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Review

Complement in clinical medicine: Clinical trials, case reports and therapy monitoring

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ABSTRACT

Research during past decades made it evident that complement is involved in more tasks than fighting infections, but has important roles in other immune surveillance and housekeeping functions. If the balance between complement activation and regulation is out of tune, however, complement can quickly turn against the host and contribute to adverse processes that result in various clinical conditions. Whereas clinical awareness was initially focused on complement deficiencies, excessive activation and insufficient regulation are frequently the dominant factors in complement-related disorders. The individual complement profile of a patient often determines the course and severity of the disease, and the pathophysiological involvement of complement may be highly diverse. As a consequence, complement assays have evolved as essential tools not only in initial diagnosis but also for following disease progression and for monitoring complement-targeted therapies, which become increasingly available in routine clinical use. We herein review the current state of complement-directed drug candidates in clinical evaluation and provide an overview of extended indications considered for the FDA-approved inhibitor eculizumab. Furthermore we review the literature describing cases reports and case series where eculizumab has been used "off-label". Finally, we give a summary of the currently available tests to measure complement profiles and discuss their suitability in diagnostics and treatment monitoring. With complement finally entering the clinical arena, there are intriguing opportunities for treating complement-mediated diseases. However, this progress also requires a new awareness about complement pathophysiology, adequate diagnostic tools and suitable treatment options among clinicians treating patients with such disorders.

1. The changing landscape of complement in disease and therapy

The past few decades have led to a profound shift in our perception of the human complement system. Commonly known as an innate immunity segment of the host defense system that protects our bodies from invading microbes and other threats, it has become increasingly evident that complement has important roles in other immune surveillance and housekeeping tasks but also contributes to a wide and diverse range of clinical disorders (Ricklin et al., 2010; Ricklin et al., 2016). The reason for this ambivalence of complement in physiological and pathophysiological processes is founded in its functional organization. In order to provide instant and effective protection against foreign intruders, complement invokes an elaborate network of soluble and cell surface-bound components, pattern-recognition proteins (PRP), proteases, receptors, effectors and regulators (Fig. 1) (Merle et al., 2015a; Ricklin et al., 2016). Under normal circumstances, specialized PRP detect danger-associated patterns on particle surfaces and initiate an enzymatic cascade that leads to the covalent attachment of opsonins (*i.e.* C4b and C3b proteins) via one of three principal initiation routes (termed classical, lectin and alternative pathway; CP, LP and AP, respectively). On the surface, C3b and C4b form enzyme complexes (termed convertases) that cleave the abundant plasma protein C3 into C3b than can again be deposited and form convertases, thereby fueling the rapid amplification of opsonization on unprotected surfaces. In many cases, this AP-mediated amplification acts as the major driving force and triage point of complement activation via any initiation pathway (Harboe et al., 2009; Harboe et al., 2004).

