement proteins and activation products are typically based on turbidimetry, nephelometry, and ELISAs. However, there is the need and demand for more specific examination to also identify variants with defective functions that show no deviation from normal concentrations. New diagnostic test developments are therefore moving towards faster, simpler, and more standardized tests for application in both clinical laboratories and high-throughput facilities, with the ultimate goal of precision and personalized medicine. In recent years, significant progress has been made, particularly in functional assays for fluid-phase and in flow cytometry for membrane-bound regulatory complement proteins. Functional ELISAs and liposome assays may soon replace traditional hemolytic assays, while genetic analysis has yielded important new insights into complement-related diseases; overall, there is a strong trend toward multiplexing. This review discusses current advances in analytical methods of regulatory complement proteins, covering commercial assays from industry and newly developed assay strategies from research, and assesses their impact on the field.

1. Introduction

1.1. The complement system and its regulation

The complement system is a complex protein network consisting of more than 50 soluble, peripheral and integral proteins and protein fragments. It is part of the innate humoral immune system and as such mainly responsible for the defense against pathogens and the clearance of immune complexes, apoptotic, necrotic or tumor cells [1,2]. In addition, many connections also exist to the adaptive immune system and the coagulation system [3–5]. Immune defense by the complement system relies on a cascade of enzymatic cleavages and rearrangements of proteins and their cleavage products (Fig. 1). This cascade can be initiated by three different activation pathways that are based on the binding of antibodies or the recognition of specific surface patterns: the classical, the lectin, and the alternative pathway.

The classical pathway begins when C1q binds to immunoglobulins (IgG and IgM) in antigen-antibody immune complexes or other ligands, such as C-reactive protein (CRP) [6]. C1q is associated with two molecules of C1r and two molecules of C1s, forming the C1 complex ($C1qr_2s_2$). After binding to the target, conformational changes lead to activation of C1r, which in turn cleaves and thereby activates C1s. The lectin pathway, on the other hand, involves collectins, such as mannose-binding lectin (MBL), collectin 10 (CL-10 or CL-L1), or collectin 11 (CL-11 or CL-K1), and ficolins, such as ficolin-1 (or M-ficolin), ficolin-2 (or L-ficolin), or ficolin-3 (or H-ficolin), which recognize certain carbohydrate structures on the surfaces of pathogens or altered host cells [7]. Similar to the C1 complex, these collectins and ficolins form a complex with MBL-associated serine proteases (MASP-1,

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List of abbreviations

AAE Acquired angioedema
AAV Anti-neutrophil cytoplasmic autoantibody-associated vasculitis
AD Alzheimer's disease
AH50 Alternative pathway hemolytic assay
AI Artificial intelligence
ALP alkaline phosphatase
AMD Age-related macular degeneration
ANCA Anti-neutrophil cytoplasmic autoantibody
AP Alternative pathway
ASO Antisense oligonucleotides
Ba/Bb Fragments of Factor B
C1–INH C1-esterase inhibitor
C1q, C1r, C1s Components of the C1 complex
C2–C9 Complement proteins 2–9
C3b, C4b Fragments of C3 and C4
C3G C3 glomerulopathy
C3NeFs **C3 nephritic factors**
C4BP **C4b-binding protein**
CAD **Cold agglutinin disease**
CD35/CD46/CD55/CD59 Cluster of differentiation 35/46/55/59
CFB Complement Factor B (gene)
CFH Complement Factor H (gene)
CFHR Complement Factor H-related proteins (gene)
CFI Complement Factor I (gene)
CH50 Classical pathway hemolytic assay
CHAPLE Complement hyperactivation, angiopathic thrombosis and protein-losing enteropathy
CHO Chinese hamster ovary
CLU Clusterin
CL-10 (CL-L1)/CL-11 (CL-K1) collectin 10/collectin 11
CM-HUS Complement-mediated hemolytic uremic syndrome
CP Classical pathway
CPN/CPU Carboxypeptidase N/U
CR1/CR2/CR3/CR4 Complement receptor 1/2/3/4
CRIg Complement receptor immunoglobulin
CRP C-reactive protein
DAF Decay-accelerating factor
DDD Dense deposit disease
DEAP-HUS Deficiency of CFHR plasma proteins and autoantibody positive form of hemolytic syndrome
DNP Dinitrophenyl
ECL Electrochemiluminescence
EDTA Ethylenediaminetetraacetic acid
EGTA Ethylene glycol tetraacetic acid
EID Electroimmunodiffusion
ELISA Enzyme-linked immunosorbent assay
EP Effector pathway
EVH Extravascular hemolysis
EQA External Quality Assurance
FB Factor B
FD Factor D
FDA Food and Drug Administration
FH Factor H
FHL-1 FH-like protein 1
FHRs FH-related proteins (1–5)
FI Factor I
FLAER Fluorescently labeled aerolysin
FRET Förster resonance energy transfer
GA Geographic atrophy
GPI Glycosylphosphatidylinositol
G6P Glucose-6-phosphate
G6PDH Glucose-6-phosphate dehydrogenase
HAE Hereditary angioedema
HPLC High-performance liquid chromatography
ICS International Complement Society
IUIS International Union of Immunological Societies
IgA/IgG/IgM Immunoglobulin isotype A/G/M
IgAN IgA nephropathy
LP Lectin pathway
LPS Lipopolysaccharide
MAC Membrane attack complex
MASPs MBL-associated serine proteases
MBL Mannose-binding lectin
MCP Membrane cofactor protein
MG Myasthenia gravis
NAD^+/NADH Nicotinamide adenine dinucleotide (oxidized/reduced)
NMOSD Neuromyelitis optica spectrum disorder
P Properdin
PCR Polymerase chain reaction
PIGA Phosphatidylinositol N-acetylglucosaminyltransferase subunit A (gene)
PNH Paroxysmal nocturnal hemoglobinuria
RBC Red blood cells
RID Radial immunodiffusion
SA-AKI Sepsis-associated acute kidney injury
shRNA short hairpin RNA
siRNA Small interfering RNA
SLE Systemic lupus erythematosus
SPR Surface plasmon resonance
STEC-HUS Shiga toxin-producing *E. coli* hemolytic uremic syndrome
TCC Terminal complement complex
TMA thrombotic microangiopathy
TP Terminal pathway
TTP Thrombotic thrombocytopenic purpura
VTN Vitronectin
WHO World Health Organization

MASP-2, and MASP-3), which can undergo conformational changes after binding that activate them. The two proteins C2 and C4 are cleaved by C1s in the case of the classical pathway or by MASP-1 and MASP-2 in the case of the lectin pathway into the fragments C2a and C2b as well as C4a and C4b, respectively. C2b and C4b then combine and form the C3 convertase C4b2b, which is able to cleave C3 into C3a and C3b. The alternative pathway is fundamentally different from the other two pathways in terms of its activation: it is triggered by the hydrolysis of C3, where the molecule C3(H_2O) is initially formed. This hydrolysis can take place both on pathogen surfaces and spontaneously in the fluid phase, for which reason the alternative pathway is constantly active to a certain extent. This is also referred to as the tick-over mechanism [8]. However, it is still controversial whether this mechanism is physiologically relevant or whether the alternative pathway primarily serves to amplify the classical and lectin pathway instead [9,10]. C3(H_2O) then binds to factor B (FB), which is cleaved by factor D (FD) into the fragments Ba and Bb, with Ba being released. Like C4b2b, the complex C3(H_2O)Bb represents a C3 convertase, which can transform further C3 molecules into C3a and C3b. All three activation pathways therefore converge in the generation of C3b. C3b reacts analogously to C3(H_2O) with FB and FD to form a new C3 convertase, C3bBb, which produces further C3b. The process repeats itself in a positive feedback loop, which results in the amplification of

complement activation. Together with a C3b molecule, the C3 convertases of the classical and lectin pathway (C4b2b) or the alternative pathway (C3bBb) can finally form the corresponding C5 convertases C4b2b3b or C3bBb3b, which cleave C5 into the fragments C5a and C5b.

Once activated, the complement system can launch a series of reactions in one of its effector pathways, which can either directly or indirectly cause the destruction and elimination of pathogens. One of the most important defense mechanisms is the ability to form pores in the membranes of bacteria, yeasts and other microorganisms, which leads to lysis of these cells. Together with C6 and C7, C5b first forms the C5b67

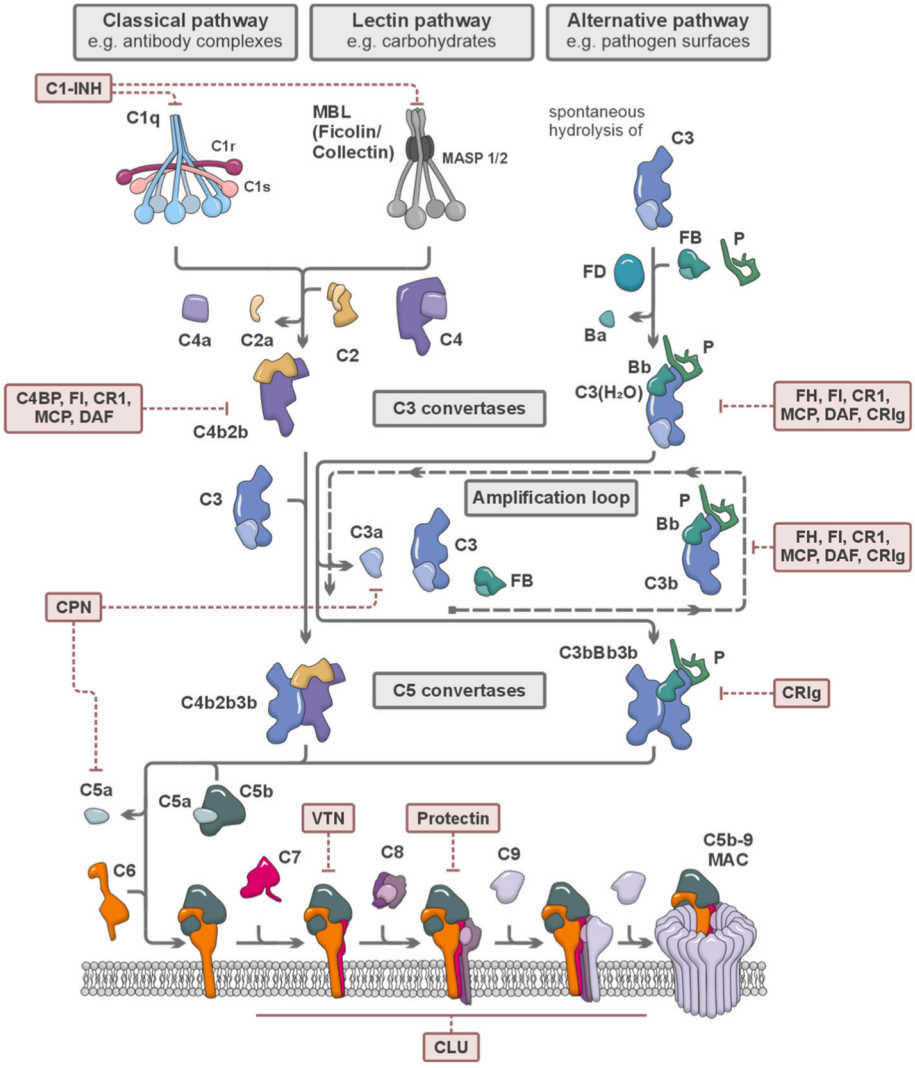

Fig. 1. Overview of the activation pathways and the terminal pathway of the complement system. Complement activation can be initiated by three different pathways: the classical, the lectin, or the alternative pathway. In the classical pathway (left), the binding of C1q to antibody complexes activates C1r and C1s, which are associated with C1q in the C1 complex. In the lectin pathway (center), collectins and ficolins recognize surface carbohydrate structures and activate MASPs upon binding to their target molecules. C1s and MASPs then cleave C4 into C4a/C4b and C2 into C2a/C2b, resulting in the formation of a C3 convertase, C4b2b. In the alternative pathway (right), C3 first hydrolyzes to C3(H_2O), which then binds to FB. The cleavage of FB by FD forms another C3 convertase, C3(H_2O)Bb. These C3 convertases cleave further C3 into C3a/C3b, creating an amplification loop. The C3 convertases finally combine with a C3b molecule and form the respective C5 convertases of the classical and lectin pathway, C4b2b3b, and alternative pathway, C3bBb3b. The C5 convertases cleave C5 into C5a/C5b, with C5b representing the starting point for the terminal pathway. C5b first combines with C6 and C7 to form the C5b67 complex, which incorporates into the target membrane. The C5b67 complex ultimately binds to C8 and several C9 molecules to assemble in the C5b-9 complex (MAC), a transmembrane pore that causes lysis of the target cell. Regulatory complement proteins intervene at various points in complement activation. P is the only positive regulator and can serve as a platform that stabilizes the alternative pathway C3 and C5 convertases. All other regulatory complement proteins are negative regulators with one or more inhibitory functions. C1–INH inhibits C1r/C1s and MASPs, thereby preventing the cleavage of C2 and C4. C4BP accelerates the decay of the classical and lectin pathway C3 convertase and serves as a cofactor for FI. FH, together with its alternative splice variant FHL-1, accelerates the decay of the alternative pathway C3 convertase and serves as a cofactor for FI. The exact function of FHRs is still controversial, but they probably fine-tune the modulation of the alternative pathway through competition with FH and FHL-1. FI inhibits the assembly of C3 convertases by cleaving and thereby inactivating C3b and C4b in the presence of a cofactor. CPN inactivates the anaphylatoxins C3a and C5a by removing arginine. CLU and VTN inhibit the formation and insertion of the MAC into the target membrane. CR1 and MCP bind to C4b/C3b, destabilize the C3/C5 convertases, and serve as cofactors for FI. DAF binds to the C3/C5 convertases and accelerates their decay. Protectin binds to C8 and C9 and prevents the formation of the MAC. CRIg binds to the alternative pathway C3/C5 convertases and thereby inhibits the cleavage of C3/C5. Abbreviations used in this figure: C1–INH, C1-esterase inhibitor; C4BP, C4b-binding protein; CLU, clusterin; CPN, carboxypeptidase N; CR1, complement receptor 1; CRIg, complement receptor immunoglobulin; DAF, decay-accelerating factor; FB, Factor B; FD, Factor D; FH, Factor H; FHL-1, FH-like protein 1; FHRs, FH-related proteins 1-5; FI, Factor I; MAC, membrane attack complex; MASPs, MBL-associated serine proteases; MBL, mannose-binding lectin; MCP, membrane cofactor protein; P, Properdin; and VTN, vitronectin.

complex, which inserts itself into the lipid bilayer of the cell membrane. C5b67 then combines with C8 and several C9 molecules to form the complete C5b-9 complex, a transmembrane pore, also known as membrane attack complex (MAC) or terminal complement complex (TCC) [11,12]. Furthermore, C3b and C4b have the ability to label pathogen surfaces, foreign particles, or cellular debris (opsonization), facilitating their recognition by phagocytic cells. The exposure of a reactive thioester group ensures that C3b and C4b can bind covalently to nucleophilic groups on these surfaces [13]. C3a and C5a, in turn, serve as anaphylatoxins, which can bind to specific receptors, promoting inflammatory reactions and recruiting immune cells to the site of infection or injury (chemotaxis) [14]. Overall, the complement system can thus be regarded as a potent and effective defense barrier. At the same time, however, tight regulation is required to prevent excessive complement activation and protect healthy self-tissue from inflammation and complement-mediated cell injury. It is therefore only logical that most regulators of the complement system primarily have inhibitory functions.

The regulation of the canonical complement system is ensured by a variety of both fluid-phase and membrane-bound proteins, which together maintain the delicate balance between the highest possible defense against pathogens and the least possible damage to self-tissue (immune homeostasis) (Fig. 1). The fluid-phase regulatory complement proteins are soluble proteins that are mainly distributed in the blood, but also occur in other body fluids (e.g. synovial fluid, cerebrospinal fluid, or aqueous humor [15–18]), and include: C1-esterase inhibitor (C1–INH), C4b-binding protein (C4BP), Factor H (FH), FH-like protein 1 (FHL-1), FH-related proteins 1-5 (FHR-1, FHR-2, FHR-3, FHR-4, FHR-5), Factor I (FI), Properdin (also known as Factor P), carboxypeptidase N (CPN, also known as kininase I or anaphylatoxin inactivator), clusterin (CLU, also known as SP-40,40 or Apolipoprotein J) and vitronectin (VTN, also known as S-protein) [19]. Among the membrane-bound regulatory proteins are: complement receptor 1 (CR1, also known as CD35), membrane cofactor protein (MCP, also known as CD46), decay-accelerating factor (DAF, also known as CD55), protectin (also known as CD59, MAC-inhibitory protein, or membrane inhibitor of reactive lysis) and complement receptor immunoglobin (CRIg, also known as VSIG4 or Z39Ig) [19]. Although these proteins are primarily cell surface proteins, soluble forms also exist that can be found in certain body fluids (e.g. intraocular fluid [20]).

C1–INH and C4BP are regulatory complement proteins of the classical and lectin pathway. C1–INH is a serine protease inhibitor that is able to bind to C1r and C1s as well as MASP-1 and MASP-2 and inactivate them. Thus, C1–INH already interferes at the initiation of these two activation pathways and ensures that C2 and C4 are not cleaved. In addition, C1–INH also plays an important role in coagulation, fibrinolysis, and the kinin pathway [21]. C4BP, on the other hand, regulates C4b, which is produced further downstream in the enzymatic cascade, by binding to C4b, accelerating the decay of the classical and lectin pathway C3 convertase C4b2b, and serving as a cofactor for FI. C4BP recognizes altered host cells through complex formation with protein S, which binds to phosphatidylserine on apoptotic and necrotic cells [22]. FH, in turn, is the key regulator of the alternative pathway and, similar to C4BP, has three important functions: it binds to C3b, accelerates the decay of the C3 convertase C3bBb, and also serves as a cofactor for FI. Through its C-terminal region, FH recognizes certain negatively charged molecules on the surface of host cells and of the extracellular matrix, such as sialic acids and glycosaminoglycans [23]. In sum, C4BP and FH together protect healthy host cells from complement [24]. A shorter, alternative splice variant of FH is FHL-1, which has the same regulatory N-terminal domains as FH but lacks some of the C-terminal domains responsible for host surface recognition. As a result, it shares similar activities with FH by contributing to the regulation of the alternative pathway on host cells, but with a much lower efficiency in discriminating between self and non-self surfaces, especially with regard to sialylated surfaces [25]. However, due to its lower molecular weight and lack of glycosylation, it has another important function in the eye where, unlike FH, it is able to passively diffuse through Bruch's membrane, bind to heparan sulfate, and enable tissue-specific regulation of the extracellular matrix in the eye [26]. The FHR proteins, on the other hand, are proposed to be responsible for fine-tuning the modulation of the alternative pathway by partially counteracting the inhibitory activity of FH and FHL-1 [27]. While the functions of FH and FHL-1 have already been well characterized, the exact functions of FHR proteins have not been fully elucidated. Recent studies have shown that they can enhance complement activation and opsonization, but some early studies also observed an attenuating effect [28]. In addition, functions outside the canonical complement cascade involving the regulation of inflammation as well as the interaction with various immune cells have also been described for them [29]. FH, FHL-1, and the FHR proteins are closely related in terms of their evolutionary origin, location in the genome, structure and function, for which reason they are referred to collectively as the FH protein family [30]. FI is an important regulatory complement protein that acts on all three activation pathways. FI is also a serine protease and is responsible for converting C3b and C4b into their respective inactive forms, thereby preventing the assembly of the C3 convertases. The cleavage of C3b and C4b by FI leads to the formation of iC3b (with release of C3f), which can then be further degraded to C3dg (with release of C3c) and C3d, or to the formation of C4d (with release of C4c). For this process, FI requires a cofactor: FH, CR1, or MCP for C3b, and C4BP, CR1, or MCP for C4b [31,32]. Properdin is the only regulatory complement protein with an activating function. Specifically, it mediates the formation of the alternative pathway C3 and C5 convertases, stabilizes them, and thereby increases their activity. Studies have also shown that it is able to initiate the alternative pathway by binding to pathogen surfaces and by acting as a platform for the assembly of the alternative pathway C3 convertase [33]. CPN is a metalloprotease capable of removing basic amino acid residues from various peptides and proteins in the blood. Its role as a regulatory complement protein is to cleave C-terminal arginine from the anaphylatoxins C3a and C5a, thereby inactivating them [34]. Vasodilatory kinins (e.g. bradykinin) are also among the substrates of CPN, which increase vascular permeability. Hence, CPN participates in the regulation of inflammation in several aspects [35]. Equally multifunctional are CLU and VTN, which are both ubiquitous proteins distributed throughout the human body. Besides their many physiological functions, such as the removal of misfolded proteins, transport of lipids, tissue remodeling or cell adhesion, their role in complement regulation is to inhibit the terminal pathway through interaction with the late complement proteins (C6, C7, C8, and C9) and MAC precursor complexes [36–40]. Since CLU and VTN bind to the exposed hydrophobic regions of the nascent C5b-7 complex, a site where C5b-7 typically attaches to the cell membrane, they effectively interfere with C5b-7 deposition and incorporation into the lipid bilayer. Due to steric hindrance, they also prevent the polymerization of C9 into a ring-like structure [41]. Overall, CLU and VTN block the formation of the MAC, thus protecting host cells from damage through lytically active pores.