With progressing amplification, the convertases start cleaving the

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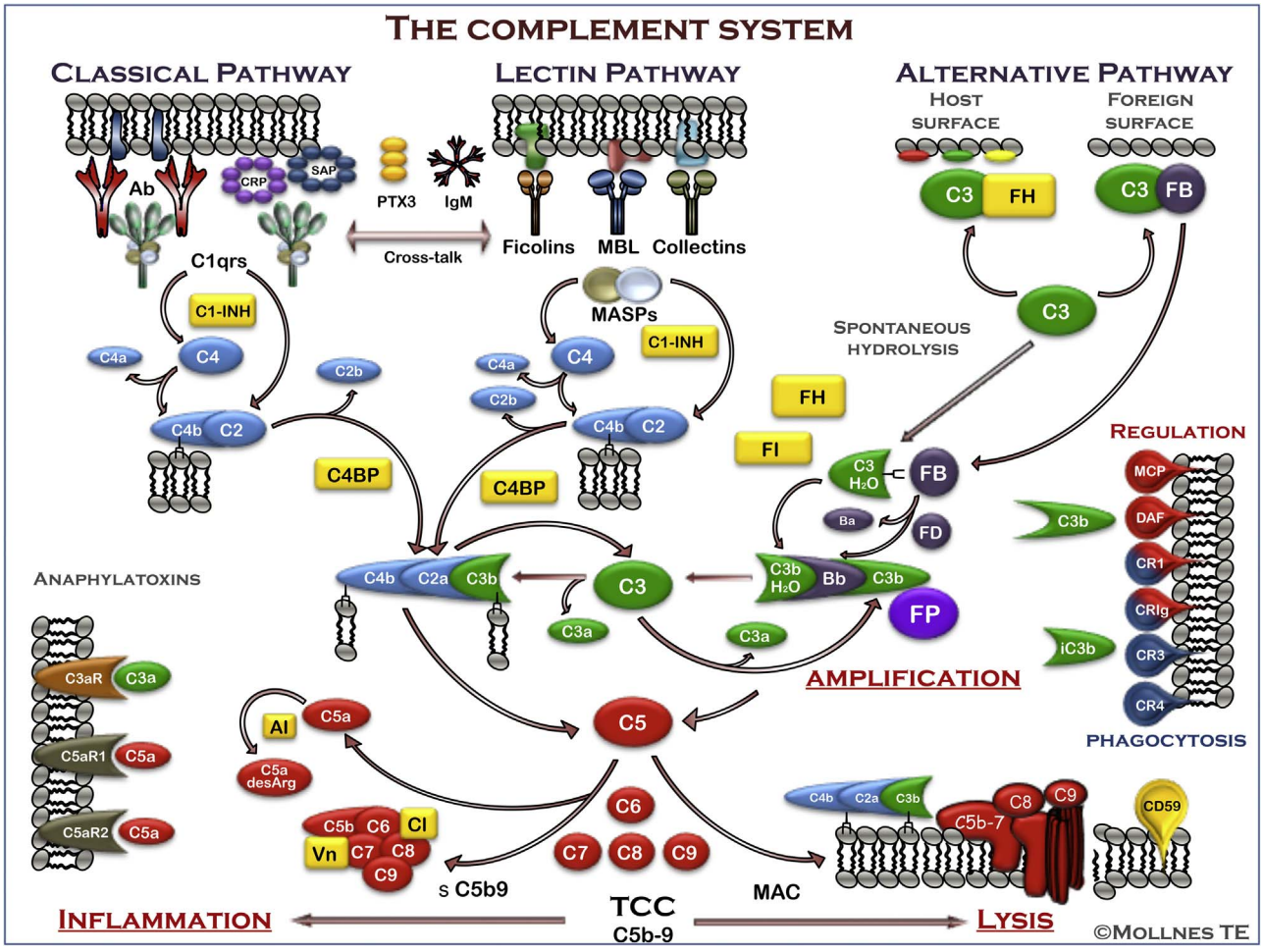

Fig. 1. *Schematic overview of complement activation on foreign particles or damaged host cell surfaces.* Binding of pattern recognition proteins (PRP) to danger markers initiates the cascade via the classical pathway (CP), where C1q recognizes antibodies but also mediators such as C-reactive protein (CRP) and serum amyloid P (SAP), or the lectin pathway (LP) with the main PRPs being MBL, ficolins and collectins. This leads to formation of CP/LP C3 convertases that cleave the abundant plasma protein C3 to C3a and C3b. Whereas C3a has immunomodulatory functions, the opsonin C3b can be covalently deposited on the triggering surface and form alternative pathway (AP) C3 convertases that cleave more C3 and, in absence of regulators, fuel an amplification loop that leads to rapid opsonization stabilized by properdin (FP), the only complement regulator with enhancing effects. The assembly of C5 convertases (e.g., C3bBb3bP) allows cleavage of C5 into the pro-inflammatory anaphylatoxin C5a and C5b. C5a is a highly potent proinflammatory mediator that activates C5a receptor (C5aR) 1, but also binds to the regulatory C5aR2, which might counteract the C5aR1 effects. C5b initiates the assembly of the terminal C5b-9 complex (TCC) which exists in two forms: the membrane attack complexes (MAC) when inserted into a membrane and the soluble sC5b-9 when formed in the fluid-phase. Whereas MAC may exert lysis of certain bacteria (e.g. *Neisseria)* or cell death (typically red cells), it also induces inflammation during sub-lytic cell activation. The opsonins C3b and its degradation products iC3b and C3dg bind to complement receptors (CR) on immune cells and facilitate adherence (C3b to CR1), phagocytosis (iC3b to CR3 and CR4), or mediate adaptive immune responses (iC3b and C3dg to CR2). Complement regulators are crucial to keep the system under control. Soluble regulators include C1-INH, C4b-bining protein (C4BP), Factor I (FI), Factor H (FH), anaphylatoxin inhibitors (AI, comprising carboxypeptidases), vitronectin (Vn) and clusterin (Cl). Membrane regulators include CR1, membrane cofactor protein, (MCP), and decay accelerating factor (DAF) at the level of C4 and C3, by destabilizing convertases and degrading opsonins, and CD59 at the level of C8 and C9 by preventing MAC formation.

C5 component, a fragment of which initiates the formation of lytic membrane attack complexes (MAC; C5b-9_n) that directly destroy or damage susceptible cells. The activation of C3 and C5 also liberates potent chemotactic fragments (*i.e.*, the anaphylatoxins C3a and C5a) that recruit immune cells to the site of activation and prime them. Professional phagocytes recognize opsonins on the attacked particles via complement receptors (CR), thereby mediating their phagocytic removal. Finally, complement effectors are shaping the downstream immune reaction by lowering the threshold of B cell activation and mediating T cell responses, among others (Merle et al., 2015b; Ricklin et al., 2016). In order to provide such broad and instant reactivity in case of threats, the complement system must not be cell-specific and opsonization may occasionally occur on host tissue. Our cells are generally protected from amplification and effector insult by a panel of complement regulators, which are expressed on their surface or recruited from circulation (Schmidt et al., 2016). However, if the balance between complement activation and regulation is out of tune, complement can quickly turn against the host and trigger and/or exacerbate adverse processes that result in diseases and clinical complications (Ricklin and Lambris 2013; Ricklin et al., 2016).

Whereas the pathophysiological involvement has long been recognized, it is only now that the extent of this 'dark side of complement' becomes apparent. Initial clinical awareness primarily focused on complement deficiencies, yet excessive activation and insufficient regulation are by far more common drivers of complement-mediated disorders. In principle, any foreign or altered/damaged host cell surface can trigger a complement response (Ricklin et al., 2016). The sudden exposure to a massive amount of danger pattern, as in the case of sepsis or trauma, can overwhelm the system and fuel a vicious cycle that causes tissue damage and systemic inflammation. Similarly, transplants or biomedical materials often trigger an adverse complement response that affect both the clinical and functional outcome of the treatment. And even though complement typically helps clearing cellular debris, the inability to do so efficiently can trap complement in an inflammatory state, as obvious in cases such as atherosclerosis or Alzheimer's disease. Any imbalance between activating and regulatory mechanism,

often caused by genetic alteration in individual components, can facilitate and accelerate disease processes and even act as causal factor of some (Harris et al., 2012; Ricklin et al., 2016). The eyes and kidneys appear to be particularly susceptible to the consequences of dysregulated complement as evidenced by examples such as age-related macular degeneration (AMD) and atypical hemolytic uremic syndrome (aHUS), respectively. Due to the upstream placement of complement in danger recognition and defense coordination, inappropriate complement activation typically starts involving downstream crosstalk processes that contribute to inflammation and thrombosis (Foley 2016).

With increasing genetic and molecular insight into complement-mediated pathological processes we realize that the exact involvement of complement pathways and components is highly distinct between individual diseases (Ricklin et al., 2016). Even more importantly, the same disease spectrum may be caused and shaped by a broad variety of different alterations in complement activators and/or regulators. The individual complement profile of a patient (sometimes referred to as "complotype") often determines the course and severity of the disease (Harris et al., 2012). Disorders such as AMD, aHUS or C3 glomerulopathy (C3G) are among the most well-described examples in this context (Nester et al., 2015; van Lookeren Campagne et al., 2016; Zipfel et al., 2015). Profound and quantitative knowledge of the plasma levels and genetic profile of individual components and the overall activity of the cascade therefore become increasingly important for facilitating clinical diagnoses and allow for an efficient monitoring of disease progression. The recent surge of complement-targeted therapies in a variety of diseases (see below) puts an even stronger emphasis on the need for reliable diagnostic tools (Morgan and Harris 2015; Ricklin and Lambris 2016b). In the case of clinical trials, they allow for the much-needed patient stratification and provide a critical tool for assessing the achievement of clinical endpoint criteria. Even after approval of complement drugs, proper diagnostics remains essential for monitoring treatment progress and deciding about adjustments of the medication. The following sections therefore provide an overview and specific examples of the use of complement diagnostics in the clinical and therapeutic context and summarize established and emerging concepts of assessing a patient's complement profile.

2. Complement-targeted treatments entering clinical evaluation

The system's upstream involvement in a broad range of disease processes renders complement an intriguing and promising target for therapeutic intervention, with indications ranging from inflammatory and age-related diseases to biomaterial- and transplant-induced complications (Morgan and Harris 2015; Ricklin and Lambris 2016b). Whereas early efforts in complement drug discovery were hampered by limited molecular insight, technical challenges and safety concerns, profound progress in all these areas has paved the way to a newfound confidence in this approach and led to a surge in novel complement-targeting concepts (Morgan and Harris 2015; Ricklin and Lambris 2016b). Key to this development has certainly been the availability of first complement drugs in the clinic, in particular the therapeutic anti-C5 antibody eculizumab (Soliris®, Alexion Pharmaceuticals), which allowed to gain clinical experience and extend the spectrum of indications. Although the clinical and commercial success of eculizumab has fueled an interest in developing other C5 inhibitors, the knowledge about the highly distinct involvement of complement in disease processes described above has led to the realization that the diversity of pathomechanisms requires a similar diversity in therapeutic intervention points.