CR1, the first of the in total four complement receptors (next to CR2, CR3, and CR4), is an integral membrane protein expressed on erythrocytes, myeloid cells and a few other cells, such as B-lymphocytes and brain cells [42]. It generally binds to C3b and C4b, thereby inhibiting complement activation by destabilizing the C3 and C5 convertases and inactivating C3b and C4b through its function as a cofactor for FI. Depending on the cell type on which it is present, it also has various other functions: on granulocytes and monocytes, CR1 promotes the recognition and phagocytosis of immune complexes and particles, and on erythrocytes, CR1 facilitates the clearance of opsonized immune complexes from the bloodstream [43]. Moreover, the proliferation of B-lymphocytes is influenced by the occupancy of CR1 on these cells, hence CR1 also plays a crucial role in B-lymphocyte regulation [44]. MCP, another integral membrane protein, is widely expressed on almost all cell types, except for erythrocytes. Similar to CR1, it binds to C3b and

C4b and serves as a cofactor for FI, participating in the degradation of C3b and C4b and preventing unwanted complement activation on host cells. Apart from its role in complement regulation, it also has an important function in the modulation of T-lymphocyte activation, proliferation and differentiation [45]. Since it is exploited by many bacteria and viruses as a receptor for their attachment and entry into host cells, it is often referred to as a "pathogen magnet" [46]. DAF and protectin are regulatory complement proteins found on the surface of a broad range of cell types, including erythrocytes, leukocytes, endothelial and epithelial cells, where they protect these cells from complement attack [47–49]. In contrast to CR1 and MCP, they are both peripheral membrane proteins anchored to the outer membrane side by glycosylphosphatidylinositol (GPI). As the name suggests, DAF inhibits the progression of the activation pathways and the formation of C3b and C5b by binding to the C3 and C5 convertases and accelerating their decay [50], while protectin acts at the terminal pathway stage and prevents the formation of the MAC by binding to C8 (α-chain) and C9 and inhibiting the polymerization of C9 [51,52]. CRIg is another integral membrane protein that is only expressed on tissue resident macrophages and dendritic cells [53,54]. It exists in both a short and a long isoform, consisting of either one or two immunoglobin domains, respectively. By binding to C3b and iC3b, it promotes phagocytosis of opsonized particles, pathogens, and altered host cells [54], and by binding to the C3b subunit of the alternative pathway C3 and C5 convertases, it inhibits the cleavage of C3 and C5 and blocks further complement activation [55]. This dual function of CRIg also enables an anti-inflammatory removal of altered host cells, as with C4BP and FH, and thus prevents the development of autoimmune diseases.

1.2. Pathologies associated with regulatory complement proteins

Regulatory complement proteins, circulating in the blood and other body fluids or located on the surface of various cell types, are distributed across the entire human body. In a healthy state, regulatory complement proteins influence both the activation pathways and the effector pathways of the complement system. Fluid-phase regulatory complement proteins distinguish healthy self-tissue from altered or non-self-tissue and perform their functions on cell surfaces or in the extracellular matrix, while membrane-bound regulatory complement proteins label cells of the host tissue. Together, they operate in a complex interplay with each other, with other components of innate and acquired immunity, and even beyond that, with other central physiological mechanisms of the body. Consequently, disorders of regulatory complement proteins can give rise to a range of pathologies, which can occur for several reasons: i) single or multiple mutations in the genes encoding regulatory complement proteins (e.g. *CFH*) or in stem cell clones (e.g. phosphatidylinositol N-acetylglucosaminyltransferase subunit A gene, *PIGA*) can lead to deficiency or dysfunction of these proteins, ii) autoimmune diseases can cause the production of autoantibodies (e.g. anti-C1-INH, anti-FH) that limit the activity of certain regulatory complement proteins, and iii) impaired synthesis (e.g. due to liver diseases), overconsumption (e.g. due to chronic inflammatory and autoimmune diseases), or increased loss (e.g. due to kidney or gastrointestinal diseases) can result in reduced plasma levels of regulatory complement proteins [56]. Accordingly, these pathologies can be either inherited or acquired during the lifetime of the patient. Interfaces that are in constant contact with the complement system are particularly sensitive to disorders of regulatory complement proteins. These pathologies therefore mostly affect organs such as the kidneys and blood, but can also affect the skin, eyes, brain, and organs of the respiratory and gastrointestinal tracts [57]. In the following section, we discuss some of the most prominent clinical conditions associated with regulatory complement proteins. Each pathology, the respective regulatory complement proteins involved, and the most relevant genetic variations related to the disease are summarized in Table 1.

A central function of the canonical complement system is the defense against pathogens (i.e. bacteria, viruses, fungi, and parasites). Deficiency of the regulatory complement proteins FH and FI is often associated with low FB and C3 plasma levels, as these alternative pathway proteins are consumed excessively due to uncontrolled complement activation [108]. In combination with a deficiency or dysfunction of properdin as well as the pathogen recognition proteins (i.e. C1q, MBL),

Table 1
Selection of pathologies associated with regulatory complement proteins. Symbols used in this table: *deletion has a protective effect; †expression decreased; ‡expression increased; §expressed and secreted or recruited to cell surface; and N/A: not applicable.

Pathology	Symptoms	Associated regulators	Genetic variations	References
Recurrent infections	recurrent infections with pyogenic bacteria, such as *Neisseria meningitidis* and *Streptococcus pneumoniae*	FH, FI, and properdin	C1qA/B/C, C2, C3, C4A/B, C5, C6, C7, C8A/B/G, C9, MBL2, CFH, CFI, CFP	[58,59]
Hereditary angioedema (HAE)	acute, recurrent edema of the dermis, subcutis, and submucosa in the area of the face, extremities, genitals, or in the upper respiratory and gastrointestinal tract	C1–INH	SERPING1, F12, PLG, ANGPT-1, KNG1, MYOF, HS3ST6	[60,61]
Age-related macular degeneration (AMD)	impaired central vision to complete vision loss, drusen formation, choroidal neovascularization	FH, FHL-1, FHR-1/FHR-3, FHR-4‡, FI, and VTN	C2/CFB, C3, C9, CFD, CFH, CFHR1/CFHR3*, CFI, ARMS2, HTRA1, VTN	[62–75]
Geographic atrophy (GA)	progressive macular degeneration, vision loss	MCP†	C3	[76,77]
Atypical hemolytic uremic synodrome (aHUS)	microangiopathic hemolytic uremia, thrombocytopenia, impaired renal function	FH, FHR-1/FHR-3, FI, and MCP	C3, CFB, CFH, CFHR1/CFHR3, CFI, MCP/CD46, THBD/CD141	[78–82]
Dense deposit disease (DDD)	hematuria, proteinuria, hypertension, facial and peripheral edema	FH, FHR-1/FHR-3, and FHR-5	C3, CFH CFHR1/CFHR3*, CFHR5	[83–86]
IgA nephropathy (IgAN)	frequently asymptomatic, intermittent hematuria or proteinuria possible, impaired renal function	FH, FHR-1/FHR-3, FHR-1/FHR-5‡, and MCP†	CFH, CFHR1/CFHR3*, CFHR5	[87–94]
Paroxysmal nocturnal hemoglobinuria (PNH)	hemolytic crises, thrombosis and thromboembolism	DAF and protectin	PIGA	[95–98]
Complement hyperactivation, angiopathic thrombosis, and protein-losing enteropathy (CHAPLE)	complement hyperactivation, angiopathic thrombosis, and protein-losing enteropathy	DAF	DAF/CD55	[99]
Alzheimer's disease (AD)	gradual decline of cognitive functions	CR1 and CLU	APOE, APP, PSEN1, PSEN2, CR1, CLU, BIN1, PICALM	[100–105]
Cancer	type-specific symptoms, neoplasm, fatigue, fever, weight loss, edema	C4BP§, FH§, FHL-1§, MCP‡, DAF‡, and protectin‡	N/A	[106,107]

early pathway proteins (i.e. C2-4) and the proteins of the MAC (C5-9), this leads to an increased susceptibility to recurrent infections, which applies in particular to infections with pyogenic bacteria, such as *Neisseria meningitidis* and *Streptococcus pneumoniae* [109]. Predispositions are therefore located on the genes that encode these complement proteins (i.e. *C1qA/B/C*, *C2*, *C3*, *C4A/B*, *C5*, *C6*, *C7*, *C8A/B/G*, *C9*, *MBL2*, *CFH*, *CFI*, *CFP*) [58].

A disease that, although not triggered by complement dysregulation, is closely related to a single regulatory complement protein (C1–INH) is hereditary angioedema (HAE). HAE is characterized by acute, recurrent edema of the dermis, subcutis, and submucosa in the area of the face, extremities, genitals, or in the upper respiratory and gastrointestinal tract [60]. There are three types of HAE: in type I, the plasma level of C1–INH is low; in type II, the plasma level of C1–INH is normal, but the C1–INH present is dysfunctional; and in type III (also referred to as nlC1-INH-HAE), both the plasma level and function of C1–INH are normal, but the production of bradykinin is increased. In type I and II, this is due to a mutation in the *SERPING1* gene (which encodes C1–INH) while in type III (nlC1-INH-HAE), mainly the *F12* gene of coagulation factor XII, but also other genes, are altered by mutations [110]. There is also an acquired form of angioedema (AAE), which can be caused by underlying lymphoproliferative disorders (e.g. lymphoma) or autoimmune diseases (e.g. systemic lupus erythematosus, SLE) due to excessive consumption of C1–INH or autoantibodies against C1–INH [111]. The development of edema can be attributed to the role of C1–INH in the kinin pathway, whereby a defective C1–INH can lead to the activation of the contact system and an overproduction of the vasodilator bradykinin. Furthermore, C1–INH deficiency or dysfunction in HAE are typically accompanied by an overactivation of the C1 complex and a depletion of C2 and C4 [61].

Regulatory complement proteins are also strongly linked to ophthalmological diseases such as age-related macular degeneration (AMD). Patients with AMD suffer from impaired central vision, which can culminate in complete vision loss in the course of the disease [112]. Over time, changes occur in the retinal pigment epithelium and Bruch's membrane, leading to inflammation, hypoxia, and cell death. Consequently, cellular debris accumulates and results in the formation of drusen. In the advanced, neovascular form of AMD (so-called wet AMD), new abnormal blood vessels additionally grow in the choroid and leak fluid, contributing to faster and more severe vision loss [62,113,114]. Behind the condition lies a complex interaction of various causes, including genetic, lifestyle, environmental, and age-related factors [115–117]. However, the complement system appears to play a particularly crucial role, as several variations in the genes of complement proteins (e.g. *C2/CFB*, *C3*, *C9*, *CFD*, *CFH*, *CFI*, *VTN*) can influence both the risk of AMD and its progression [64–66]. The most important example is the Y402H polymorphism in the *CFH* gene, which alters the binding of FH to its ligands and causes excessive complement activation as well as reduced clearance of cellular debris [118,119]. Interestingly, a common *CFH* haplotype with a deletion of *CFHR1* and *CFHR3* is associated with a decreased risk for AMD [73,74], while other studies suggest a prominent role of FHRs in AMD in general [63,120]. Moreover, deficiencies or dysfunctions of complement proteins, such as C3, FB, FD, or FI, may also contribute to the dysregulation of the alternative pathway in the eye [67,70,71,75]. The advanced, non-neovascular form of AMD (so-called dry AMD) is more common than the neovascular form [121]. As it progresses, sharply circumscribed atrophic lesions may occur on the macula, which is associated with further macular degeneration and vision loss. This condition is then also referred to as geographic atrophy (GA) [76]. As another regulatory complement protein, MCP was found to play an important role in GA, with altered expression of MCP leading to an increased susceptibility of retinal pigment epithelial cells towards complement attack [77].

Atypical hemolytic uremic syndrome (aHUS) is a form of thrombotic microangiopathy (TMA, also including thrombotic thrombocytopenic purpura) and is characterized by a combination of three conditions, namely microangiopathic hemolytic anemia, thrombocytopenia, and impaired renal function [81]. These conditions often occur in acute episodes, which can be triggered by certain events, such as infections, pregnancy, or medications [80]. In contrast to typical HUS (also referred to as STEC-HUS), which primarily affects children under 5 years and is caused by an infection with Shiga toxin-producing *Escherichia coli*, the age at onset of the disease is more broadly distributed in aHUS (also referred to as CM-HUS) [122,123]. The disorder is mainly caused by an uncontrolled activation of the alternative pathway on endothelial cells, followed by damage to these cells, activation of the coagulation system, thrombus formation, and disruption of erythrocytes [124]. Especially in glomeruli, microvascular thrombi can block sensitive blood vessels and, in severe cases, lead to kidney failure [124]. Pathogenesis in aHUS patients is often related to a combination of acquired and congenital factors, such as the development of autoantibodies, which are mostly directed against FH, and genetic defects [125]. Specifically, these genetic defects can be either gain-of-function mutations (e.g. *C3* and *CFB*) or loss-of-function mutations (e.g. *CFH*, *CFI*, *MCP/CD46*, and *THBD/CD141*): mutations in the genes encoding C3 and FB can generate defective (i.e. more stable) C3 convertases with increased activity, while mutations in the genes encoding regulatory complement proteins (i.e. *CFH*, *CFI*, *MCP/CD46*) and thrombomodulin result in reduced plasma levels or decreased activity of the respective protein and thus unrestricted complement activation [78,79]. A special case is a form of aHUS characterized by anti-FH autoantibodies together with a deletion in *CFHR1/CFHR3*, which encode FHR-1 and FHR-3 (DEAP-HUS) [126].

Other disorders that severely affect the kidneys include dense deposit disease (DDD, formerly classified as membranoproliferative glomerulonephritis type II) and IgA nephropathy (IgAN or Berger's disease). DDD is a form of C3-glomerulopathy (C3G, also including C3-glomerulonephritis and CFHR5-nephropathy) associated with symptoms such as hematuria, proteinuria, hypertension, and edema in different areas of the body [84,127]. Patients with IgAN, by contrast, frequently remain asymptomatic, although the course of the disease is very variable [89]. Intermittent hematuria or proteinuria are possible, but some patients also suffer from severe impaired renal function or even kidney failure [128]. After blood and urine analyses, the diagnosis of both diseases is typically confirmed by a kidney biopsy. Subsequent histological examination of the biopsy allows the identification of C3 deposits at the glomerular basement membrane with electron microscopy for DDD, whereas for IgAN, IgA immune complexes in combination with C3 and properdin deposits in the glomerular mesangium are detected via immunofluorescence [129,130]. Despite the two cases both being characterized by an immune overreaction with inflammation, the pathophysiological mechanism of the two diseases is fundamentally distinct: DDD involves uncontrolled activation of the alternative pathway, mainly in the fluid phase (rather than at the endothelial cell surface), leading to an accumulation of C3 in the glomeruli. This can be caused by a deficiency or dysfunction of FH due to genetic defects, but also by the development of autoantibodies such as anti-FH, anti-FB, anti-C3b, or C3 nephritic factors (C3NeFs), which impair FH functional activity or stabilize the C3 convertase and prevent its regulation by FH [84,131,132]. In IgAN, the glycosylation of IgA1 is often defective, with IgA1 *O*-glycans being deficient of galactose. This causes the immune system to recognize the IgA antibodies as antigens, resulting in the production of *O*-glycan-specific autoantibodies directed against them. These autoantibodies then form immune complexes with the IgA1 and accumulate in the glomeruli, where they activate mesangial cells. As a result, mesangial cells begin to proliferate, secrete extracellular matrix components, and release cytokines and chemokines, inducing a state of local inflammation [94,133]. While the complement system may not be directly responsible for IgAN pathogenesis, it substantially participates in the destructive process, as it gets activated mainly via the lectin or alternative pathway, thereby overall enhancing the inflammatory reaction [92,134]. Moreover, predispositions in the genes encoding FH, FHR-1, FHR-3, and FHR-5 as well as decreased expression of MCP may

contribute to IgAN risk and progression [87,90,91,93]. Hence, the underlying disorder in DDD can be considered a complement-mediated disease with dysregulation of the alternative pathway, whereas in IgAN, it is an immune complex-mediated disease with an autoimmune reaction that engages the complement system and its regulators.

Apart from fluid-phase regulatory complement proteins, membrane-bound regulatory proteins are also linked to a range of pathologies. A condition that mainly affects erythrocytes is paroxysmal nocturnal hemoglobinuria (PNH) [96]. The name is misleading, as PNH is a chronic condition (not paroxysmal) in which erythrocytes are continuously lysed by the complement system (not only at night). Despite being a genetic disorder, PNH is usually acquired during the lifetime of the patient. This is because the disease is typically triggered by a spontaneous somatic mutation in the *PIGA* gene within a single (or multiple) hematopoietic stem cell followed by its clonal expansion [97,98]. The mutation alters the expression of DAF and protectin, causing these proteins to lack their GPI anchor. As a result, DAF and protectin cannot properly attach to the cell membrane, leaving erythrocytes (and all other blood cells derived from the stem cell) deficient in these two key regulators and thus more vulnerable to complement attack [95]. This leads to increased complement-mediated lysis of erythrocytes, which can manifest itself in hemolytic crises, but also dangerous to life-threatening thromboses and thromboembolisms due to cross-activation of the coagulation system [135]. Another genetic disorder of membrane-bound regulatory proteins is CHAPLE syndrome [136,137]. The name is an acronym and describes the conditions that characterize CHAPLE syndrome: complement hyperactivation (CH), angiopathic thrombosis (A), and protein-losing enteropathy (PLE). Unlike PNH, it is caused by a germline mutation in the *CD55* gene (which encodes DAF) and is therefore hereditary. The mutation causes a deficiency of DAF, which leads to dysregulation of the complement system and cross-activation of the coagulation system [137]. For this reason, patients with CHAPLE syndrome also carry a high risk of thrombosis [137]. Other symptoms include abdominal pain, nutrient deficiency, and edema of the face and extremities [99]. This demonstrates that the symptoms of CHAPLE primarily focus on the gastrointestinal tract, while PNH mainly affects the blood and bone marrow.