After years of development, more than a dozen candidate drugs covering a wide range of the complement cascade are meanwhile being evaluated in clinical trials (Tables 1 and 2). They not only show a broad variety in their therapeutic target but also in the use of molecular entities ranging from small molecules and peptides to proteins, antibodies and oligonucleotides (Ricklin and Lambris 2016a). Whereas most of the currently evaluated drug candidates act as protein–protein interaction inhibitors, some are based on physiological regulators or impair the production of complement component on the genetic level. Even though established complement diseases such as AMD, aHUS and paroxysmal nocturnal hemoglobinuria (PNH) remain in the focus of the indication spectrum, several candidates explore novel or hitherto challenging disease areas (*e.g.*, transplantation or sepsis), thereby helping to foster our knowledge about the possibilities and limitation of therapeutic complement modulation. In this context, it is important that more than ten years after its approval, eculizumab is still enrolled in a variety of clinical trials to assess the potential of expanding the drug's indication list (Table 2). Similar is true for C1-inhibitor (C1-INH) preparations (Table 1). Notably, C1-INH should be considered a "plasma cascade inhibitor" rather than a complement-specific therapeutic, since the inhibitory actions of this physiological regulator reach beyond complement; indeed they might be even more important in the kallikrein-kinin system by reducing formation of bradykinin and in the FXII-mediated intrinsic activation of the coagulation system (Zeerleder 2011).

In its current state, therapeutic complement inhibition covers an impressive breath of concepts and approaches. This includes the treatment of chronic, episodic and/or acute disorders, the local or systemic administration of therapeutic entities, the targeting of initiation, amplification or effector stages, and the intervention at the protein and gene level. It appears logical, therefore, that such a diversity of indications and approaches requires highly specific considerations when it comes to the planning of clinical development plans and the evaluation of therapeutic success. Whereas many of the previous obstacles in complement drug discovery have meanwhile been resolved, the selection of suitable indications and patient populations has emerged as a new challenge (Ricklin and Lambris 2016b). An impressive example in this context is AMD. Genome-wide association studies identifying the complement regulator factor H (FH) as major risk factor for disease progression (Hageman et al., 2005), sparked a sudden interest in this indication, initial complement-focused intervention studies were of limited success. Intriguingly, a recent phase 2 trial with the anti-Factor D (FD) antibody lampalizumab (Genentech) revealed that patients carrying an additional complement variant on top of the known FH Y402H polymorphism were benefiting much more from the treatment (Ehmann and Regillo 2016) (see details below). This underscores the critical significance of patient stratification in planning successful clinical trials. In this context, diagnostic and genetic methods and biomarker analysis become increasingly important as decision-making, stratification, and monitoring tools (Mollnes et al., 2007). At some point, they may even pave the way to precision medicine approaches for complement-targeted therapies. Fortunately, the field of complement diagnostics has drastically evolved in the wake of the recent progress in complement research, and offers a wide range of tools to assess the genetic, molecular and functional complement profile of a patient.

3. The importance of genetic biomarkers in clinical trials: the case of lampalizumab

The above-mentioned case of Genentech's lampalizumab illustrates several important aspects of current complement-targeted drug discovery. The drug candidate targets the serine protease FD, which constitutes a bottle neck in the AP-driven amplification loop due to its role to transform the pro-convertase (C3bB) into the active AP C3 convertase (C3bBb). Even though serine proteases are considered druggable targets and FD has early been on the radar of therapeutic complement modulation, initial efforts have been challenging due to limited septicity of small molecule candidates and pharmacokinetic challenges caused by the rapid systemic turnover of FD (Ricklin and Lambris 2007). Lampalizumab originated from one of the antibody development programs by Tanox, with their anti-FD candidate (TNX-

Table 1
Complement therapeutics targeting complement initiation and amplification in clinical trials (with an emphasis on ongoing or recently completed trials listed in the ClinicalTrials.gov database; range 2015-present).

Target	Drug/Candidate (Company)	Entity[1]	Clinical Phase (Trial No.)[2]	Indications[3]
C1r, C1s, MASPs	Cinryze (Shire)[4]	Pro	P1 (NCT02435732)	Transplantation
			P3 (NCT02547220)	Transplantation
	Berinert (CSL Behring)[4]	Pro	P1/2 (NCT02134314)	Transplantation
			P2 (NCT02936479)	Transplantation
	Cetor (Sanquin)[4]	Pro	P3 (NCT01275976) term	Trauma/Sepsis
			P2 (NCT02251041)	Transplantation
	Ruconest/Conestat alfa[4] (Pharming)	Pro	P2 (NCT02869347)	Contrast-induced nephropathy
C1s	TNT009 (True North)	Ab	P1 (NCT02502903)	CAD, BP, AIHA, ESRD
MASP-2	OMS721 (Omeros)	Ab	P2 (NCT02222545)	TMA
			P2 (NCT02682407)	IgAN, LN, MN, C3G
FP	CLG561 (Novartis)	Ab	P2 (NCT02515942)	AMD (GA)
C3	AMY-101 (Amyndas)	Pep	P1 (N/A)	C3G, Transplantation
	APL-1 (Apellis)	Pep	P1 (N/A)	COPD
	APL-2 (Apellis)	Pep	P1 (NCT02588833)	PNH
			P1 (NCT02264639)	PNH (add-on)
			P1 (NCT02461771) comp	AMD (CNV)
			P2 (NCT02503332)	AMD (GA)
Conver-tases	Mirococept (*MRC*)	Pro	P3 (EMPIRIKAL, ISRCTN49958194)	Transplantation
FD	Lampalizumab (Genentech)	Ab	P2 (NCT02288559)	AMD (GA)
			P3 (NCT02745119)	AMD (GA)
			P3 (NCT02247531)	AMD (GA)
			P3 (NCT02247479)	AMD (GA)
	ACH-4471 (Achillion)	SM	P1 (ACTRN12616000082404p)	PNH
			P2 (NCT03053102)	PNH

[1]1Abbreviations: Ab, antibody; Pep, peptide; Pro, protein; SM, small molecule; [2]Abbreviations: P1-3, clinical phase 1–3; comp, completed; term, terminated. [3]Abbreviations: AIHA, autoimmune hemolytic anemia; AMD, age-related macular degeneration; BP, bullous pemphigoid; C3G, C3 glomerulopathy; CAD, cold agglutinin disease; CNV, choroidal neovascularization; COPD, chronic obstructive pulmonary disease; ESRD, end-stage renal disease; GA, geographic athrophy; IgAN, IgA nephropathy; LN, lupus nephritis; MN, membranous nephropathy; PNH, paroxysmal nocturnal hemoglobinuria; TMA, thrombotic microangiopathies. [4]C1-INH preparations are already approved for hereditary angioedema; potential post-approval trials for the primary indications of the C1-INH drugs are not shown.

224) being transferred to Genentech after the company's acquisition in 2007 (Ricklin and Lambris 2007). The clinical candidate is a humanized antigen-binding fragment (Fab) of an IgG1 antibody.

It was later described that lampalizumab, rather than acting on the catalytic center of the protease, tightly binds to an exosite of FD that prevents its binding to the pro-convertase (Katschke et al., 2012). Geographic atrophy in connection with dry forms of AMD is the only indication for which lampalizumab is currently in clinical development, which may at least partially be related to pharmacokinetic restrictions. Whereas the anti-FD Fab was shown to be cleared from systemic circulation within a few hours, the elimination half-life in the eye extends beyond two days after intravitreal injection (Loyet et al., 2014b). The local administration of lampalizumab into the eye also takes away some emphasis on safety considerations, since the low plasma concentrations after intravitreal injections are not expected to significantly affect systemic complement activity (Loyet et al., 2014a).

In a phase 1 study (NCT00973011), lampalizumab was found to be safe and well tolerated, and was subsequently evaluated in phase 2 trials. In the MAHALO study (NCT01229215, NCT01602120), 129 patients were enrolled to receive the drug or a sham treatment either monthly or bimonthly. Although the official results remain to be published (Boyer et al., 2016), reports showed that the treatment success (measured as reduction of geographic atrophy progression) was approximately 20% overall, whereas a subgroup of patients carrying a Factor I polymorphism showed a significantly stronger effect (44%) (Holz et al., 2014). Interestingly, genotyping analysis of enrolled patients receiving sham treatment revealed that almost all patients were carriers of FH and/or C2/FB polymorphisms; whereas an additional C3 polymorphism did not notably affect disease progression, those patients carrying the FI polymorphism experienced an almost 50% more rapid growth of the geographical atrophy region during the study (Yaspan et al., 2014). Two large, identical phase 3 studies (Chroma, Spectri; Table 1) involving close to 1000 patients are currently ongoing with results expected by the end of 2017. It will be important to see how much the genetic background of patients influenced treatment success and whether this may have implications for therapeutic recommendations in case of approval.