The role of the complement system and its regulators in neurological and oncological disorders has gained increasing attention in recent years and continues to be the focus of ongoing research. Alzheimer's disease (AD) is a neurodegenerative disease in which cognitive abilities gradually decline, manifesting primarily in symptoms such as memory loss, disorientation, and an inability to perform daily tasks independently [103]. The pathophysiology of AD in the brain is complex, but significant changes in the neurons involve the formation of extracellular plaques from amyloid-β (Aβ) peptides and intracellular neurofibrillary tangles from tau proteins, which results in the disruption of neuronal function and the destruction of synapses, followed by neuronal cell injury and death [100]. With the progression of the disease, certain regions of the brain undergo increasing degeneration (brain atrophy). A dysfunction of glial cells, specifically microglia and astrocytes, which are responsible for the clearance of protein aggregates, may also cause a state of chronic inflammation, leading to further damage to brain cells and driving the condition forward [138,139]. The complement system is part of this neurodegenerative and inflammatory process [101], with complement proteins and activation products being found in the vicinity of Aβ plaques [140–142]. Due to the low expression of regulatory complement proteins on their surface, brain cells are particularly vulnerable to complement-mediated cell injury [143,144]. Moreover, genome-wide association studies have identified variations in the genes of two regulatory complement proteins, namely CLU and CR1, that confer an increased susceptibility for AD [102,104,105]. Several studies have also suggested CLU in plasma and cerebrospinal fluid as a biomarker for AD [145–148]. The role of CLU could be associated with its function as an extracellular chaperone, as it binds to Aβ peptides and impacts their aggregation and the clearance of Aβ plaques [149]. Results from other studies indicate that CLU can interact with tau protein and BIN1 [150] and affect tau protein fibril formation [151]. The expression of CR1 on glial cells implies an influence of CR1 on the phagocytosis of these cells, which could also be related to the removal of Aβ plaques [152]. However, the exact mechanisms behind the role of CR1 and CLU in AD are still poorly understood and remain subject to further investigations.

A similarly broad field of research is the relationship between the complement system and cancer. The complement system is generally ascribed a dual role in cancer, as it both assists in the recognition and elimination of malignant host cells and promotes anti-tumor immune responses, while also facilitating an immunosuppressive tumor microenvironment and tumor metastasis [106,107]. Membrane-bound regulatory complement proteins have been linked to a variety of cancer types, including gastrointestinal cancers [153–159], lung cancer [160], liver cancer [161], breast cancer [162], ovarian cancer [163], cervical cancer [164], bladder cancer [165], uveal cancer [166], thyroid cancer [167], blood cancer [168,169] as well as head and neck cancer [170,171]. In general, upregulated expression of these regulatory complement proteins on the surface can improve the ability of cancer cells to evade complement-mediated cytotoxicity, which supports their survival and proliferation [172]. Cancer cells expressing high amounts of MCP on their surface are also able to modulate T-lymphocytes, causing them to transform into a regulatory phenotype. This phenotype then exhibits a decreased production of the pro-inflammatory cytokine IL-2 and an increased production of the anti-inflammatory cytokine IL-10, which allows cancer cells to dampen adaptive immune responses [173]. In addition, C4BP, FH, and FHL-1 have been shown to be expressed and secreted or recruited to the surface by ovarian [174,175] and lung cancer cells [176], attenuating complement activation and opsonization in their tumor microenvironment. These resistance mechanisms of tumors can significantly interfere with the effectiveness of certain anti-cancer treatments that rely on antibodies and the complement system and need to be considered in the development of novel immunotherapies [177].

1.3. The need for analytical methods of regulatory complement proteins

The field of complement-directed therapy has experienced an immense surge in recent years: after the approval of eculizumab by the Food and Drug Administration (FDA) in 2007, another 15 complement drugs for a total of 10 indications have now followed (Table 2), and many more are already in clinical trials. At present, the only therapeutics available in the clinic that are related to regulatory complement proteins are preparations for the supplementation of C1–INH [178,179]. However, due to the large number of pathologies associated with regulatory complement proteins, it can be expected that, in the coming years, further regulatory complement proteins will be added to the list of complement therapeutics, either as a target or for replacement [180]. This is evidenced by the fact that many approaches to develop complement therapeutics are already being pursued, in which regulatory complement proteins are either plasma-derived or produced recombinantly (Table 3). Moreover, engineered proteins are being investigated whose structure is based on natural regulatory complement proteins but whose therapeutic effect is boosted by targeted modification. This can be achieved either through an enhanced regulatory activity or by adjusting the affinity for specific target surfaces. These engineered proteins can be truncated versions of the natural regulatory complement protein or fusion proteins from different complement proteins, their fragments, and other biomolecules (e.g. artificial peptides or parts of antibodies). Antibodies can also be employed to inhibit the activity of regulatory complement proteins by blocking them [223] or to potentiate their activity by binding to them and stabilizing them in a more active conformation [212,213]. Another strategy is to use a vector (e.g. an adeno-associated virus) to deliver the gene for a regulatory complement protein into the cells of a specific target tissue in the patient, which

Table 2
Currently available, FDA-approved complement therapeutics on the market.

Drug name	Trade name	Company	Approval date	Target	Pathway	Modality	Indication
Cinryze	-	Takeda Pharmaceuticals	October 2008	**C1r, C1s, MASPs**	**CP**	Purified protein	Hereditary angioedema (HAE)
Berinert	-	CSL Behring	October 2009	**C1r, C1s, MASPs**	**CP**	Purified protein	Hereditary angioedema (HAE)
Ruconest	-	Pharming Group NV	July 2014	**C1r, C1s, MASPs**	**CP**	Recombinant protein	Hereditary angioedema (HAE)
Haegarda	-	CSL Behring	June 2017	**C1r, C1s, MASPs**	**CP**	Purified protein	Hereditary angioedema (HAE)
Sutimlimab	Enjaymo	Sanofi	February 2022	**C1s**	**CP**	Antibody	Cold agglutinin disease (CAD)
Pegcetacoplan	Empaveli	Apellis	May 2021	**C3**	**AP/AL**	Peptide	Paroxysmal nocturnal hemoglobinuria (PNH)
Pegcetacoplan (for injection)	Syfovre	Apellis	February 2023	**C3**	**AP/AL**	Peptide	Geographic atrophy (GA)
Iptacopan	Fabhalta	Novartis	December 2023	**FB**	**AP/AL**	Small molecule	Paroxysmal nocturnal hemoglobinuria (PNH), IgA nephropathy (IgAN)[b]
Danicopan	Voydeya	AstraZeneca	April 2024	**FD**	**AP/AL**	Small molecule	Extravascular hemolysis (EVH) in paroxysmal nocturnal hemoglobinuria (PNH)
Eculizumab	Soliris	Alexion	May 2007	**C5**	**TP**	Antibody	Paroxysmal nocturnal hemoglobinuria (PNH), atypical hemolytic uremic syndrome (aHUS), myasthenia gravis (MG), neuromyelitis optica spectrum disorder (NMOSD)
Ravulizumab	Ultomiris	Alexion	December 2018	**C5**	**TP**	Antibody	Paroxysmal nocturnal hemoglobinuria (PNH), atypical hemolytic uremic syndrome (aHUS), myasthenia gravis (MG), neuromyelitis optica spectrum disorder (NMOSD)
Avacincaptad Pegol	Izervay	Iveric Bio	August 2023	**C5**	**TP**	Aptamer	Geographic atrophy (GA)
Pozelimab	Veopoz	Regeneron Pharmaceuticals	August 2023	**C5**	**TP**	Antibody	(CD55-deficient) Complement hyperactivation, angiopathic thrombosis and protein-losing enteropathy (CHAPLE)
Zilucoplan	Zilbrysq	UCB Pharma	October 2023	**C5**	**TP**	Peptide	Myasthenia gravis (MG)
Crovalimab	Piasky	Genentech	June 2024	**C5**	**TP**	Antibody	Paroxysmal nocturnal hemoglobinuria (PNH)
Avacopan	Tavneos	Chemocentryx	October 2021	**C5aR1**	**EP**	Small molecule	Anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV)
Vilobelimab	Gohibic	InflaRx	June 2023[a]	**C5a**	**EP**	Antibody	Critical COVID-19

[a] FDA emergency use authorization.
[b] Accelerated approval, **CP:** classical pathway, **AP:** alternative pathway, **AL:** amplification loop, **TP:** terminal pathway, **EP:** effector pathway.

then express the corresponding protein themselves, potentially eliminating the need for regular administration. For oncological diseases, there are also approaches to block the function of regulatory complement proteins overexpressed on the surface of malignant cells. This can be achieved either with neutralizing antibodies or RNA-based methods using antisense oligonucleotides (ASO) or small interfering RNA (siRNA).

The growing awareness of the role of the complement system in inflammatory and immunological diseases in the clinic has been accompanied by an increase in complement testing [243]. Nevertheless, many complement-related diseases are still often recognized too late or not treated appropriately. Especially in the case of anemic and renal pathologies associated with regulatory complement proteins (i.e. HAE, PNH, aHUS, DDD, or IgAN), these are rare conditions in which patients frequently have to undergo a long and frustrating process before receiving the correct diagnosis. Here, the development of faster, simpler, and more cost-effective assays for application in clinical laboratories could help accelerate this process and, after successful diagnosis, may also serve to track disease progression [244]. In contrast, research and development of new complement therapeutics primarily require sensitive, reliable, and robust assays for characterizing drug candidates and evaluating their efficacy. For high-throughput screening, assays should also be easy to implement, with the possibility of automation and miniaturization.

In general, the treatment of many complement-related diseases demands regular monitoring to check the activity of the drug and adjust its medication if necessary. For example, patients with aHUS are currently treated with infusions of C5 inhibitors (i.e. eculizumab or ravulizumab), which block the terminal pathway and decrease overall complement activity. By checking inhibitor blood concentration, complement activity, and other markers (e.g. platelet count, LDH, or creatinine), doses or intervals between individual infusions can be fine-tuned. However, a common side effect of C5 inhibitors is an increased susceptibility to upper respiratory tract infections, particularly with meningococci. An alternative to this could be the treatment with iptacopan, which specifically inhibits FB and can also be administered orally [245]. In the case of aHUS, targeting a complement protein further upstream in the enzymatic cascade, thereby exclusively blocking a component of the alternative pathway, represents a step toward more selective treatment of the cause. As new complement therapeutics become available, the movement in this direction will continue. Drugs for use in replacement therapy of regulatory complement proteins will therefore drive the need for companion diagnostics to ensure and monitor their correct effect. Here, assays that can be conducted by family doctors and hospital staff, or that may even be suitable for at-home testing, would be desirable. Ideally, it would also be great to have assays in the future that could distinguish between individual variants of regulatory complement proteins or that could analyze several regulatory complement proteins simultaneously (multiplexing). These demands for novel analytical methods for regulatory complement proteins are coupled to challenges that must be overcome through collaboration between medical doctors, analytical researchers, and assay developers from the clinic, academia, and industry. In the next section, we present analytical methods that have already been established in the clinic in order to gain an insight into the current state. After that we will focus on recent advances in the development of analytical methods for regulatory complement proteins from research.

Table 3
Selection of complement therapeutics related to regulatory complement proteins on the market and under development.

Drug name	Developer/Producer	Modality	Associated regulator(s)	Clinical stage	Reference
Cinryze	Takeda Pharmaceuticals	Purified full-length protein	C1–INH	Approved	[181, 182]
Berinert	CSL Behring	Purified full-length protein	C1–INH	Approved	[183, 184]
Ruconest	Pharming Group NV	Recombinant full-length protein	C1–INH	Approved	[185, 186]
Haegarda	CSL Behring	Purified full-length protein	C1–INH	Approved	[187]
PRP6-HO7	Academic research	Recombinant truncated and oligomerized protein	C4BP	Preclinical stage	[188]
MAP-1/C4BP	Academic research	Fusion protein (with MAP-1)	C4BP	Preclinical stage	[189]
C4BP-IgM	Academic research	Fusion protein (with antibody fragment)	C4BP	Preclinical stage	[190, 191]
GEM103	Disc Medicine	Recombinant full-length protein	FH	Phase II	[192, 193]
CPV-104	Eleva Biologics	Recombinant full-length protein	FH	Phase I	[194, 195]
AMY-201/Mini-FH	Amyndas Pharmaceuticals	Recombinant truncated protein	FH	Preclinical stage	[196, 197]
TT30	Alexion Pharmaceuticals	Fusion protein (with CR2)	FH	Phase I	[198, 199]
TriFu	Takeda Pharmaceuticals	Fusion protein	FH, DAF, CR1	Preclinical stage	[200, 201]
FH18-20	Academic research	Recombinant truncated protein	FH	Preclinical stage	[202]
FH-IgG	Academic research	Fusion protein (with antibody fragment)	FH	Preclinical stage	[203, 204]
KP104	Kira Pharmaceuticals	Fusion protein (with antibody)	FH	Phase II	[205]
C3d-mAb-2fH	Academic research	Fusion protein (with antibody)	FH	Preclinical stage	[206]
MAp44-FH	Academic research	Fusion protein (with MAP-1)	FH	Preclinical stage	[207]
sMAP-FH	Academic research	Fusion protein (with sMAP)	FH	Preclinical stage	[208]
AAV_FH1-419-20 AAV_FH1-719-20	Academic research	Gene vector expressed truncated protein	FH	Preclinical stage	[209]
AAV5-VMD2-CR2-fH	Academic research	Gene vector expressed fusion protein (with CR2)	FH	Preclinical stage	[210, 211]
GEM307	Disc Medicine	Potentiating monoclonal antibody	FH	Preclinical stage	[212, 213]
GT103	Grid Therapeutics	Inhibiting antibody	FH	Phase I	[214, 215]
MFHR1/CPV-102	Eleva	Fusion protein	FH, FHR-1	Preclinical stage	[216]
MFHR13	Academic research	Fusion protein	FH, FHR-1	Preclinical stage	[217]
CB4332	Catalyst Biosciences	Recombinant enhanced protein	FI	Preclinical stage	[218]
GT005	Novartis	Gene vector expressed full-length protein	FI	Phase II	[219, 220]
CLG561	Alcon/Novartis	Inhibiting monoclonal antibody	Properdin	Phase II	[221]
NM3086	NovelMed Therapeutics	Inhibiting monoclonal antibody	Properdin	Phase I	[222]
mAb 1340	Academic research	Inhibiting monoclonal antibody	Properdin	Preclinical stage	[223]
TP10/CDX-1135	Celldex Therapeutics	Recombinant full-length protein (extracellular domain)	sCR1	Phase II	[224, 225]
TP20/sCR1-sLex	Celldex Therapeutics	Recombinant full-length protein (extracellular domain with sLex carbohydrate)	sCR1	Preclinical stage	[226, 227]
CSL040	CSL Behring	Recombinant truncated protein	CR1	Phase I	[228]
MAP-1:CD35	Academic research	Fusion protein (with MAP-1)	CR1	Preclinical stage	[229]
Mirococept/APT070	Inflazyme Pharmaceuticals	Fusion protein (with lipopeptide)	CR1	Phase III	[230, 231]
anti-CD46 S-ODN	Academic research	Antisense phosphorothioate oligonucleotides	MCP	Preclinical stage	[232]
anti-CD46 siRNA	Academic research	Small interfering RNA	MCP	Preclinical stage	[233, 234]
CAB-2/MLN-2222	Millennium Pharmaceuticals	Fusion protein	MCP, DAF	Phase I	[222]
DCP (Decay-Cofactor Protein)	Academic research	Fusion protein	MCP, DAF	Preclinical stage	[235]
MAP-1:CD55	Academic research	Fusion protein (with MAP-1)	DAF	Preclinical stage	[229]

(*continued on next page*)

Table 3 (*continued*)

Drug name	Developer/Producer	Modality	Associated regulator(s)	Clinical stage	Reference
scFv-35-DAF	Academic research	Fusion protein (with antibody fragment)	DAF	Preclinical stage	[236, 237]
anti-CD55 S-ODN	Academic research	Antisense phosphorothioate oligonucleotides	DAF	Preclinical stage	[232]
anti-CD55 siRNA	Academic research	Small interfering RNA	DAF	Preclinical stage	[233, 238]
anti-CD59 siRNA	Academic research	Small interfering RNA	Protectin	Preclinical stage	[233, 238]
CRIg-Fc	Genentech/Academic research	Fusion protein (with antibody fragment)	CRIg	Preclinical stage	[239, 240]
CRIg/FH	Academic research	Fusion protein	CRIg, FH	Preclinical stage	[241]
CG001	Academic research	Fusion protein (with antibody)	CRIg, FH	Preclinical stage	[242]

2. The current status of established analytical methods

Analytical methods established in the clinical lab for examining the complement system and its regulators can be divided into four test categories: a) functional assays for the activity of individual activation pathways, b) quantitative assays for the concentration of specific complement proteins, activation products, and autoantibodies, c) functional assays for the activity of regulatory complement proteins, and d) analysis of complement genes.