4. Expanding indications case by case: the extended off-label use of eculizumab

Eculizumab, the humanized monoclonal IgG2/4-antibody targeting C5 was approved by the United States Food and Drug Administration (FDA) and the European Medicines Agency for the treatment of PNH in 2007 and aHUS in 2011. During the time this drug has been available for these two rare diseases, the eculizumab usage for off-label indications has been extensive. A recent report from France revealed that the off-label administration of eculizumab exceeded 50% of the drugs's total consumption in the period from 2011 to 2013 (Castaneda-Sanabria et al., 2016). Although this number is large and probably not representative for all countries, it clearly demonstrates a growing medical knowledge, faith and willingness to adopt complement inhibitory therapy to other patients groups.

Based on a literature search in PubMed including case reports and case series we compiled an overview of the off-label use of eculizumab, categorized into different disease entities (Table 3). The search revealed 106 papers documenting off-label use in at least 25 different conditions (a complete list of the conditions and corresponding references is found in Supplementary Table 1). Even without considering the ongoing clinical trials with eculizumab, listed in Table 2, or showing all published reports and case series, this overview provides an impression of the comprehensive off-label use of this anti-C5 antibody. Although the question may arise whether such extensive off-label administration without fully relying on evidence-based medicine represents good medical practice, the reasons for this development are diverse. Indeed part of the eculizumab use is implemented in clinical trials (*e.g.* myastenia gravis and cold agglutination diseases; Table 2), whereas the casuistry-based usage often occurs in critical clinical situations where the patients have nothing to lose and the clinicians are standing with their back against the wall (Barratt-Due et al., 2016). Furthermore,

Table 2
Complement therapeutics targeting terminal complement pathways in clinical trials (with an emphasis on ongoing or recently completed trials listed in the ClinicalTrials.gov database; range 2015-present).

Target	Drug/Candidate (Company)	Entity[1]	Clinical Phase (Trial No.)[2]	Indications[3]
C5	Soliris/Eculizumab [4]	Ab	P2 (NCT01303952) comp	CAD
	(Alexion)		P2 (NCT02093533)	MPGN
			P2 (NCT01567085)	Transplantation
			P2 (NCT01919346)	Transplantation
			P2 (NCT01895127) term	Transplantation
			P2 (NCT01399593) term	Transplantation
			P2/3 (NCT02145182) comp	Transplantation
			P2/3 (NCT01106027)	Transplantation
			P3 (NCT02301624)	Myasthenia gravis
			P3 (NCT01997229) comp	Myasthenia gravis
			P3 (NCT01892345)	Neuromyelitis optica
	ALXN1210 (Alexion)	Ab	P1/2 (NCT02598583)	PNH
			P2 (NCT02605993)	PNH
			P3 (NCT02946463)	PNH (naïve)
			P3 (NCT03056040)	PNH (treated)
			P3 (NCT02949128)	aHUS (naïve)
	Tesidolumab/LFG316 (Novartis/Morphosys)	Ab	P1 (NCT02878616)	Transplantation
			P2 (NCT02763644)	TMA
			P2 (NCT01527500) comp	AMD (GA)
			P2 (NCT02515942)	AMD
			P2 (NCT02534909)	PNH
			P2 (NCT01526889)	Uveitis/Panuveitis
	Coversin (Akari)	Pro	P2 (NCT02591862)	PNH
	RA101495 (Ra Pharma)	Pep	P1 (ACTRN12615001143516)	PNH
			P2 (NCT03030183)	PNH (poor responders)
			P2 (NCT03078582)	PNH
	Zimura	Nuc	P2 (NCT02397954) comp	IPCV
	(Ophthotech)		P2/3 (NCT02686658)	AMD
	ALN—CC5	Nuc	P1/2 (NCT02352493)	PNH
	(Alnylam)			
C5a	IFX-1	Ab	P2 (NCT02246595) comp	Sepsis
	(InflaRx)		P2 (NCT02866825) comp	SIRS
			P2 (NCT03001622)	Hidradenitis suppurativa
	ALXN1007	Ab	P2 (NCT02245412) term	GVHD
	(Alexion)		P2 (NCT02128269) comp	APS
C5aR1	Avacopan/CCX168 (Chemocentryx)	SM	P2 (NCT02222155) comp	ANCA-vasculitis
			P2 (NCT01363388) comp	ANCA-vasculitis
			P2 (NCT02464891) term	aHUS
			P2 (NCT02384317) comp	IgAN
			P3 (NCT02994927)	ANCA-vasculitis

[1]Abbreviations: Ab, antibody; Nuc, nucleotide; Pep, peptide; Pro, protein; SM, small molecule; [2]Abbreviations: P1-3, clinical phase 1–3; comp, completed; term, terminated. [3]Abbeviations: aHUS, atypical hemolytic uremic syndrome; AMD, age-related macular degeneration; APS, antiphospholipid syndrome; CAD, cold agglutinin disease; GA, geographic athrophy; GVHD, graft versus host disease; IgAN, IgA nephropathy; IPCV, idopathic polypoidal choroidal vasculopathy; PNH, paroxysmal nocturnal hemoglobinuria; SIRS, systemic inflammatory response syndrome; TMA, thrombotic microangiopathies. [4]Eculizumab is already approved for PNH and aHUS; potential post-approval trials for the primary indications are not listed.

almost all diseases listed in Table 3 are conditions in which complement appears to play a key role in the pathophysiology, giving a theoretical foundation for testing the efficacy of eculizumab. Randomized clinical trials will for most of these diseases not be possible because they are rare and featured by a complex heterogeneity. Thus, case series and case reports definitely represent a valuable contribution to new and important knowledge. Still, it is important to be aware that case-based literature generally reflects a substantial publication bias, since those cases with positive outcome are much more likely both to be submitted and to be accepted for publication, than those with negative results. Especially in exploratory cases, the proper assessment of a patient's complement status and monitoring of therapeutic progress is essential for ensuring a high quality and clinical value of extended-use case studies.

5. An evolving need for reliable diagnostic and monitoring tools

The arsenal of diagnostic tools to evaluate complement profiles and activities have greatly expanded over the past few decades. Several comprehensive reviews on complement tests and diagnostics have been published in recent years the last years and we here only refer to some selected reviews published after 2005 for further reading (Harboe et al., 2011; Mollnes et al., 2007; Nilsson and Ekdahl 2012; Oppermann and Wurzner 2010; Prohaszka et al., 2016). An overview of the major classes of complement diagnostic approaches is presented in Fig. 2.

Herein we will focus on assays that might be useful in diagnosis of diseases where complement is suggested to be a substantial part of the pathogenesis, and thus are candidates for future complement inhibitory therapy. Furthermore, we will emphasize on those assays that might be best suited for therapeutic monitoring. Since eculizumab is the only FDA-approved specific complement inhibitory drug on the marked, we will focus on assays related to C5 function. Notably, these assays may be used on a broad basis also for future drugs, both those targeting C5 and other central components including the main component C3, as well as specific targets within the CP, LP and AP branches of the cascade.

The considerable number of assays that are currently used for investigating the complement system can be divided into the following groups: 1) screening of total functional complement activity; 2) quantification of single components; 3) measuring functional activity of single components; 4) quantification of complement activation products; 5) detection of autoantibodies to complement components; 6) assessing cell surface expression and tissue deposition of complement proteins or fragments; 7) determining genetic profiles, and finally; 8) the use of novel, though not yet well established and standardized,

Table 3
Off-label use of eculizumab – case reports and case series[1,2].

Disease or disease group	Medical condition	Number of papers[1]
Hemolytic uremic syndrome	Typical HUS	7
	Drug-induced HUS	3
Thrombotic microangiopathies (TMA)	Posttransplant TMA	8
	Stem cell transplantation-associated TMA	6
	Thrombotic thrombocytopenic purpura	4
	Drug- or viral-induced TMA	5
Kidney diseases	C3 glomerulopathies (C3G)	17
	IgA nephropathy	3
	Cryoglobulin-induced glomerulonephritis	1
	Lupus nephritis	1
	Scleroderma renal crisis	1
Antibody mediated rejection	Kidney transplantation	17
	Pancreas-kidney transplantation	2
	Lung and intestine transplantation	2
	Liver transplantation	1
	Face transplantation	3
Antiphospholipid syndrome (APS)	Catastrophic APS	10
	Prophylactic use	2
Neuroimmunological diseases	Neuromyelitis optica	2
Vasculopathy and vasculitis	Malignant atrophic papulosis	2
	ANCA-associated vasculitis	1
Haematological diseases	Cold agglutinin disease	3
	Warm IgG/M-mediated hemolytic anemia	2
	Sickle cell disease	2
Other	Inherited CD59 deficiency	1

[1]See Supplementary Table 1 with all references included.
[2]The table is based on the Pubmed search *(Terminal Complement Blockade OR C5-inhibition OR eculizumab) (therapy OR treatment) NOT (atypical haemolytic uremic syndrome OR aHUS OR paroxysmal nocturnal haemoglobinuria OR PNH)*. 459 hits were identified 23rd of March 2017. Reviews, clinical trials, comments, animals and preclinical studies, reports regarding PNH or aHUS and non-English written papers, were not included.

assays to monitor eculizumab treatment (Table 4). Note, from this table it is crucially important how the samples are obtained and handled for each of the assay groups.