Functional assays that determine the activity of the individual activation pathways typically utilize distinct surfaces, inhibitors, buffer compositions, or sample dilutions to specifically trigger either the classical, the lectin, or the alternative pathway. The successful completion of the activation pathway, from its initiation along the enzymatic cascade to the end of the terminal pathway, is achieved by the detection of the C5b-9 complex (MAC or TCC). This is realized via hemolysis in the case of the CH50 and AH50 assays (Fig. 2), via liposome lysis in the case of the CH50 Autokit (Fig. 3), or via enzyme-conjugated antibodies in the

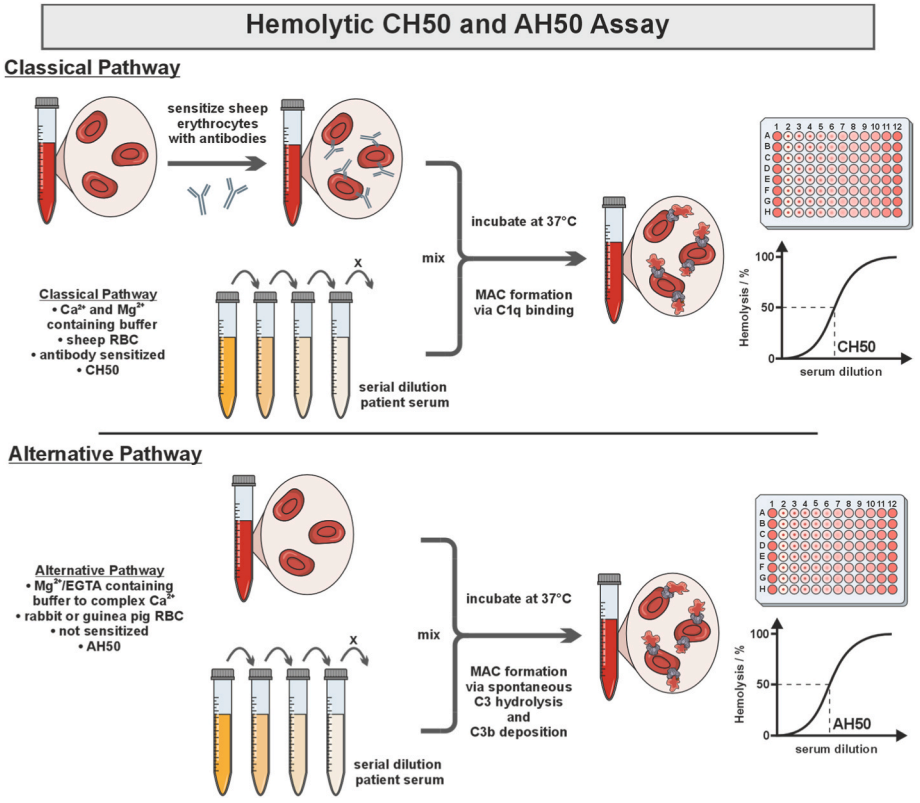

Fig. 2. Procedure of the hemolytic CH50 and AH50 assays. A) In the CH50 assay, sensitized sheep erythrocytes are mixed and incubated with a serial dilution of patient sample at 37 °C in a Ca^{2+}/Mg^{2+} containing buffer. The complement system is activated via C1q binding, which results in hemolysis due to the insertion of C5b-9 complexes in the erythrocyte membrane. The released hemoglobin is subsequently separated from the erythrocytes via centrifugation and the absorbance of hemoglobin is measured at 415 nm or 540 nm as a readout. The reciprocal dilution at which 50% of the sheep erythrocytes are lysed is reported as the CH50 value. B) In the AH50 assay, rabbit, chicken, or guinea pig erythrocytes are used which lack sialic acids on their surface and therefore do not need to be sensitized beforehand. The erythrocytes are mixed and incubated with serial dilutions of patient sample at 37 °C in an Mg^{2+}/EGTA containing buffer. The complement system is activated via spontaneous C3 hydrolysis and C3b deposition on the erythrocyte surface. Hemolysis caused by the insertion of C5b-9 complexes in the erythrocyte membrane leads to the release of hemoglobin, which can again be detected in the supernatant after centrifugation via its absorbance at 415 nm or 540 nm. Like the CH50 value, the AH50 value is the reciprocal dilution at which 50% of the rabbit, chicken, or guinea pig erythrocytes are lysed. Abbreviations used in this figure: MAC, membrane attack complex; RBC, red blood cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

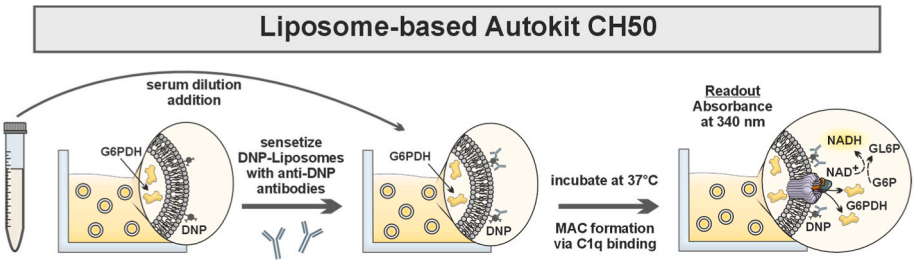

Fig. 3. Procedure of the Autokit CH50 assay. Liposomes containing a DNP-modified lipid in their membrane and G6PDH in their inner cavity are sensitized with anti-DNP antibodies from the reagent and incubated with different sample dilutions. MAC formation via C1q binding lyses the liposomes and brings G6PDH in contact with its substrates, G6P and NAD^+. G6PDH catalyzes the reaction of G6P with NAD^+, generating NADH as a product. The readout is performed via the absorbance of NADH at 340 nm, which correlates to the classical pathway activity. Abbreviations used in this figure: DNP, dinitrophenol; G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; GL6P, 6-phosphogluconolactone; MAC, membrane attack complex; NAD^+, nicotinamide adenine dinucleotide (oxidized form); NADH, nicotinamide adenine dinucleotide (reduced form).

case of the complement system screen ELISA (Fig. 4).

In the CH50 assay (Fig. 2), sheep erythrocytes sensitized with rabbit anti-sheep erythrocyte stroma antibody (also referred to as hemolysin) are mixed and incubated with a serial dilution of patient sample at 37 °C for a specific period of time (usually 30 min) [246]. The sensitizing antibody activates the complement system by recruiting C1q, which in turn leads to the formation of the C5b-9 complex in the erythrocyte membrane and the release of hemoglobin from the erythrocytes (hemolysis). The erythrocytes are subsequently centrifuged, and the supernatant of each sample dilution is measured based on the absorbance of hemoglobin at either 415 nm or 540 nm. The reaction of the erythrocytes with the patient sample takes place in a buffer which contains Ca^{2+} and Mg^{2+} ions, allowing all activation pathways to proceed. However, contributions from the alternative pathway are excluded, since the surface of the sheep erythrocytes is decorated with sialic acids, which recruit FH and thus inhibit the alternative pathway. From the percentage of lysis obtained for each sample dilution, the CH50 value can then be derived, which represents the reciprocal sample dilution at which 50% of the sheep erythrocytes are lysed. Different from the CH50 assay, the AH50 assay (Fig. 2) is performed using rabbit, chicken, or guinea pig erythrocytes [247,248]. These erythrocytes bear little to no sialic acids on their surface, thereby preventing inhibition of hemolysis by FH. Consequently, these erythrocytes do not need to be sensitized, as they already inherently induce complement activation. Combining their unique surface properties with a special buffer composition initiates only the alternative pathway, whereby the formation of the C5b-9 complex in the erythrocyte membrane is mediated by spontaneous C3 hydrolysis and the deposition of C3b on the surface of the erythrocytes. The reaction buffer contains Mg^{2+} ions and ethylene glycol tetraacetic acid (EGTA), which selectively chelates Ca^{2+} ions that are necessary for the activation of the classical and lectin pathway, but not the alternative pathway. As with the CH50 assay, the erythrocytes are mixed and incubated with a serial dilution of patient sample at 37 °C. After centrifugation, hemolysis is measured again based on the absorbance of released hemoglobin in the supernatant. The AH50 value is determined using the reciprocal sample dilution at which 50% of the rabbit, chicken, or guinea pig erythrocytes are lysed. Both hemolytic assays (CH50 and AH50) often employ a sample containing erythrocytes but no serum as a negative control (0% lysis), a sample containing erythrocytes and distilled water as a positive control (100% lysis), and the sample dilutions without erythrocytes as blanks. Furthermore, these assays have been simplified using fixed sample dilutions with an excess of erythrocytes [249] or have been adopted in such a way that the erythrocytes are embedded in an agarose gel medium in a Petri dish [250]. The sample is then transferred into circular holes punched into the gel, usually measuring about 3 mm in diameter. After incubation and radial diffusion of the sample components into the gel, the degree of hemolysis is determined based on the size of the hemolytic zones that form around

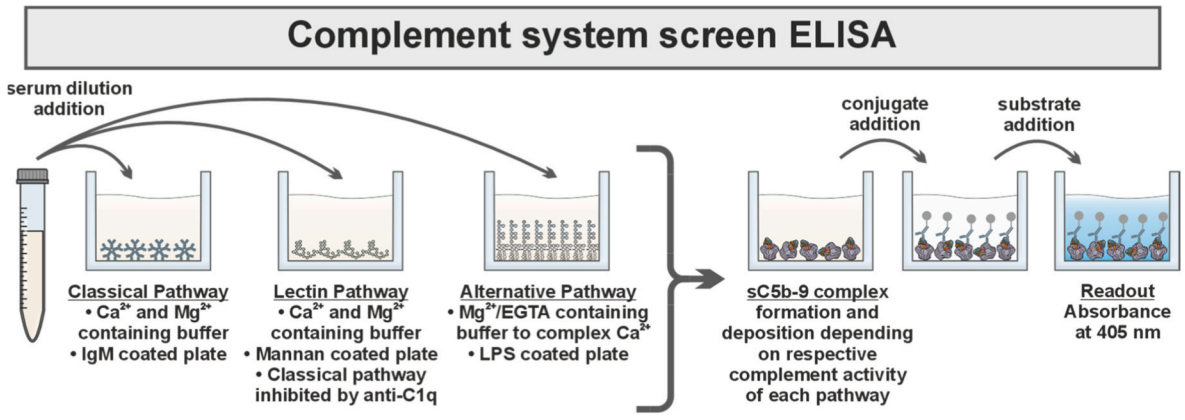

Fig. 4. Procedure of the complement system screen ELISA. Sample dilutions are added to three types of wells of a microtiter plate, which are characterized by their different surface coatings: IgM antibodies are immobilized in the wells of the classical pathway, mannan in the wells of the lectin (MBL-dependent) pathway, and LPS in the wells of the alternative pathway. Specificity for each activation pathway is ensured by high sample dilutions and the presence of Ca^{2+} and Mg^{2+} ions in the case of the classical pathway, high sample dilutions and the addition of anti-C1q antibodies in the case of the lectin (MBL-dependent) pathway, and the absence of free Ca^{2+} ions via an Mg^{2+}/EGTA containing buffer in the case of the alternative pathway. After the sample dilutions have been applied to the wells, the microtiter plate is incubated at 37 °C for 1 h. The microtiter plate is then washed, and an ALP-conjugated anti-human C5b-9 antibody is added. Following another incubation step at room temperature for 30 min, the microtiter plate is washed again, and the substrate solution is added. The hydrolysis of the substrate catalyzed by ALP generates p-nitrophenolate as a product, whose absorbance at 405 nm is used for the readout. The signal obtained for each type of well correlates to the corresponding activity of the respective activation pathway. Abbreviations used in this figure: IgM, immunoglobulin M; LPS, lipopolysaccharides.

the holes.

Other assays originally designed for the diagnosis of PNH include Ham's test [251], a sucrose hemolysis test [252,253], a complement hemolysis sensitivity test [254,255], and a sephacryl gel card test [256–258], most of which are now rarely used in clinical practice anymore and have been largely replaced by flow cytometry (detecting DAF and protectin on erythrocytes and several other markers on immune cells) as the standard method [259–261]. In this process, several different GPI-anchored proteins expressed on the surface of erythrocytes, neutrophils, monocytes, and lymphocytes are analyzed using fluorescently labeled aerolysin (FLAER) or fluorescently labeled monoclonal antibodies (Table 4). Additional proteins are measured to identify and differentiate individual cell types and to exclude cellular debris, platelets, and unlysed erythrocytes [259,260]. However, there is also a modified Ham's test, which can be used to distinguish aHUS from other thrombotic microangiopathies, such as acquired thrombotic thrombocytopenic purpura (TTP). In the modified Ham's test, the cell viability of cultured cells from a *PIGA*-null TF-1 cell line is compared in a chromogenic WST-1 assay, once incubated in serum from aHUS patients and once in normal human serum or TTP serum, with the cells undergoing cell death significantly faster in the case of aHUS serum [262]. The cell line has since been further modified so that cell viability can be monitored via bioluminescence [263].

The principle of the liposome-based CH50 Autokit assay (Fig. 3) is very similar to that of the hemolytic CH50 assay, except that erythrocytes are substituted with liposomes. This offers several advantages, as liposomes are artificially produced vesicles with a predefined lipid composition, which are highly stable and easy to store for months or even years. Erythrocytes, on the other hand, are derived from animals, which can be associated with significant inter-individual variability and batch-to-batch variations. In addition, erythrocyte preparations are generally prone to lesions and can only be stored for a few weeks. Together, this gives hemolytic assays the overall disadvantage of being difficult to automate and standardize. The liposomes in the Autokit CH50 assay are prepared to contain a synthetic dinitrophenyl (DNP)-modified lipid (hapten) in their membrane and the enzyme glucose-6-phosphate dehydrogenase (G6PDH) in their inner cavity [264]. When the liposomes are exposed to an anti-DNP antibody in the reagent, they become sensitized, enabling them to recruit C1q and activate the complement system. The formation of the C5b-9 complex in the liposome membrane leads to liposome lysis and causes glucose-6-phosphate (G6P) and oxidized nicotinamide adenine dinucleotide (NAD^+) from the surrounding reagent to come into contact with G6PDH from inside the liposomes. This allows the reaction of NAD^+ with G6P, catalyzed by G6PDH, to take place, generating reduced nicotinamide adenine dinucleotide (NADH) as a product. While it is the classical pathway that is predominantly initiated, contributions from the alternative pathway are not explicitly excluded in this assay. The degree of liposome lysis and thus the total complement (mainly classical pathway) activity is represented by the absorbance signal of NADH and can be measured at 340 nm.

Since liposome-based assays do not require centrifugation or washing steps, they can be classified as homogeneous assays. In contrast, the complement system screen ELISA (Fig. 4) (as well as the CH50 or AH50 assay) is a heterogeneous assay. In this assay format, the surface of the wells of a microtiter plate is coated with different pathway-related trigger molecules. IgM antibodies are immobilized in the wells for the classical pathway, mannan for the lectin (MBL-dependent) pathway, and lipopolysaccharides (LPS) for the alternative pathway [265–267]. The specificity for the alternative pathway is guaranteed by a low sample dilution and an Mg^{2+}/EGTA buffer, while buffers containing Ca^{2+} and Mg^{2+} ions are used for the other two pathways. To avoid remaining contributions from other activation pathways, high sample dilutions are applied for the classical and lectin pathways to suppress the alternative pathway, and anti-C1q antibodies are added for the lectin pathway to inhibit the classical pathway [268]. All sample dilutions are applied to the wells of the microtiter plate and incubated at 37 °C for 1 h, whereby the respective activation pathway is initiated. When the complement system is intact, the respective enzymatic cascades of the individual activation pathways proceed to the terminal pathway and the assembly of the C5b-9 complex, which deposits on the solid-phase surface coatings of the wells. After washing, an alkaline phosphatase (ALP)-conjugated anti-human C5b-9 detection antibody (directed against a neoepitope of C9 that is only exposed after the formation of C5b-9) is added and incubated at room temperature for 30 min. The microtiter plate is washed again, and the substrate solution is added, which starts the hydrolysis of *p*-nitrophenyl phosphate catalyzed by ALP. The amount of generated C5b-9 complexes can finally be detected by measuring the absorbance of the hydrolysis product *p*-nitrophenolate at 405 nm, which also reflects the activity of the respective activation pathway. A serum pool from several healthy individuals can be employed as a positive control and heat-inactivated serum (at 56 °C for 20 min) as a negative control. The complement system screen ELISA is now widely used in research and drug development. Since its development, it has been increasingly improved [269] and also extended to the ficolin-dependent pathway with acetylated bovine serum albumin as the trigger molecule [270].

The Autokit CH50 assay is currently the only liposome-based complement assay established in clinical settings, yet it only provides information on the total complement (mainly classical pathway) activity. Hence, the only method currently available for determining the activity of all activation pathways is the complement system screen ELISA. Liposome-based strategies covering each pathway separately are researched with increasing effort and may be available to the community at large in the near future [271]. Other academic approaches are moving toward cell-based methods (other than erythrocytes), such as an assay for measuring complement convertase activity using a human lymphoma cell line [272] or an assay system for evaluating peptide coatings capable of recruiting regulatory complement proteins to endothelial cell surfaces [273]. These cell-based assays could also be adapted to measure the activity of individual activation pathways, either by detecting cell lysis or using flow cytometry. In part, this has already been implemented in a cell-based assay, which integrates bioluminescent human embryonic kidney cell lines in an array and was used to identify a classical pathway stimulus in aHUS [274]. The array combines three cell lines established by different knockout strategies, resulting in the cells lacking different regulatory complement proteins on their surface (MCP, DAF, and protectin). The complement-mediated cytotoxicity induced by normal human serum and aHUS serum is detected by monitoring cell viability via the bioluminescence signal.

With the functional, commercially available assays (CH50, AH50, Autokit CH50, and ELISA), it is possible to assess after just one run

Table 4
Overview of surface markers used for each cell type in the diagnosis of PNH by flow cytometry. Abbreviations used in this table: B, B-lymphocytes; CD, cluster of differentiation; DAF, decay-accelerating factor; FLAER, fluorescently labeled aerolysin; PNH, paroxysmal nocturnal hemoglobinuria; T, T-lymphocytes.

	Erythrocytes	Neutrophils	Monocytes	Lymphocytes
Surface markers mostly used for PNH diagnosis	DAF (CD55) Protectin (CD59)	FLAER CD16 CD24 CD157	FLAER CD14 CD157	-
Surface markers partially used for PNH diagnosis	CD58 CD71	CD66b CD66c CD87 CD177	CD48 CD87	FLAER/CD48/CD73 B: CD24/DAF (CD55) T: CD52/DAF (CD55)
Surface markers for cell gating	CD235a	(CD10) CD15 (CD33) CD45	(CD33) CD45 CD64	CD45 B: CD19/CD20 T: CD3

whether there is an abnormality in one of the activation pathways or the terminal pathway. However, they do not allow conclusions to be drawn about the site within the pathway or the regulatory complement protein that is affected. A typical approach of testing for complement deficiency is to mix patient serum with human serum that has been depleted of a specific complement protein (e.g. C3). In a hemolytic titration assay, it is then determined whether the patient serum is able to restore the functional activity (CH50 or AH50) of the complement-depleted serum. If this is not the case, the patient serum is probably missing the same protein as the complement-depleted serum. To verify this, the hemolytic test can be repeated by mixing the patient serum with the suspected protein in purified form and with confirmed function as a reconstitution assay [275]. Complement testing at the level of entire pathways usually requires a sophisticated workflow protocol to identify the correct cause of the underlying condition and make a targeted diagnosis or prognosis. Examples of practical guidelines for the most important complement deficiencies and complement-related diseases have been published by various groups and institutions [276–278].

Quantitative assays for complement testing are in most cases immunochemical assays, which include traditional techniques, such as radial immunodiffusion (RID) and electroimmunodiffusion (EID), as well as their advancements, such as turbidimetry, nephelometry, and ELISA-type assays.

In RID, the patient sample is placed into circular holes in an agarose gel containing the analyte-specific antibodies [279,280]. The analyte diffuses into the agarose gel and forms antigen-antibody immune complexes (precipitin) with the antibodies. These complexes become visible as white rings. The size of the rings is measured, and a standard curve is used to determine the concentration of the analyte in the patient sample. EID is a technique that combines RID with electrophoresis and is more sensitive than conventional RID [281,282]. It also employs an agarose gel with analyte-specific antibodies, into which circular holes are punched in a row. The patient samples are placed in these holes and, when a voltage is applied, the analyte migrates into the agarose gel, where it encounters the antibodies and forms aggregates of precipitin. The higher the concentration of the analyte, the further it can migrate before being retained by the antibodies. Instead of rings, white arcs form in the agarose gel. The height of these arcs is compared to a standard curve, from which the concentration of the analyte can eventually be derived.

Turbidimetry and nephelometry are among the techniques that are still widely used today and are closely related in terms of their underlying principles. Both immunoturbidimetry and -nephelometry exploit the fact that the turbidity of a solution changes as a result of a reaction between antibodies and the analyte [283,284]. The antibodies attach themselves to the analyte and form aggregates that scatter the incident light. Polyclonal antibodies are preferred over monoclonal antibodies, as they can bind to several epitopes on the analyte. The main difference between the two techniques lies in the instrumental setup for detection. In turbidimetry, the reduced intensity of the transmitted light (i.e. the light that passes through the sample) compared to the incident light intensity (reference) is measured. Here, the photodetector is placed behind the sample in line with the light beam. In nephelometry, on the other hand, the intensity of the scattered light is measured at a specific angle to the light beam, for which reason the photodetector is positioned at this angle to the light source (usually a laser) and sample. Some microtiter plate readers also employ an integrating sphere (Ulbricht sphere), which collects the scattered light uniformly and directs it to the photodetector.

Together with ELISA-type assays, these methods enable the detection and quantification of a whole range of complement proteins, with C3 and C4 being established in routine analysis. The emergence of new complement therapeutics as well as a deeper understanding and new discoveries regarding the role of the complement system in various pathologies have extended routine complement testing beyond these two complement proteins, with concentrations of C1q, C1–INH, FB, MBL, and properdin now also frequently being determined on a regular basis [285]. To study the degree of complement activation, ELISAs for specific protein quantification of activation products were developed and are commercially available using monoclonal antibodies directed against neoepitopes from individual components or soluble complexes of C3 cleavage (C3a, iC3b/C3b/C3c, C3d), the classical and lectin pathway (C1rs/C1–INH, C4a, C4b/C4c, C4d), the alternative pathway (Ba, Bb, C3bBbP), and the terminal pathway (C5a, sC5b-9) [286–288]. In addition, a multiplex detection array for measuring complement activation based on C3a, C5a, and C5b-9 has been developed [289]. Because of their longer half-lives, the detection of C3d (ca. 4 h) and sC5b-9 (ca. 1 h) is usually preferred in clinical settings over other activation products (e.g. C5a: ca. 1 min) [275]. When comparing complement activation states, it is also recommended to normalize the concentration of the activation product (e.g. C3d) to the concentration of its corresponding native protein (e.g. C3), although this is probably more important in chronic than in acute diseases [286,290,291]. This ensures that a state of increased complement activation is detected even if the concentration of the native protein is significantly lower due to deficiency or overconsumption [292] or if it is significantly higher, as is possible in obese patients [293]. Furthermore, a wide variety of commercially available or in-house ELISAs exist for autoantibody detection, including autoantibodies against C1q, C3, MBL, C1–INH, FB, FH, and FI [294]. The concentration and functional activity of C3NeFs can also be measured using ELISAs, hemolytic assays, or specialized functional assays, which measure C3 activation products or C3 convertase stabilization [295].

Complement analysis is indicated for differential diagnosis when a disorder of the complement system or a clinical condition involving the complement system is suspected [296–298]. It can assist in refining the diagnosis, making prognostic statements, and monitoring disease progression or treatment. However, it should be noted that in practice, complement analysis is often only part of a series of clinical examinations. The most common laboratory tests of complement parameters for a selection of pathologies are summarized in Table 5 and a generalized sequence of laboratory tests for complement analysis is given in Fig. 5. The procedure for complement analysis comprises the following steps and is frequently performed in the order:

Fluid-phase regulatory complement proteins

1) **Activation pathway assays** (function)
2) **Activation products** (concentration)
3) **Individual complement components** (concentration and function)
4) **Autoantibodies** (concentration)
5) **Genetic analysis**

Membrane-bound regulatory complement proteins and complement receptors

1) **Flow cytometry** (surface concentration)
2) **Genetic analysis**

The first step is usually a screening of the individual activation pathways using functional assays to gain insight into which of the three activation pathways may be affected and where a possible disorder may be located inside the complement cascade. Quantitative assays of the activation products can then reveal whether the problem is more likely to be a complement deficiency or increased complement consumption. Depending on the outcome of this preliminary complement analysis, appropriate follow-up tests are usually performed.

As an example, active SLE is often characterized by low classical pathway activity (CH50, functional ELISA) and low concentrations of C1q, C3, and C4 [300]. In severe cases, such as proliferative lupus nephritis, autoantibodies against C1q can also be detected [301]. In HAE, functional assays of individual components are crucial for distinguishing between the different types of HAE. While the diagnosis of

Table 5
Selection of common laboratory tests for complement analysis of pathologies associated with regulatory complement proteins. Abbreviations used in this table: aHUS, atypical hemolytic uremic syndrome; AP, alternative pathway; C1–INH, C1 esterase inhibitor; C3G, C3-glomerulopathy; CP, classical pathway; DAF, decay-accelerating factor; FB, Factor B; FD, Factor D; FH, Factor H; FI, Factor I; HAE, hereditary angioedema; LP, lectin pathway; MBL, mannose-binding lectin; PNH, paroxysmal nocturnal hemoglobinuria; SLE, systemic lupus erythematosus. Table adapted from Prohászka et al., 2016 [277].

Pathology	Functional assays		Quantitative assays			Flow cytometry	Genetic analysis
	Entire pathways	Individual components	Individual components	Autoantibodies	Activation products		
Recurrent infections	CH50, AH50, ELISA (CP, LP, AP)	-	C1q, C3, C4, C5, C6, C7, C8, C9, MBL, FD, Properdin	-	C3a/C3d, sC5b-9	-	-
SLE	CH50, ELISA (CP)	-	C1q, C3, C4	anti-C1q	C3a/C3d, sC5b-9	-	-
Angioedema	CH50, ELISA (CP)	C1–INH (HAE type I & II)	C1–INH (HAE type I), C1q (AAE), C4	anti-C1-INH (AAE)	-	-	*SERPING1* (HAE type I & II), *F12, PLG*, *ANGPT-1*, *KNG1*, *MYOF*, *HS3ST6* (HAE type III)
aHUS	CH50, AH50, ELISA (CP, AP)	FH, FI	C3, FB, FH, FI	anti-FH	C3a/C3d, sC5b-9	-	*C3*, *CFB*, *CFH*, *CFI*, *MCP/CD46*
C3G	CH50, AH50, ELISA (CP, AP)	FH, FI	C3, C4, FH, FI	C3NeFs, anti-FH, anti-FB	C3a/C3d, sC5b-9	-	*C3*, *CFB*, *CFH*, *CFI*, *MCP/CD46*
PNH	-	DAF, Protectin	-	-	-	DAF, Protectin, FLAER	-

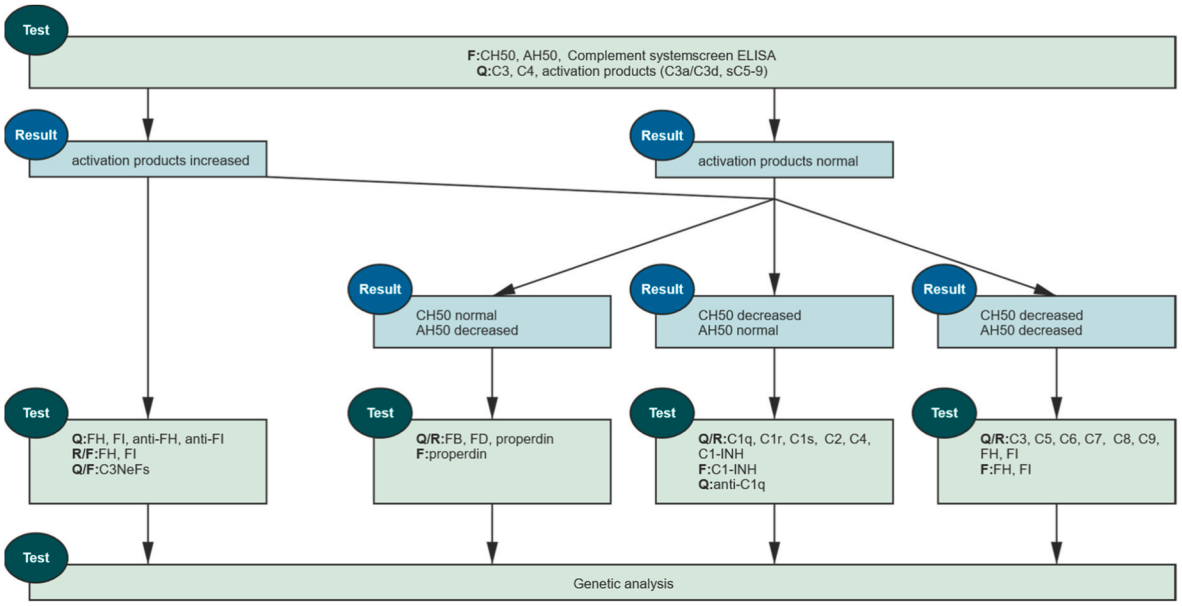

Fig. 5. Generalized sequence of laboratory tests for complement analysis. In the first step, the activities of the individual complement pathways and the concentrations of the activation products are determined. Elevated activation product levels should be investigated further even if the activities of the activation pathways are normal, as these can be compensated for in acute phase reactions. Depending on the results of the functional assays, targeted tests can be used in a second step to search more specifically for the presumed cause. These tests include quantitative, reconstitution, and functional assays of individual components of the activation pathways and the terminal pathway, as well as regulatory complement proteins and autoantibodies. If lectin pathway activity is determined by ELISA and found to be low, additional tests for MBL, MASP-1, MASP-2, and ficolin should be considered. In a third step, if the results are still inconclusive or to confirm the diagnosis, genetic analysis can be performed to identify genetic causes. Abbreviations used in this figure: C1–INH, C1-esterase inhibitor; C3NeFs, C3 nephritic factors; F, functional assay; FB, Factor B; FD, Factor D; Q, quantitative assay; R, reconstitution assay. Figure adapted from Shih et al., 2015 [299] and Schröder-Braunstein et al., 2019 [275].

type I HAE can be confirmed by measuring low C1–INH concentrations in a quantitative assay, type II HAE requires functional analysis of C1–INH (normal to elevated C1–INH concentrations with reduced activity). For this reason, functional assays, in particular for C1–INH, have been greatly advanced in recent years and decades [302,303]. This also applies to functional assays of FH and FI, which can, for example, play a role in the diagnosis and treatment of aHUS or AMD where variants with normal concentration but missing function are described [304,305]. C3G and IgAN are typically diagnosed with kidney biopsy and subsequent histological examination. Especially in cases of C3G, complement analysis can help clarify the diagnosis and decide on treatment options, as histological findings may overlap [306]. For the assessment of membrane-bound regulatory complement proteins, such as in the diagnosis of PNH, flow cytometry has become the standard method [259–261]. An assay based on fluorescently labeled aerolysin (FLAER) for the detection of PNH-related leukocytes is often used for this purpose, which offers increased sensitivity (Table 4) [307–309]. FLAER is a fluorophore-conjugated bacterial toxin that specifically binds to the GPI moiety of GPI-anchored proteins on normal leukocytes. Since FLAER is a mutated, inactive form of aerolysin, it does not cause cell lysis. For erythrocyte analysis, EDTA-anticoagulated peripheral blood is typically stained directly (e.g. with fluorescently labeled monoclonal antibodies

targeting DAF, protectin, and CD235a). For leukocyte analysis, however, the blood sample is first treated with a lysis solution to remove erythrocytes. Following a washing step, the leukocytes are incubated with a staining cocktail (e.g. containing FLAER and fluorescently labeled monoclonal antibodies targeting CD14, CD15, CD24, CD45, and CD64) [310].

Genetic analysis generally aims to identify defects in complement genes associated with specific diseases [311]. Dye terminator cycle sequencing (also known as Sanger sequencing) is employed for single nucleotide variants or massively parallel sequencing (also known as next-generation sequencing) together with PCR-based techniques, such as multiplex ligation-dependent probe amplification, for single nucleotide, small deletion-insertion, and copy number variants for serial single gene testing or panel testing of complement genes [312–316]. Patients with suspected aHUS or C3G, for example, can be tested for mutations and polymorphisms in the genes of the complement components and regulators of the alternative pathway [312,317,318]. Quantitative and functional assays are usually sufficient for the diagnosis of type I and II HAE, so that sequencing of *SERPING1* is not absolutely necessary. However, genetic analysis can facilitate the diagnosis of type III HAE (nlC1-INH-HAE) and provide information about possible mutations in *F12*, *PLG*, *ANGPT-1*, *KNG1*, *MYOF*, and *HS3ST6*, as no biochemical assays are currently available for this purpose [319,320]. In recent years, genetic analysis has become increasingly important in complement testing. As sequencing techniques will continue to evolve in the future, approaches such as whole-genome and whole-exome sequencing are likely to become more widely applied and accessible [321,322].

3. Research-based progress in the development of new analytical methods

3.1. Fluid-phase regulatory proteins

3.1.1. C1-esterase inhibitor (C1–INH)

Overall, assays for C1–INH can serve to distinguish between early complement activation via the classical or lectin pathway. This can be achieved using ELISAs by Hurler et al., which quantify complexes of C1s/C1–INH and MASP-1/C1–INH [323]. Yet, probably the most common disease associated with C1–INH has been HAE, so that the initial analytical methods for determining C1–INH were developed for the diagnosis of HAE. Reduced CH50 values and C4 plasma levels can be a first sign in the diagnosis of HAE. Further confirmation can subsequently be obtained through quantitative and functional C1–INH tests [324]. In type I HAE, low C1–INH concentrations and low C1–INH activities are detected, whereas type II HAE is characterized by normal to elevated C1–INH concentrations but low C1–INH activities. To confirm the diagnosis of type III HAE (nlC1-INH-HAE), genetic analysis for mutations in genes like *F12* is usually required. Genetic analysis may also be considered if C1–INH values are ambiguous [325]. Due to the different types of HAE, which vary in their cause but are quite similar in their symptoms, functional assays for C1–INH were necessary at an early stage in addition to quantitative assays [60,302]. Hence, the first analytical methods for C1–INH were functional assays, mainly for diagnosing HAE and studying complement regulation by C1–INH. These early assays were enzyme inhibition assays based on the inhibitory function of C1–INH on C1r or C1s activity [326,327]. Later advances included the development of immunochemical assays [328,329], followed by chromogenic assays and ELISA. In chromogenic assays, the sample is typically mixed with an excess of purified C1s. This allows functional C1–INH in the sample to bind to C1s. The activity of the residual C1s is then determined by adding a chromogenic substrate, which is cleaved by C1s. In the case of the ELISA, the functional C1–INH in the sample is incubated with a biotinylated C1s instead. The complex formed from C1–INH and biotinylated C1s is then captured on an avidin-coated microtiter plate and detected with an enzyme-conjugated anti-C1-INH antibody. The addition of a chromogenic substrate, which is converted by the enzyme, finally enables optical readout. Since their development, chromogenic assays and ELISAs for the diagnosis of HAE have been compared by various research groups [330–333]. Current developments are heading in the direction of dried blot spot assays, which overcome the complex sample drawing, processing, and storage/transportation requirements of the other tests. Here, signature peptides after proteolytic cleavage of C1–INH by trypsin for quantitative analysis of C1–INH [334,335] or enzymatic reaction products of C1s for functional analysis of C1–INH [336,337] are detected using liquid chromatography tandem mass spectrometry. However, this also makes these techniques instrumentally demanding. Chockalingam et al. recently introduced a lateral flow assay with fluorescence readout for point-of-care diagnostics of HAE, which offers a simpler, faster, and more widely accessible test for quantifying functional C1–INH [338]. Another approach involves assays based on the interaction of C1–INH with proteases of the contact system (i.e. Factor XIIa or kallikrein) rather than those of the complement system (i.e. C1r or C1s). An ELISA developed by Joseph et al. detects the complexes formed by C1–INH with Factor XIIa and kallikrein [339] and a chromogenic assay by Ghannam et al., in turn, exploits the inhibition of the enzymatic activity of the contact-phase proteases by C1–INH [340]. In addition, Kajdácsi et al. introduced an ELISA that quantifies C1–INH complexes with various proteases, including thrombin of the coagulation system [341]. These assays represent an alternative to the commercial tests available to date and shift the focus in the diagnosis of HAE more towards the physiologically relevant sites of the disorder, namely the formation of bradykinin. Given the significance of C1–INH in diagnosing HAE, it is likely that analytical methods for C1–INH (such as mass spectrometry using dried blood spots, point-of-care lateral flow assays, and new ELISAs and chromogenic assays) will continue to be improved and optimized in the future (Fig. 6).

3.1.2. C4b-binding protein (C4BP)

Despite the many functions of C4BP, few diseases are currently known to be directly related to C4BP. Some studies indicated a link between mutations in the genes encoding C4BP and aHUS as well as recurrent pregnancy loss, although these results remain controversial [342–344]. For this reason, most C4BP assays are purely quantitative or highly specialized, serving mainly academic purposes. In the clinic, C4BP can generally be investigated by directly measuring plasma levels of C4BP or by genetic analysis, but they are not part of routine diagnostics, as cases of C4BP deficiency are extremely rare [345]. Among the first analytical methods for C4BP were those developed by Dahlbäck et al. and included immunological, electrophoretic, and hemolytic assays to characterize the function of C4BP and to investigate the interaction of C4BP with protein S, as well as electron microscopy revealing the spider-like structure of C4BP [346–348]. Later, turbidimetric assays [349] and ELISA [350,351] were developed to determine the plasma levels of C4BP and the C4BP-S complex more rapidly and with higher sensitivity. To assess the FI cofactor activity of C4BP, C4b degradation can be examined by SDS-PAGE in combination with autoradiography or Western blot [352]. This technique was implemented, for example, to study the regulatory activities of individual truncated forms of C4BP [353] or to identify the binding site of *Bordetella pertussis* on C4BP [354]. Customized assays are often employed to study the interaction of C4BP with specific ligands. For example, Trouw et al. used a band shift assay in agarose gel, a dot blot assay, and SPR analysis to characterize the binding of C4BP to DNA in relation to the influence of C4BP on necrotic cells [355]. Agarwal et al. used a direct binding assay in a microtiter plate, SPR analysis, and an in-house ELISA to characterize the binding of C4BP to plasminogen, which enhances plasminogen activation [356]. Flow cytometry measurements were also employed to study the interactions of C4BP with immune cells, such as the activation of B-lymphocytes by C4BP [357] or the influence of C4BP on the phagocytosis of apoptotic cells by macrophages [358]. Furthermore, approaches based on mass spectrometry are being pursued more frequently to investigate

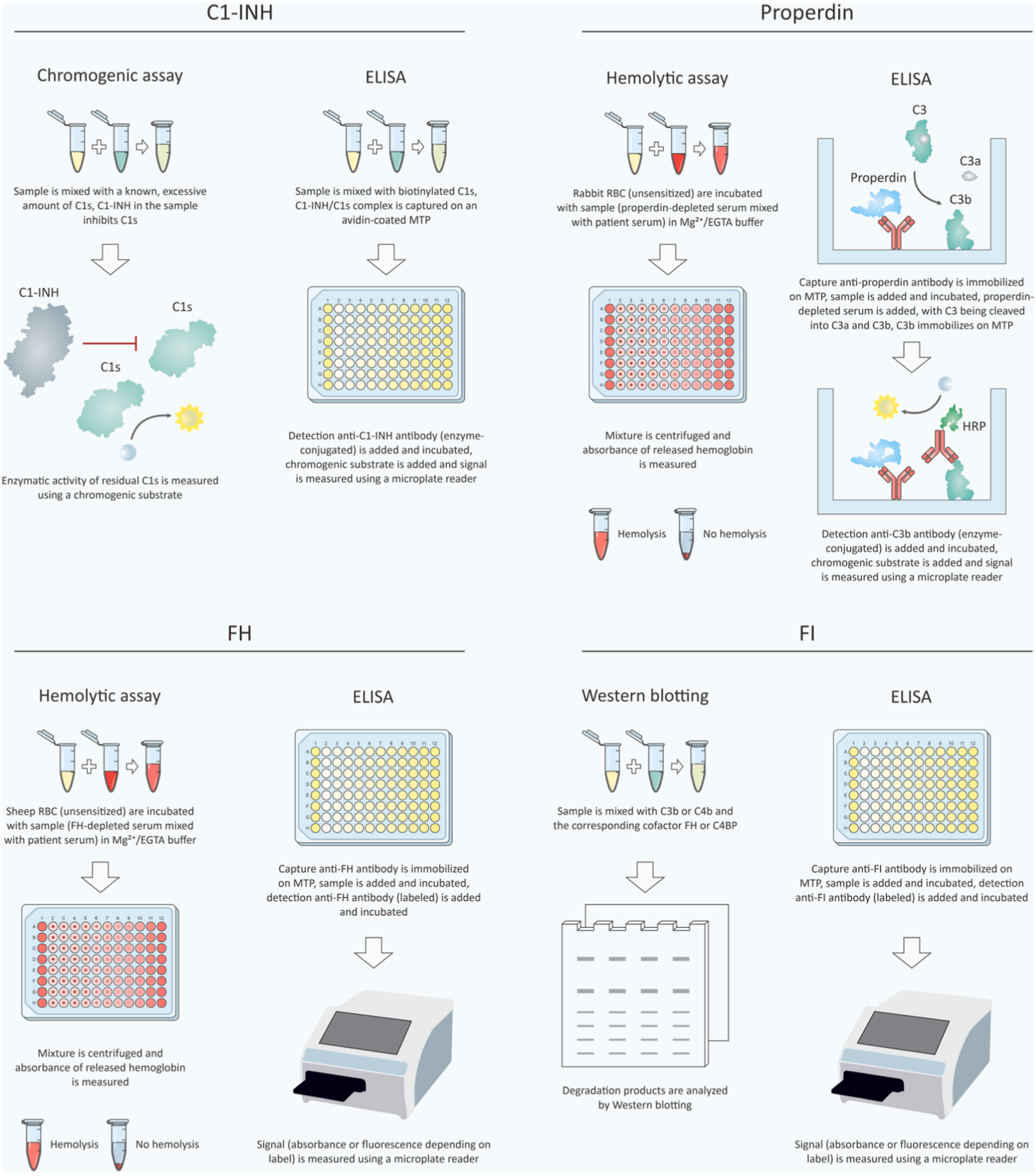

Fig. 6. Advances in functional assays for regulatory complement proteins from research and industry. For C1–INH, functional chromogenic assays and a functional ELISA are available, which measure the enzymatic activity of remaining, uninhibited C1s or determine the concentration of C1–INH/C1s complexes, respectively (top left). Properdin can be functionally assessed either by using a hemolytic assay with rabbit RBC or by using a functional ELISA based on the properdin-promoted cleavage of C3 (top right). The functions of FH and FI can be evaluated using a combination of a hemolytic assay with sheep RBC (in the case of FH) or a Western blot (in the case of FI) and a quantitative ELISA for concentration determination. The hemolytic assay is based on the binding of FH to sialic acids on the surface of sheep RBC and the protective properties of FH against hemolysis (bottom left), whereas the Western blot detects the cleavage products of C3b or C4b formed by FI in the presence of a suitable cofactor (bottom right). Abbreviations used in this figure: C1–INH, C1-esterase inhibitor; C4BP, C4b-binding protein; FH, Factor H; FI, Factor I; HRP, horseradish peroxidase; MTP, microtiter plate; RBC, red blood cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

C4BP. Using selected reaction monitoring mass spectrometry, Enjalbert et al. were able to improve the detection specificity for the quantification of C4BP (α-chain) and other proteins [359]. More recent progress was made in studies by Kadava et al., who provided structural insights and quantitative information on the proteoform profile of C4BP using techniques such as mass spectrometry, mass photometry, and atomic force microscopy [360]. However, the further development of analytical methods for C4BP will depend largely on the possible, as yet unknown, roles that C4BP may play in pathologies, and consequently on its clinical impact.

3.1.3. Factor H (FH) and FH-related proteins (FHRs)

Due to its ability to recognize host structures and avert complement attack on these surfaces by inhibiting the alternative pathway and the amplification loop, FH is one of the most significant regulators of the complement system [28,361]. This is also reflected in the various approaches to using purified FH or recombinant forms of FH for therapeutic purposes. Along with its role in various pathologies such as AMD, aHUS, and C3G, this makes analytical methods for FH, together with FHRs, valuable in research, the development of complement therapeutics, and the diagnosis of complement-related diseases including companion diagnostics [30,362]. Common clinical tests for FH include CH50 and AH50 assays for an initial assessment, whereby low AH50 values are to be expected in the case of an FH abnormality, but low CH50 values can also frequently be observed due to C3 consumption [363]. By measuring FH plasma levels and activity, determining the presence of FH autoantibodies, or performing genetic analysis, assumptions about whether a certain disorder is caused by an FH abnormality can be verified [275].

After the first purification and characterization of FH by immunochemical and hemolytic assays in 1965, studies investigated the C3b binding and FI cofactor activity of FH by ELISA or SDS-PAGE in combination with autoradiography or Western blot, respectively [364,365]. Since then, hemolytic assays have become the standard for the functional assessment of FH in research and can also be applied in the clinic to monitor disease progression [366]. One common assay allows to determine the decay acceleration activity of FH [367], while another can be used to study the cell surface protection activity of FH [368,369]. As described above, both assays rely on the fact that the membrane of sheep erythrocytes contains sialic acids that act as natural ligands of human FH and are summarized in Ref. [370]. In the first assay, alternative pathway C3 convertases are generated on C3b-coated sheep erythrocytes by adding FB and FD. After incubation of these sheep erythrocytes with FH from plasma and subsequent incubation with rat serum containing ethylenediaminetetraacetic acid (EDTA), the decay acceleration activity of FH can be determined based on the hemolysis of the sheep erythrocytes. In the second assay, sheep erythrocytes are directly mixed and reacted with FH from human serum. The incubation is stopped by adding buffer that contains EDTA, and the cell surface protection activity of FH is calculated from measurements on the hemolysis of the sheep erythrocytes. It should be noted that these assays may not specifically address an FH abnormality or the presence of FH autoantibodies in the patient serum, as an increased hemolysis could also be explained by the presence of C3 convertase-stabilizing C3NeFs.

As with most soluble complement proteins, the quantification of FH is typically performed by immunoassays such as ELISAs, although many of these assays determine only the total FH concentration. However, FH complexes with autoantibodies in aHUS patients or with C3b in patients with FI deficiency can interfere in the measurement. In addition, it should be noted that distinguishing between free FH and complex-bound FH is crucial for the diagnosis of aHUS, as only free FH is usually biologically active. As a result, patients with aHUS may be found to have supposedly normal FH concentrations, although in fact there is a critical deficiency of functional FH. Here, an ELISA developed by Nozal et al. with special monoclonal antibodies is able to differentiate between free and complex-bound FH [371]. This possibility of determining free FH concentrations is likely to not only better reflect the disease course of aHUS but also allow for better predictions of potential relapses in aHUS patients with high anti-FH autoantibody titers [372]. Variant-specific monoclonal antibodies that detect the polymorphic Y402H variant of FH employed in an ELISA by Hakobyan et al. were developed to evaluate the risk of AMD or to identify null alleles in aHUS patients [373,374].

Various techniques and assay formats have been conceived to reveal the interactions between FH and its ligands. For example, analytical ultracentrifugation, SPR and NMR spectroscopy, and X-ray scattering were applied to examine the self-association of FH and its binding to C3 cleavage products and glycosaminoglycans [375,376]. By using affinity chromatography and microtiter plate binding assays, Clark et al. could demonstrate the tissue-specific binding properties of FH [377]. Comprehensive structural analysis by X-ray crystallography and saturation transfer difference NMR spectroscopy furthermore enabled Blaum et al. to gain deep insights into the complex interactions between FH and sialic acid glycans [378].

Multiplexed mass spectrometry is becoming increasingly important in clinical laboratories for general complement testing, but also for regulatory complement proteins. A multiplexed assay has been published by Zhang et al., enabling the simultaneous quantification of FH variants and all FHRs in plasma samples using mass spectrometry [379]. Moreover, genetic analysis of the *CFH* gene has become a valuable tool for the diagnosis and individualized treatment of patients with FH-associated diseases. One such method, developed by Velissari et al., allows real-time PCR-based genotyping of the Y402H polymorphism in the *CFH* gene [380]. After genotyping and mutation screening, Merinero et al. additionally performed a complete functional characterization of the different FH variants from aHUS and C3G patients, including cell surface protection (hemolytic assay), decay acceleration (SPR), FI cofactor activity (proteolysis assays), and C3b binding activity (SPR) to identify and categorize new pathological variants in the *CFH* gene [381].

The role of FHRs in health and disease is increasingly being uncovered, yet it has been elusive for a long time and remains a subject of ongoing research [28,30]. Consequently, the development of specific assays for FHRs were mostly limited to research purposes. However, the discovery that certain FHRs are associated with diseases such as AMD, aHUS, and IgAN has sparked interest in analytical methods for FHRs [382]. For example, ELISAs by van Rossum et al. for the quantification of FHR-2, FHR-3, FHR-4, and FHR-5 were introduced [383]. Thus, it can be assumed that innovations in the field of analytical methods for FHRs will be accelerated in the future. The methods for FH/FHR analysis described above are summarized again in Table 6.

Table 6
Overview of analytical methods for FH and FHRs. Abbreviations used in this table: FH, Factor H; FHR, FH-related proteins; FI, Factor I; PCR, polymerase chain reaction; SPR, surface plasmon resonance.

Hemolytic assays	ELISA	Mass spectrometry	Others
functional FH assay for measuring decay acceleration activity [367]	ELISA that differentiates between free and complex-bound FH [371]	multiplexed mass spectrometry assay for several FH variants and all FHRs [379]	real-time PCR-based genotyping of the polymorphic Y402H variant of FH [380]
functional FH assay for measuring cell surface protection activity [368,369]	ELISA that specifically detects the polymorphic Y402H variant of FH [373,374]		decay acceleration (SPR), FI cofactor activity (proteolysis assay), C3b binding activity (SPR) [381]
	ELISAs for FHRs (FHR-2, FHR-3, FHR-4, FHR-5) [383]		

3.1.4. Factor I (FI)

When considering pathologies such as AMD, aHUS, and C3G, it becomes apparent that clinical conditions caused by defects in the *CFH* gene are often also influenced by defects in the *CFI* gene. This reflects the fact that FH and FI work together in the inactivation of C3b, and hence, abnormalities in either protein can impair proper complement regulation [384,385]. Similar to FH, if an FI deficiency or dysfunction is suspected, the CH50 and AH50 values can be determined as an initial assessment, with low CH50 and AH50 values indicating an FI abnormality [275]. Further testing such as FI plasma levels, activity, or genetic analysis can be performed to confirm whether an FI abnormality is indeed present.

When FI was first described in 1968, many studies attempted to explore and measure its function in complement regulation [386–388]. Due to the critical role of FI in the breakdown of C3b and C4b from all activation pathways, which was uncovered shortly thereafter, analytical methods for the evaluation of FI functional activity gained in significance. Three important assays that were established are summarized by Sim et al. [389]. The first assay is a hemolytic assay that can be used to assess the degradation of C3b by FI on the surface of sheep erythrocytes in the presence of a cofactor, such as FH. It can be adapted as needed by either keeping the FI concentration or the cofactor concentration constant and varying the other concentration accordingly [390]. The second assay is also a hemolytic assay and relates more to the cofactors of FI and their decay acceleration activity. Here, C3bBb-coated sheep erythrocytes are used to determine either FI functional activity or FI cofactor activity under appropriate circumstances [391]. The third assay can again be used to measure the degradation of C3b in the fluid phase, as already described for C4BP and FH. Purified (optionally radiolabeled) C3b is incubated in the presence of FI and a cofactor, and the degradation products are then analyzed using SDS-PAGE together with autoradiography or Western blot.

Over the last years, there have been multiple efforts to assess the functional activity of pathological FI variants. Kavanagh et al. investigated various FI mutants associated with aHUS in C3b and C4b degradation assays using SDS-PAGE and Western blot [392]. Nilsson et al. also examined the binding sites that are necessary for the functional activity of FI and performed a broad characterization of FI mutants from patients with FI deficiency, sequencing the corresponding DNA segments and analyzing the FI variants structurally and functionally in C3b and C4b degradation assays with the help of SDS-PAGE and autoradiography [393,394]. By using X-ray crystallography, Roversi et al. established a structural model for the mechanism behind the proteolytic activity of FI in inactivating C3b and C4b in the presence of a cofactor and for the influence of polymorphisms in the *CFI* gene associated with diseases on this activity [395]. In the study by Gerogianni et al., three different techniques were applied to investigate the inactivation of C3b by FI variants associated with aHUS and AMD and the degradation products of the reaction. SDS-PAGE and ELISA were used to detect the degradation of C3b and the generation of iC3b in the fluid phase, while a bead-based assay measured the degradation of surface-bound C3b of FI variants. FH and soluble CR1 (sCR1) were employed as cofactors [396]. An assay, in which patient serum is highly diluted and incubated with purified C3b and FH, was described by Java et al. and employed to characterize FI variants from AMD patients [385,397]. After the incubation step, the sample is further diluted in ELISA buffer and C3b inactivation by FI is measured using an ELISA for the detection of iC3b. A similar approach was taken by Jong et al. to determine and classify the functional activity of FI variants in AMD and aHUS [398]. Instead, Zhang et al. developed a hemolytic assay for FI variants from patients with aHUS or C3G in which C3b-coated sheep erythrocytes were mixed and incubated with diluted patient plasma and one specific cofactor (FH, sCR1, or sMCP) [384]. Purified FB and FD were then added, incubated again, and hemolysis of the sheep erythrocytes was induced with rat EDTA serum. The degree of hemolysis was used as a measure of the amount of residual C3b on the sheep erythrocytes and thus indirectly for FI functional activity. Other concepts include an assay developed by Lachmann et al. based on conglutination [399], a real-time SPR assay that measures the formation of the C3b/FH/FI complex by Hallam et al. [400], and a recently published minigene splicing assay by Donelson et al. based on reverse transcriptase polymerase chain reaction [401], all of which are *in vitro* assays. An interesting approach is therefore the surrogate *in vivo* assay by Tan et al., which was applied to detect and quantitatively score retinal vascularization in zebrafish embryos and to study the relationship between rare alleles in the *CFI* gene and AMD [402].

Overall, it will be particularly important for routine testing in the future to determine which of the functional FI assays developed in research can be translated to clinical settings. Simpler and more rapid functional assays for both FH and FI will be needed for the diagnosis of AMD, aHUS, and C3G, going beyond hemolytic assays and the quantification of degradation products via SDS-PAGE and Western blot (Fig. 6).

3.1.5. Properdin (Factor P, FP)

Deviations from normal plasma levels of properdin have been associated with various clinical conditions [403]. The most prominent connection is the significantly increased susceptibility to meningococcal infections caused by properdin deficiency [404,405]. Properdin deficiencies are generally divided into three types. In type I, there is a complete lack of properdin in plasma; in type II, the properdin level in plasma is low; and in type III, the properdin levels in plasma are normal, but the properdin present is dysfunctional [406]. Consequently, types I and II can be detected using quantitative assays, while type III requires a functional test of properdin. In the clinic, CH50 and AH50 assays can be performed to determine whether an abnormality of properdin is present. In cases of properdin deficiency, the CH50 values are usually within the normal range, while the AH50 values are reduced [407]. Nevertheless, these tests cannot clearly distinguish between type I/II and type III properdin deficiency. For this purpose, a combination of quantitative assays to measure the plasma levels of properdin, functional assays to assess the properdin activity in serum, and genetic analysis of the *CFP* gene should be used to identify the exact underlying cause.

Pillemer et al. first discovered properdin in 1954 and described it as a component of a new activation pathway that, unlike the classical pathway, was antibody-independent [408]. The concept of the alternative pathway, which was introduced with this discovery, sparked a debate about whether properdin was really a protein distinct from antibodies or components of the classical pathway, but these doubts were conclusively dispelled in 1968 by Pensky et al. through the isolation and characterization of properdin in highly purified form [409]. An initial analytical method for quantifying properdin, the zymosan assay, was already provided by Pillemer et al. with their demonstration of properdin [408,410]. This assay is based on the activation of the alternative pathway by zymosan, which is enhanced in the presence of properdin. By readout of the hemolysis of sensitized sheep erythrocytes, it can be determined how a sample can restore this enhancing activity in properdin-deficient serum. Later, further quantitative assays were developed, such as a solid phase radioimmunoassay [411], a radial hemolysis assay [412], and a sandwich ELISA [413]. Moreover, hemolytic assays for the study of the convertase stabilizing activity of properdin using sensitized sheep erythrocytes [414,415] or for the study of the complement activation promoting activity of properdin using rabbit erythrocytes or neuraminidase-treated sheep erythrocytes [416] were conceived.

Antibodies targeting properdin may have potential therapeutic use in complement-related diseases and have been employed for the development of quantitative and functional ELISAs [223,417,418]. Under physiological conditions, properdin forms oligomers, mainly dimers, trimers, and tetramers. Moore et al. applied a functional ELISA to demonstrate the specific distribution of neutrophil-derived properdin oligomers [417]. Tsyrkunou et al. determined the properdin concentration in serum samples from patients receiving chemotherapy using

ELISAs [419]. Since properdin is produced and stored by neutrophils, among other cells, chemotherapy-induced neutropenia can be accompanied by a significant decline in circulating properdin levels, as Tsyrkunou et al. found.

Structural and functional studies were conducted by various groups to lay the foundation for understanding the role of properdin in amplifying the alternative pathway. With the help of electron microscopy, Alcorlo et al. provided a model of how properdin mediates the assembly of C3 and C5 convertases and stabilizes them [420]. This was expanded upon by Pedersen et al., who investigated the properdin-convertase complex using X-ray crystallography and the interaction of properdin with C3 products using surface plasmon resonance spectroscopy. They also revealed the role of mutations and oligomerization on the conformation, binding properties, and functional activity of properdin in extensive studies involving small-angle X-ray scattering, hemolytic assays, a C3b deposition assay on zymosan, and a serum bactericidal assay [421]. Furthermore, Michels et al. elucidated the influence of properdin on C5 convertase activity and MAC formation using a hemolytic assay. This assay represents a previously developed convertase activity assay with rabbit erythrocytes and the addition of a C5 inhibitor [422,423], which was adapted with properdin-depleted serum supplemented with purified properdin [424]. The assay can also be performed in a similar format to differentiate between patient samples containing properdin-dependent and properdin-independent C3NeFs [425].

Similar to FH and FI, functional assays for properdin will become increasingly important for the diagnosis and classification of properdin deficiencies, as evidenced by the first products already entering the market (Fig. 6 and Table 7). Moreover, oligomer-specific assays will help researchers explore the influence of the oligomerization state on the functional activity of properdin, thereby opening new avenues for its therapeutic modulation.

3.1.6. Carboxypeptidase N (CPN)

There are only a few known cases of CPN deficiency, with no reported cases of complete deficiency, but low blood concentrations of CPN have been associated with symptoms such as recurrent angioedema and chronic urticaria [426,427]. Its testing is therefore largely limited to research settings or specific investigative purposes.

Following its discovery and description of its enzymatic activity as a peptidase and esterase for the inactivation of bradykinin by Erdös et al. [428–430], its role in the inactivation of anaphylatoxins was soon elucidated by Bokisch et al. [34,431]. Due to this function, various assays have been developed in the past, mainly to measure its enzymatic activity. Initial approaches were spectrophotometric assays based on absorbance measurements of hydrolytic cleavage products of substrates such as furylacryloyl-alanyl-arginine/-lysine [432] and hippuryl-arginine/-lysine [433]. These spectrometric assays were later further refined, either through HPLC-assisted techniques, in which the hydrolytic cleavage products were separated by HPLC prior to measurement [434,435], or through improved substrates that allowed for easier colorimetric measurement of the hydrolytic cleavage products [436,437]. One such substrate is *p*-hydroxyhippuric acid--L-arginine/-L-lysine, which is converted by CPN to *p*-hydroxyhippuric acid and L-arginine/L-lysine. In the presence of hippuricase, *p*-hydroxybenzoic acid is then produced, which reacts further with 4-aminoantipyrine and sodium periodate to form a quinoneimine dye whose absorbance can be easily detected. Another more recent colorimetric assay by Willemse et al. employs benzoyl-L-alanyl-L-arginine as a substrate and arginine kinase, pyruvate kinase, and lactate dehydrogenase as auxiliary enzymes in a coupled enzymatic reaction to finally generate NADH and detect its absorbance signal [427]. Willemse et al. also investigated the influence of the enzymatic activity of procarboxypeptidase U (CPU) on CPN assays. As they encountered interferences, they developed highly selective substrates for CPU to distinguish between the two enzymatic activities (CPU and CPN) in plasma samples [438,439].

Table 7
Selection of commercially available assays for fluid-phase regulatory complement proteins. Functional assays are marked in bold.

Product	Assay technology	Analyte	Company	Webpage (www.)
Human C1–INH ELISA Kit	ELISA	**C1–INH**	Hycult Biotech	hycultbiotech.com
Human C1 Inhibitor ELISA Kit	ELISA	**C1–INH**	Creative Biolabs	creative-biolabs.com
Human C1 Inhibitor ELISA Kit	ELISA	**C1–INH**	abcam	abcam.com
Technochrom C1–INH	Chromogenic	**C1–INH**	Technoclone	technoclone.com
Berichrom C1-Inhibitor	Chromogenic	**C1–INH**	Siemens	siemens-healthineers.com
MicroVue C1-Inhibitor Plus	ELISA	**C1–INH**	QuidelOrtho	quidelortho.com
Human Complement C4BP ELISA Kit	ELISA	**C4BP**	abcam	abcam.com
Complement Factor H Functional Assay	Combination of ELISA and Hemolytic assay	**FH**	Creative Biolabs	creative-biolabs.com
Human Complement Factor H ELISA Kit	ELISA	**FH**	Hycult Biotech	hycultbiotech.com
Human Complement Factor H ELISA Kit	ELISA	**FH**	abcam	abcam.com
MicroVue FH EIA	ELISA	**FH**	QuidelOrtho	quidelortho.com
Human Complement Factor H ELISA Kit	ELISA	**FH**	Invitrogen	thermofisher.com
Human Complement Factor H DuoSet ELISA	ELISA	**FH**	R&D Systems	rndsystems.com
Human CFHR1 ELISA Kit	ELISA	**FHR-1**	abcam	abcam.com
Human CFHR1 ELISA Kit	ELISA	**FHR-1**	Invitrogen	thermofisher.com
Human FHR-2 ELISA Kit	ELISA	**FHR-2**	Hycult Biotech	hycultbiotech.com
Human CFHR2 ELISA Kit	ELISA	**FHR-2**	abcam	abcam.com
Human FHR-3 ELISA Kit	ELISA	**FHR-3**	Hycult Biotech	hycultbiotech.com
Human CFHR3 ELISA Kit	ELISA	**FHR-3**	abcam	abcam.com
Human FHR-4 ELISA Kit	ELISA	**FHR-4**	Hycult Biotech	hycultbiotech.com
Human CFHR4 ELISA Kit	ELISA	**FHR-4**	abcam	abcam.com
Human FHR-5 ELISA Kit	ELISA	**FHR-5**	Hycult Biotech	hycultbiotech.com
Human CFHR5 ELISA Kit	ELISA	**FHR-5**	abcam	abcam.com
Human CFHR5 ELISA Kit	ELISA	**FHR-5**	Invitrogen	thermofisher.com
Complement Factor I Functional Assay	Combination of Western blot and ELISA	**FI**	Creative Biolabs	creative-biolabs.com
Human Complement Factor I ELISA Kit	ELISA	**FI**	Hycult Biotech	hycultbiotech.com

(*continued on next page*)

Table 7 (*continued*)

Product	Assay technology	Analyte	Company	Webpage (www.)
Human Complement Factor I ELISA Kit	ELISA	**FI**	abcam	abcam.com
Human Complement Factor P ELISA Kit	ELISA	**Properdin**	Svar Life Science	svarlifescience.com
Complement Factor P Functional Assay	ELISA	**Properdin**	Svar Life Science	svarlifescience.com
Complement Factor P Functional Assay	Combination of ELISA and Hemolytic assay	**Properdin**	Creative Biolabs	creative-biolabs.com
Human Complement Factor P ELISA Kit	ELISA	**Properdin**	Creative Biolabs	creative-biolabs.com
Human Properdin ELISA Kit	ELISA	**Properdin**	Hycult Biotech	hycultbiotech.com
ELISA MAX Deluxe Set Human Properdin Kit	ELISA	**Properdin**	BioLegend	biolegend.com
Human Properdin ELISA Kit	ELISA	**Properdin**	Invitrogen	thermofisher.com
Human Clusterin ELISA Kit	ELISA	**CLU**	Hycult Biotech	hycultbiotech.com
Human Clusterin ELISA Kit	ELISA	**CLU**	R&D Systems	rndsystems.com

3.1.7. *Clusterin (CLU) and vitronectin (VTN)*

Along with immunohistochemical examination of CLU and VTN in tissue samples, clinical testing primarily focuses on determining their concentration in blood, plasma, serum, or urine samples for research and specialized diagnostics. CLU and VTN deficiencies are uncommon and not as clinically recognized as other complement deficiencies. While genetic variations leading to CLU deficiency have been associated with Alzheimer's disease [149], no genetic deficiency of VTN has been reported so far [440]. Reduced CLU plasma levels have been detected in cases of sepsis [441], whereas reduced VTN serum levels have been linked to autoimmune, kidney, and liver diseases [440]. CLU is being discussed as a biomarker for various clinical conditions, such as autoimmune diseases [442,443], impaired renal and hepatic function [444,445], cancer [446–449], and Alzheimer's disease [450]. In addition, CLU inside erythrocyte membranes has been proposed as a biomarker for cellular stress and senescence [451]. VTN has also been suggested as a biomarker in cancer [452–454], but also for fibrosis in patients with kidney transplants [455,456] as well as for portopulmonary hypertension [457].

3.1.8. *Clusterin (CLU)*

A sandwich ELISA specifically for CLU was developed by Choi et al. to determine CLU concentrations in seminal plasma and serum samples [458] and a competitive ELISA by Morrissey et al. used biotinylated recombinant human CLU to compete with CLU in serum samples for binding [459]. Later, an optimized sandwich ELISA was established by Jongbloed et al. and employed for the quantification of CLU in cerebrospinal fluid and plasma samples [460].

A combination of several biomarkers, including CLU, measured in urine samples using ELISAs has been shown to be effective in assessing kidney injury status. This biomarker panel can be used to assess the risk of chronic kidney disease and could prove valuable in the diagnosis, prognosis, or monitoring of IgA nephropathy, kidney stones, or kidney transplant rejection [461–464]. Moreover, using proteome analyses by liquid chromatography tandem mass spectrometry, transcriptome analyses by qRT-PCR, CLU immunofluorescence staining of kidney biopsies, and ELISA for CLU urine levels, Baird et al. found that the ratio of CLU to creatinine in urine could be a suitable biomarker for kidney cell senescence, representing a non-invasive alternative to histological analysis [465].

Analytical methods for CLU other than ELISA include, for example, a reverse phase microarray and a multiplexed homogeneous proximity ligation assay. The reverse-phase protein microarray developed by Aguilar-Mahecha et al. detects CLU in plasma and serum spots on epoxy-coated slides, allows high-throughput measurements, and requires smaller sample volumes compared to ELISA [466]. The multiplexed homogeneous proximity ligation assay by Lundberg et al. analyzes CLU in plasma samples as one of a total of 74 potential biomarkers. The binding of antibody pairs, each modified with a unique nucleic acid sequence, to their common target analyte results in the formation of amplicons after a ligation step. The amplicons are then amplified in multiplex by qRT-PCR, enabling high-throughput quantification of each target analyte [467].

Due to its role in Alzheimer's disease, various efforts have been made to examine CLU in the human brain, including a study by Chen et al., in which CLU was quantified using mass spectrometry [468]. Mass spectrometry was also implemented in quantitative assays of CLU as part of a panel of six urinary protein biomarkers, comparing different acquisition modes, namely parallel reaction monitoring, data-independent acquisition, and tandem mass tag-based data-dependent acquisition. The assays, which were recently introduced by Ponce et al., were tested on three different mass spectrometry instruments, and the data-independent acquisition mode was applied on urine samples from prostate cancer patients [469].

3.1.9. *Vitronectin (VTN)*

The first approaches to detecting VTN primarily included EID- and ELISA-based techniques [470–472]. Furthermore, an enzymatic VTN assay was developed by Korc-Grodzicki, which works by phosphorylating VTN through a catalytic subunit of protein kinase A [473]. This enzymatic reaction not only allows the VTN concentration to be determined but can also be used to radiolabel VTN and detect VTN-related proteins in animal plasma.

In an SPR-based VTN assay by Chen et al., on the other hand, the sensor gold surface is first modified with a monolayer of a calixarene derivative so that integrin can be immobilized on it as a capture protein for VTN [474]. The calixarene derivative also contains a crown ether moiety, which enables various interactions between integrin and the coated monolayer. SPR can then be used to measure the binding of VTN to integrin. Structural and mechanistic insights into the complex structure of VTN with C5b-9 (sC5b-9) were obtained by Hadders et al. using cryo-electron microscopy and provided information about the role of VTN in MAC regulation [41].

3.2. *Membrane-bound regulatory proteins*

Biopsies and histological examinations are often time-consuming. Flow cytometry has therefore become the gold standard for testing membrane-bound regulatory complement proteins [299]. Modern instruments allow single-cell sorting, multicolor or full spectrum analysis, and high-content imaging and couple flow cytometry to mass spectrometry or proteogenomic techniques [475,476]. With these new approaches, high-dimensional flow cytometry measuring multiple parameters on single cells can be achieved.

Protocols for the isolation and characterization of CR1 [477–479], MCP [480,481], and DAF and protectin [482–484] have been published by various groups. These assays mainly comprise quantitative

determination of expression levels by flow cytometry, functional analysis based on hemolysis or complement-mediated cytotoxicity, and cofactor activity assays.

Nuutila et al. investigated the expression levels of CR1, MCP, DAF, and protectin on leukocytes to determine whether these could be used as markers for the diagnosis of bacterial or viral infections [485]. From their results, they deviated and proposed two new flow cytometry indices in the diagnosis of clinical mononucleosis and clinical bacterial infection. For research on the complement system, Thielen et al. generated knockout cell lines specifically lacking MCP, DAF, or protectin using the gene-editing CRISPR/Cas9 system [486]. Sandwich ELISAs for the soluble forms of CR1, MCP, DAF, and protectin are commercially available and were applied by Mishra et al. for the assessment of leukocyte subsets in patients with coronary artery disease [487].

3.2.1. Complement receptor 1 (CR1, CD35)

Patients with SLE often present with low expression levels of CR1 on erythrocytes [488–490]. In addition, it was found that a high expression level of CR1 on neutrophils in patients with pneumonia is more likely to indicate a bacterial cause than a viral one [491]. Although flow cytometry can be used in the clinic to measure the density of CR1 on the surface of erythrocytes and leukocytes, CR1 testing is not typically part of routine diagnostics.

The first studies on CR1 were conducted by Nelson et al., in 1953, who described the immune adherence properties of CR1 on erythrocytes [492]. Early assays to characterize CR1 comprised binding assays to C3b and C4b [493–496], SPR for the interaction of CR1 with C1q, C3b, and C4b [497–499], cofactor activity and rosette formation assays for erythrocyte CR1 [477,500–502], and early flow cytometry techniques [503,504]. Since then, analytical methods in CR1 research have been continuously improved over the years. An ALP-based assay developed by Rafnar et al. measures the ability of sera and erythrocytes to clear immune complexes, which is particularly important in the investigation of diseases such as SLE [505]. Furthermore, an ELISA for the quantification of sCR1 in plasma was established by Hara et al. and Sivasankar et al. [506,507] and a method for preserving erythrocytes by fixation or freezing in order to store them for later analysis of CR1 by flow cytometry was devised by Cockburn et al. [508].

Insights into the inhibitory mechanism of CR1 were recently provided by Wymann et al. with domain duplication studies, in which they functionally evaluated CR1 truncation variants using the complement system screen ELISA, CH50 and AH50 hemolytic assays, and cofactor activity assays, and characterized their binding to C3b and C4b using SPR [509]. Other advances are moving toward genetic analysis of CR1. For example, a triplex paralogue ratio assay by Kucukkilic et al. allows to determine a copy number in the *CR1/CD35* gene, which is strongly associated with late-onset Alzheimer's disease [510]. However, more technologies and automated procedures are also emerging, such as a biosensor developed by Kliment et al. [511]. This optical biosensor uses a resonant waveguide grating to enable label-free, time-resolved monitoring of the activation kinetics of B-lymphocytes upon stimulation of various receptors (including CR1). Another example is the system developed by Spijkerman et al., which measures the expression level of activation markers (including CR1) on granulocytes and monocytes. By automating the analysis of the leukocyte phenotypes, the analysis time, which is critical for assessing the activation status of these cells, could be significantly reduced [512]. Finally, in a study by Li et al., a new proximity barcoding assay was employed to examine the surface proteome of single urinary extracellular vesicles [513]. The study found that CR1 expression on urinary extracellular vesicles was significantly decreased in patients with sepsis-associated acute kidney injury (SA-AKI), which was confirmed by super-resolution microscopy, nano-flow cytometry, Western blot, and ELISA. Moreover, the study showed that CR1 on urinary extracellular vesicles can serve as a biomarker for the diagnosis of SA-AKI.

3.2.2. Membrane cofactor protein (MCP, CD46)

To confirm the diagnosis of aHUS, genetic analysis of the *MCP/CD46* gene and measurement of MCP expression on neutrophils are commonly performed today. Besides aHUS, genetic analysis may be considered for other diseases associated with mutations in the *MCP/CD46* gene, such as SLE, C3G, and pregnancy-related disorders [514].

MCP was first identified as a C3b-binding protein on leukocytes by Cole et al., in 1985 [515] and later purified and further characterized by Seya et al. [481]. Analytical methods at that time primarily focused on the binding properties of MCP, its cofactor activity, and its expression levels on different cell types [516–520]. Binding assays were particularly valuable for understanding the interactions between MCP and pathogens such as measles and adenoviruses, and thus for understanding the role of MCP as a "pathogen magnet" [521,522]. With the rise of nucleic acid vaccines and gene therapy, in which adenoviruses are employed as important vectors, this specific role of MCP has regained significance, as a recent study could demonstrate. In this study, Danskog et al. created MCP CRISPR/Cas9 knockout clones of a human epithelial lung cell line to identify MCP as a crucial receptor for species D adenoviruses, which are currently among the most widely used adenovirus vectors [523]. They also characterized the binding of the whole virus and individual virus proteins to MCP using SPR. The study was built upon the finding that MCP acts as a receptor for most species B adenoviruses [524,525].

Soluble forms of MCP (sMCP) have been detected in various body fluids by ELISA [526,527]. In addition, increased serum levels of sMCP were measured in patients with cancer [528] and with SLE [529], while patients with glomerular diseases were found to have increased urine levels of sMCP [530]. Interestingly, sMCP has also recently been suggested as a biomarker for hepatic steatosis [531] and hepatocyte stress [532]. In these cases, the presence of the pathology was indicated by elevated circulating concentrations of sMCP.

Due to alternate splicing, MCP occurs in different isoforms that have been characterized by various groups [533–536]. A multiplex qRT-PCR assay developed by Hansen et al. enables the expression levels of each individual MCP isoform to be determined [537]. This assay, in combination with C3b binding assays, cofactor activity assays, and flow cytometry, has also been applied by Schack et al. for a detailed splice variant analysis of MCP in an aHUS patient [538]. Previous works had already elucidated the role of MCP in aHUS using techniques such as ELISA, Western blotting, and flow cytometry to measure MCP expression, as well as C3b and C4b binding and cofactor activity assays to assess MCP function [539–542].

Soon after MCP was discovered, its link to cancer was realized. Early studies recognized the upregulated expression of MCP on various leukemia as well as brain, kidney, or liver tumor cell lines [161,543–545]. Since then, MCP has become the subject of considerable research in this field. For example, a study by Hakulinen et al. showed that MCP can be released from the surfaces of certain cancer cell lines, either as sMCP or through vesicles. Here, MCP was detected by ELISA and SDS-PAGE in combination with Western blotting, while cofactor activity assays could reveal that these forms of MCP remained functionally active [546]. MCP has also been investigated as a potential biomarker in the diagnosis and prognosis of ovarian [547], breast [548], colorectal [549], and cervical cancer [550], as well as for multiple myeloma [551]. Furthermore, several studies have recently been conducted to evaluate the potential of MCP as a target for radiopharmaceuticals or antibody-drug conjugates in prostate cancer [552–556] and multiple myeloma [551,557,558]. Based on this emerging role as a biomarker or as a target for diagnostic and therapeutic anti-cancer agents, it can be assumed that MCP testing will become increasingly relevant in clinical practice, for example in the form of measuring MCP density on cancer cells for cancer prognosis, stratification, and treatment.

3.2.3. Decay-accelerating factor (DAF, CD55) and protectin (CD59)

The examination of DAF and protectin is primarily indicated for the

diagnosis and monitoring of PNH, which is performed using flow cytometry as the standard method, more specifically using FLAER, and genetic analysis in certain cases, as described above. However, a reduced expression of DAF and protectin was also observed in SLE patients with lymphopenia and neutropenia [559,560]. An assay for determining erythrocytes and reticulocytes with *PIGA* mutant phenotype, which extends flow cytometry with a preceding immunomagnetic separation step, has been introduced by Dertinger et al. [561]. Besides flow cytometry, specialized hemolytic or hemagglutination assays are available, but these are now mainly limited to research purposes [258,484].

Similar to MCP, DAF and protectin have been associated with a number of malignant diseases, including colorectal and gastric cancer [154–156,562–565], breast cancer [566–568], prostate cancer [569, 570], nasopharyngeal cancer [571], pancreatic cancer [572], uterine and ovarian cancer [573,574], as well as melanoma [575] and lymphoma [576]. Other studies have also suggested a potential connection between DAF and multiple sclerosis [577].

3.2.4. *Decay-accelerating factor (DAF, CD55)*

DAF was first described by Hoffmann et al., in 1969 [578,579]. Several years later, in 1982, human DAF was isolated for the first time by Nicholson-Weller et al. [580]. This was followed by a detailed characterization of this protein using assays to investigate its decay acceleration activity [581,582].

Various groups have examined the relationships between the structure and function of DAF by producing monoclonal antibodies directed against different DAF epitopes or recombinant mutant DAF proteins. Other approaches included the expression of mutant DAF on the surface of Chinese hamster ovary (CHO) cells or their incorporation into the membrane of sheep and rabbit erythrocytes [50,583–585]. Using flow cytometry, CHO cell binding assays, cytoprotection and hemolysis inhibition assays, C3b deposition assays, or fluid-phase C3a generation assays, the regulatory domains and ligand binding sites of DAF could be localized. Further structural insights were later obtained through X-ray crystallography and analytical ultracentrifugation [586,587].

SPR analysis was used to study the interactions of DAF with C3b, FB, and Bb [588], while Western blotting, radioimmunoassays, and ELISA were implemented to measure soluble DAF in body fluids, including tears, serum, plasma, urine, and saliva [589–591]. In addition, co-immunoprecipitation and Förster resonance energy transfer (FRET) analysis showed that DAF is part of the LPS receptor complex and is involved in LPS signaling [592,593].

Stool samples from patients with colorectal cancer were tested for DAF using ELISAs, revealing elevated concentrations compared to healthy patients and patients with other gastrointestinal diseases [594–597]. The method represents a non-invasive approach that could serve as an alternative to endoscopic procedures in the diagnosis of colorectal cancer. Genco et al. recently developed a portable device for the detection of pancreatic cancer precursor biomarkers, including DAF, in cyst fluid and plasma [598]. The device was designed for point-of-care applications and is based on a bioelectronic array that detects three different DNA or protein biomarkers at the single-molecule level in plasma samples using an organic field-effect transistor with capturing probe- or antibody-functionalized sensing gates. DAF has also been considered as a biomarker for other clinical conditions, such as active ulcerative colitis or Parkinson's disease [589,599] and DAF expressed on plasma extracellular vesicles has been proposed as a biomarker for ageing and metabolic changes [600]. Current strategies in the development of anti-cancer drugs targeting DAF comprise radionuclide-labeled chimeric monoclonal antibodies for metastatic lung cancer [601], inhibitory ligand peptides for cervical cancer [602], and silencing strategies using plasmid-delivered short hairpin RNAs (shRNA) in acute leukemia [603], whereby techniques such as flow cytometry, qRT-PCR, and cell viability assays are used to determine DAF expression levels and evaluate drug anti-tumor effects.

3.2.5. *Protectin (CD59)*

Protectin was discovered and first characterized by several groups simultaneously in 1989 [604–609]. Early binding and functional assays were employed to study the interaction of protectin with C8 (α-chain) and C9 with the help of specific peptides [51,52,610], as well as to investigate its protective role against complement attack using protectin-blocking monoclonal antibodies [611–613]. The functional assays included hemolysis and cell lysis assays, where protectin is either incorporated into animal erythrocytes or where CHO cells are transfected with human protectin. Later, several flow cytometry techniques to measure protectin on cell surfaces were established [308,309,483, 614].

A structural study providing profound mechanistic insights into MAC inhibition by protectin has recently been conducted by Couves et al. using cryo-electron microscopy, computational modeling, and molecular dynamics simulations [615]. Protectin is also exploited as a receptor for bacterial pore-forming toxins, as complex structures obtained by X-ray crystallography could show [616,617].

Protectin has also been discussed as a potential biomarker for various clinical conditions. For example, Zhou et al. proposed using protectin expressed on esophageal cancer cells to predict the resistance of these cells to radiation treatment [618]. The soluble form of protectin has also become the focus of studies searching for new biomarkers and has already been detected in a variety of body fluids, including tears, plasma, serum, urine, breast milk, amniotic fluid, and seminal fluid [619–625]. Since hyperglycemia can lead to increased glycation and thus reduced regulatory function of protectin, the less hindered MAC formation on cell surfaces causes tissue damage in patients with diabetes. Based on these findings, ELISAs have been developed by Ghosh and Sahoo et al. for soluble protectin as a biomarker for diabetes, utilizing plasma or serum samples [626]. Budding et al., on the other hand, indicate soluble protectin as a possible biomarker in the prognosis of post-transplant chronic lung allograft dysfunction [627].

Protectin is not only interesting as a biomarker, but also as a target for novel therapeutics. Its expression has already been successfully silenced in ovarian cancer cells through retrovirus-delivered shRNA interference [628]. Other strategies involved inhibitory recombinant proteins and macrocyclic peptides to increase the susceptibility of cancer cell lines to complement-mediated cytotoxicity in combination with antibody-based drugs in lymphoma and multiple myeloma [629–633]. Moreover, the macrocyclic peptides were confirmed to inhibit the activity of bacterial pore-forming toxins due to their binding to protectin [629]. The main techniques applied during the development and the assessment of their activities of these drug candidates were flow cytometry, hemolytic and cell viability assays, X-ray crystallography, and SPR analysis. Another recent study by Chaphekar et al. compared various techniques for detecting protectin on HIV-1 virions, including flow virometry, virus immunocapture assays, and Western blotting [634].

3.3. *Multiplexing*

Alongside improvements in the analysis of individual regulatory complement proteins, many efforts in the field of assay development are also moving toward multiplexing approaches [635]. For example, multiplexed antibody arrays [636], multiplexed mass spectrometry [637], and multiplexed ELISA panels [289,638] for a range of complement components have already been introduced. In mass spectrometry in particular, proteomic approaches were among the further directions pursued [639,640].

However, current multiplex panels predominantly capture soluble regulators such as FH and FI, while broader proteomic approaches additionally detect C1–INH, C4BP, CLU, VTN, and FHR proteins, indicating a clear bias toward abundant fluid-phase regulators [637–640]. In contrast, membrane-bound regulators (e.g. CD46, CD55, CD59) and low-abundance are largely underrepresented. Importantly, these

multiplex approaches are limited to quantitative measurements of protein abundance and do not provide functional readouts of regulatory activity. Thus, while multiplexing enables time- and sample-sparing profiling, limitations in antibody specificity, assay standardization, and differential sensitivity remain key constraints.

4. Progress in the development of commercial assays

To gain insight into whether there is an abnormality in a regulatory complement protein, functional assays of the activation pathways can be performed. Sheep erythrocytes for the hemolytic CH50 assay and rabbit, chicken, or guinea pig erythrocytes for the hemolytic AH50 assay can be purchased from various suppliers. The automated liposome-based Autokit CH50 for measuring total complement (mainly classical pathway) activity is a product of Fujifilm Wako, while there is also the Optilite® CH50 from The Binding Site. The complement system screen ELISA for measuring the activity of the individual activation pathways is currently marketed as the Wieslab® Complement System Screen kit by Svar Life Science or as the Human Classical/Lectin/Alternative Complement Pathway kit by Hycult Biotech. At present, immunoassays are still the standard assays for the quantitative measurement of complement components. Monoclonal and polyclonal antibodies are available for basically all regulators and can be implemented in turbidimetric, nephelometric, and ELISA-based assays. Accordingly, there are multiple commercial ELISA kits available from various manufacturers (Tables 7 and 8) and new ELISA kits are still being introduced on the market, such as the FHR quantitative ELISA by Hycult Biotech and the properdin quantitative and functional ELISAs by Svar Life Science. Despite their high sensitivity, ELISAs are often more difficult to automate than other methods, yet the use of robotic pipetting stations is increasing in large analytical facilities. In facilities with high sample numbers, other approaches may therefore be more suitable. Examples for the development of automated assays for regulatory complement proteins include an automated nephelometric immunoassay for FH [641], an automated turbidimetric immunoassay for C1–INH [642], and an automated chromogenic assay for C1–INH [643]. Instruments that are frequently used for automated turbidimetric and nephelometric protein testing in clinical settings are offered by various companies, including the Atellica® analyzer (Siemens Healthineers), the Immage® analyzer (Beckman Coulter), or the Optilite® analyzer (The Binding Site), and allow streamlined workflows.

Table 8
Selection of commercially available assays for membrane-bound regulatory complement proteins.

Product	Assay technology	Analyte	Company	Webpage (www.)
Human CR1 ELISA Kit	ELISA	**CR1**	Creative Biolabs	creative-biolabs.com
Human CD46 ELISA Kit	ELISA	**MCP**	Creative Biolabs	creative-biolabs.com
Human CD46 ELISA Kit	ELISA	**MCP**	abcam	abcam.com
Human Membrane Cofactor Protein (CD46) ELISA Kit	ELISA	**MCP**	abbexa	abbexa.com
Human Membrane Cofactor Protein (CD46) CLIA Kit	CLIA	**MCP**	abbexa	abbexa.com
Human membrane cofactor protein, MCP ELISA Kit	ELISA	**MCP**	Cusabio	cusabio.com
Human CD55 ELISA Kit	ELISA	**DAF**	Creative Biolabs	creative-biolabs.com
Human CD59 ELISA Kit	ELISA	**Protectin**	Creative Biolabs	creative-biolabs.com
Human sCD59 ELISA Kit	ELISA	**Protectin**	Hycult Biotech	hycultbiotech.com

Another relevant format that enables automated and integrated workflows is the Bioaffy® platform from Gyrolab®. This miniaturized immunoassay employs a compact disc (CD) with up to 112 microstructures. Each individual microstructure consists of a microfluidic system containing a flow-through affinity column packed with streptavidin-coated beads. The liquid handling of samples and reagents with a microfluidic system is based on a combination of capillary and centrifugal forces. By rotating the CD at different speeds and using special microfluidic chambers and channels, volumes can be reliably defined, resulting in high reproducibility and robustness. After the affinity column has been loaded with the biotinylated, analyte-specific capture antibodies, the sample is injected, and the analyte attaches to the activated affinity column. Next, the fluorescently labeled, analyte-specific detection antibody is introduced and the sandwich complex consisting of the capture antibody, analyte, and detection antibody is generated. Finally, fluorescence readout is performed by scanning the affinity column with a laser. The immunoassay is fully automated and requires very small sample volumes in the range of 0.02 to 4 µL. A C5a assay based on the Gyrolab® technology has already been established and assays for C4d and C5b-9 are being developed in cooperation with Svar Life Science [644]. Hence, extension to other complement components, including regulators, is feasible as well. A commercially available immunoassay with already two panels for multiplexing human complement components, including FH, FI, and properdin as regulators, is currently offered by Merck Millipore [15]. The Milliplex® platform is based on the Luminex® xMAP® technology and enables the detection of multiple analytes in a single well of a microtiter plate using fluorescently coded magnetic beads. All beads with the same spectrally distinct signature are precoated with an analyte-specific capture antibody. After incubation with the sample, a mixture of biotinylated, analyte-specific detection antibodies is added. The capture antibody, analyte, and detection antibody bind to each other in a sandwich complex. By adding streptavidin-conjugated phycoerythrin, the amount of sandwich complexes can be quantified. This is subsequently performed in a flow cytometry-based detection system. The beads are transferred from the well of the microtiter plate to a flow cell, where they are guided past two different lasers by sheath fluid. The first laser is used to read the respective bead signature from the spectral region and thus to identify the corresponding analyte. The second laser determines the intensity of the phycoerythrin signal, which is used to calculate the analyte concentration in the sample. This multiplexing approach requires a small sample volume and helps reduce time and costs. Concurrently, high sensitivities comparable to ELISAs can be maintained. The approach by Meso Scale Discovery, on the other hand, promises very high sensitivities with a broad dynamic range beyond that of typical ELISAs. This is achieved by electrochemiluminescence (ECL) detection instead of colorimetric detection using an enzymatic reaction. In one variant of the assay, analyte-specific capture antibodies are immobilized on a carbon electrode located at the bottom of each well of a special microtiter plate. When the sample is applied, the corresponding analyte binds to these capture antibodies. Sulfo-tag labeled, analyte-specific detection antibodies are subsequently added and form a sandwich complex with the analyte and capture antibody. A voltage applied to the electrodes in the well induces the ECL reaction of the sulfo-tag, which is based on the ruthenium (II) tris-bipyridine/tripropylamine system, and the light emission is detected. The platform also offers the possibility of multiplexing by creating patterned arrays of capture antibodies on the carbon electrode with up to 10 spots within a single well of a microtiter plate. Currently, the method only supports the measurement of complement components C3, C3a, C3b, C5a, C9, FD, and clusterin, with clusterin being the only regulator. Furthermore, Svar Life Science is developing cell-based (other than erythrocytes) complement assays using its iLite® technology. These reporter gene assays involve genetically modified cell lines that have analyte-specific receptors on their surface. When the analyte binds to the receptor, a cellular signaling cascade is triggered, resulting in the expression of a reporter gene. In the case of the iLite®

C3a or C5a assay, the interaction of C3a or C5a with C3aR or C5aR1, respectively, leads to the activation of a transcription factor, which then binds to a promoter and triggers the expression of Firefly luciferase. Bioluminescence detection is subsequently performed by adding the substrate reagent and measuring the light produced by the chemical reaction that is catalyzed by the luciferase. For normalization, Renilla luciferase is used as a control reporter gene with a separate, constitutively active protomer. Although only iLite® cells for C3a and C5a assays are currently being offered, it is quite possible that cells for regulatory complement proteins, especially for membrane-bound ones, will also become available in the future.

With the introduction of the cobas® Mass Spec system from Roche Diagnostics, mass spectrometry has now also found its way into clinical laboratories for routine analyses. The platform combines several units for sample supply, sample preparation, separation using liquid chromatography, and analyte detection using tandem mass spectrometry, enabling clinical laboratories to perform automated, integrated, and standardized high-throughput analyses [645]. Mass spectrometry not only provides high accuracy and precision but also achieves high sensitivities and specificities with the possibility of multiplexing entire analyte panels [646]. While mass spectrometry is currently limited to specialized clinical and research laboratories, it represents a promising addition to conventional immunoassays. It can therefore be assumed that mass spectrometry, once it has been established in clinical practice, will soon also be extended to routine complement testing, for example for complement profiling, complement drug monitoring, or the discovery of new complement-related biomarkers.

5. Preanalytics, standardization, and quality control

The complement system is typically tested in serum or plasma, with whole blood analysis also being possible but only rarely performed. Sample processing is extremely critical in this regard. Due to coagulation, which interferes with the analysis, blood samples must always either be mixed with an anticoagulant (often already contained in the coating of specific blood collection tubes) (for plasma) or blood coagulation and clotting must be allowed to occur in a controlled manner (for serum). Centrifugation is then applied to remove blood cells, blood cell fragments, and components of the coagulation system. EDTA and hirudin (or recombinant forms thereof, such as lepirudin) are particularly suitable anticoagulants. EDTA chelates Ca^{2+} and Mg^{2+} ions, which are essential divalent metal cation cofactors for complement activation, while hirudin prevents coagulation via its activity as a thrombin inhibitor. Since some complement proteins are extremely sensitive to post-sampling activation and can degrade over time or at elevated temperatures, complement activation should be efficiently blocked when determining the concentration of individual components and activation products. This can be ensured by using EDTA-plasma (with EDTA concentrations of at least 10 mM). Heparin and citrate, by contrast, do not sufficiently prevent complement activation and should therefore not be used. For functional assays and quantitative assays of autoantibodies, serum or hirudin-plasma are to be preferred [647].

When preparing serum samples, a correct blood processing protocol should be followed, in which blood is collected in special tubes without anticoagulants and left for coagulation in a limited time frame of less than 1 h [648]. Subsequently, direct processing by centrifugation at room temperature and storage at −80 °C ensure that the complement system remains intact and reflects the true status of the patient at the time of sampling. For the analysis of serum samples, repeated freeze-thaw cycles should be carefully avoided. If serum samples need to be transported, this should be realized on dry ice [297]. Although it is generally known in the complement field that the type of sample and sample preparation play an important role, this is still not always considered or not always correctly stated in many studies [649]. Variations in sample handling can pose a significant challenge, especially when values or methods are to be compared between different laboratories. Another challenge is the fact that the individual state of the complement system is influenced by many additional factors, including age, sex, genotype, clinical conditions, environment, and lifestyle [650–652]. All these factors must be considered when interpreting results to guarantee that values are compared with the correct reference ranges. To this end, standards are also needed to verify the accuracy and reliability of established laboratory tests and to develop and validate new methods. Although WHO standards exist for the concentrations of C1q, C4, C5, and FB, as well as for the functional activity of CH50 and C1–INH, these are not always easily accessible, and many complement components and regulators are still missing in this panel [243,297,653].

In recent years, various efforts have been made to advance complement testing in terms of standardization and quality control. The Committee for the Standardization and Quality Assessment in Complement Measurements, a subcommittee of the International Union of Immunological Societies (IUIS) and the International Complement Society (ICS), has made decisive contributions in this area, supporting laboratories in improving and standardizing their methods, developing standards and uniform measurement procedures, and organizing training courses and international external quality controls. Normal human serum standards were established for quantitative and functional analysis of complement components and activation products [654], and since the launch of the External Quality Assurance (EQA) program, significant progress has been made in quality control among diagnostic complement laboratories [655,656]. Further efforts include the standardization of methods through close collaboration between individual laboratories that have specialized in measuring specific components, as demonstrated by the standardization of the anti-FH autoantibody assay [657]. Internal and external quality controls are generally strongly recommended for effective proficiency testing in clinical laboratories. Nevertheless, there is still a lot of work to be done in this area. The newly emerging complement therapeutics will demand increased diagnostic and therapy-accompanying complement testing. However, considering that many methods have not yet been accepted by regulatory authorities, with commercial assays often being for research purposes only, this limits the number of available standardized and approved tests in the clinic. In most cases, these diagnostic tests are insufficient to obtain a complete picture of a complement-related disease. Therefore, despite the various methods based on established research techniques, clinical laboratories often still rely on in-house validated methods, which is associated with significant time, effort, and costs.

6. Conclusion and perspectives

The balance of the complement system can be disrupted by a range of pathologies. Genome-wide association studies, complement proteomics, and other research into pathophysiological mechanisms, continue to help discover previously unknown links between complement dysregulation and clinical conditions. Several complement therapeutics have already been approved for disease treatment, and many more are under development, particularly those with regulatory complement proteins as targets or for replacement therapy. In clinical practice, an increasing need for diagnostic and therapy-accompanying complement testing is thus to be expected. In addition, the evaluation of drug candidates and the discovery of new drug candidates depend on reliable analytical methods. To meet this need, faster, simpler, and more standardized assays are necessary that can be automated and those that are not dependent on expensive equipment, making them more suitable for bedside and small-scale analytical laboratories. Technical innovations in the field of method and measuring device development can facilitate and drive these advancements. Here, novel quantitative and functional assays for regulatory complement proteins or multiplexing of entire complement panels using advanced immunoassays and mass spectrometry may soon find their way into routine analysis, if obstacles such as costs, standardization, regulations, or the training of personnel in new techniques can be overcome.

Furthermore, artificial intelligence (AI) and cloud-connected systems can also be expected to lead to stronger interlinking of analytical methods in clinical laboratories [658,659]. This will include tests for regulatory complement proteins, whose results will be analyzed more quickly and consistently within complex data sets using AI, allowing for the detection of subtle disease patterns. Furthermore, these tools could assist clinicians in interpreting results and recommending further tests or treatment options for specific patients. In research and drug development, structure and binding predictions as well as molecular dynamics simulations have already been established and are now a rising field thanks to increased computational power and access to AI-based structural biology and bioinformatics tools. These techniques enable the study of complement protein conformational changes or interactions using dry-lab computational calculations, which can then be compared with wet-lab assay results [660,661].

The next big leap in testing development will be its expansion to point-of-care solutions. This is driven by companion diagnostics and the instability of complement components, and is therefore especially attractive for use at sites where blood processing or storage at −80 °C is not possible. Here, microfluidic chips could enable rapid whole blood separation either combined with direct on-chip detection or connected to the sample inlet of a measuring device [662,663]. However, while easily applicable to plasma extraction, more surface chemistry studies of microfluidic devices are needed to avoid artificial surface-induced complement activation in the case of serum [664–666]. In addition, the separation of serum from blood samples would have to be as gentle and efficient as possible, since the complement system can also be activated by damaged cells, heme released by hemolysis, or residual coagulation factors [667–669]. Nevertheless, it is conceivable that such a microfluidic sample preparation module could be integrated into a complement biosensor.

In summary, analytical methods for regulatory complement proteins will play an important role in the future in the research, diagnosis, and treatment of complement-related diseases. To address the remaining challenges and pave the way for precision and personalized medicine, close and intensive collaboration between clinicians and analytical researchers from various academic and industrial disciplines is necessary and should be pursued.

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CRediT authorship contribution statement

Ferdinand Waescher: Conceptualization, Visualization, Writing – original draft, Writing – review & editing. **Clemens Spitzenberg:** Visualization, Writing – review & editing. **Diana Pauly:** Funding acquisition, Project administration, Writing – review & editing. **Antje J. Baeumner:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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