5.1. Screening of total complement activity

The main indication for using screening assays for total complement activity is to detect complement deficiencies. Such deficiencies can be genetic, acquired or a consequence of complement inhibitory therapy.

These tests detect the total amount of active complement components present in a freshly drawn serum sample and reflect the potential of a person's serum to induce complement activation *in vitro* after adding it to a complement activator. The traditional assays in this group are the CH50 and AH50 lytic assays, based on either the lysis of antibody sensitized sheep erythrocytes (CH50 for detecting CP activity) or the lysis of untreated rabbit erythrocytes (AH50 for the of AP activity) (Joiner et al., 1983; Mayer 1958). In both cases the functional readout is the lytic destruction of erythrocytes upon membrane insertion of C5b-9 (MAC). In recent years, novel ELISA-based assays have been developed, which more specifically detect complement activation through CP, LP and AP (Seelen et al., 2005); the "complement system screen" test by Wieslab is one widely-used example (Fig. 3A). Although the readout target of these ELISAs is principally the same as for the lytic assays, it is based on detecting full assembly of C5b-9 by using an antibody to a C9 neoantigen when serum is added to micro-titer wells coated with the activation agent for the different pathways (*e.g.*, IgM for CP, mannan for LP, and lipopolysaccharides for AP).

Thus, the C5b-9 molecule is generated *in vitro* as an endpoint of the complement cascade in these assays and reflects the total amount of native components present in the sample. If all components are present and with intact function, the lytic assays will reveal normal lysis of red cells and the ELISA assays normal deposition of C5b-9. One exception is that properdin deficiency is frequently not detected in the AH50 assay in contrast to the complement screen assay (Seelen et al., 2005). If eculizumab is present C5 activation will be prevented and C5b-9 deposition in ELISA will be reduced (Fig. 3, B, a-d). If all C5 molecules are inhibited (Fig. 3B, b-d), the phenotype will be identical to a genetic C5 deficiency and the lysis respective C5b-9 deposition will be abolished in these assays. The terminal C5b-9 complement complex exists in two forms, the MAC and the soluble form (sC5b-9). The *in vitro* generated surface-bound C5b-9 must not be confused with sC5b-9, which is found in plasma and if increased is a useful marker of complement activation *in vivo* reflecting diseases with disturbed complement function. sC5b-9 can be quantified by ELISA (Fig. 3C) and is based on the same C9 neoepitope principle; in this case, the complex that is already present in plasma is captured by the antibody coated to the plate (see below). In the case of efficient C5 blockade, there should ideally be no sC5b-9 present.

5.2. Quantification of single components

Measurement of single components like C3 and C4 are usually performed with nephelometry or similar techniques using polyclonal antibodies. In such methods the total amount of the antigen present is detected without distinguishing the native component from the activation product. Thus, the sampling and storage conditions are not that critical for these assays as it is for the functional assay. The level of the single components will depend on several factors including synthesis and secretion (most components are decreased in liver failure), acute phase reaction (most components increase moderately) or *in vivo* activation (consumption with reduced levels). The concentration will reflect the sum of these factors and interpretation of the results should therefore be made with caution. A low concentration does not necessarily imply increased complement activation *in vivo*. Supplemental information from activation product tests will be needed to document a systemic increase in activation (see below).

5.3. Functional activity of single components

Normal concentrations of single components do not exclude functional defects. Thus, if a functional defect is suspected or needs to be ruled out, the functional activity can be tested. This has typically been done using sera depleted of the actual component and with the patient's fresh serum added, to see whether the activity can be restored, *e.g.* by using hemolysis as readout. In recent years, genetic tests detecting variants and mutations known to compromise the function of the protein have largely replaced the functional protein assays. These tests will not only reveal "loss-of-function" but also "gain-of-function" of single components, like C3 "gain-of-function" associated with aHUS (Roumenina et al., 2012), similar to the typical "loss-of-function of regulatory proteins (Nester et al., 2015).

5.4. Quantification of complement activation products

Monoclonal antibodies to neoepitopes exposed in the activation product but hidden in the native component enable specific quantification of activation products. Such assays are typically sandwich techniques like ELISA or luminex, using the anti-neoepitope antibody as capture antibody. A number of activation products can be tested, most of them available as commercial kits, including C4a, C4bc (*i.e.,* common epitope for C4b and C4c), C4d, C3a, C3bc (*i.e.,* common epitope for

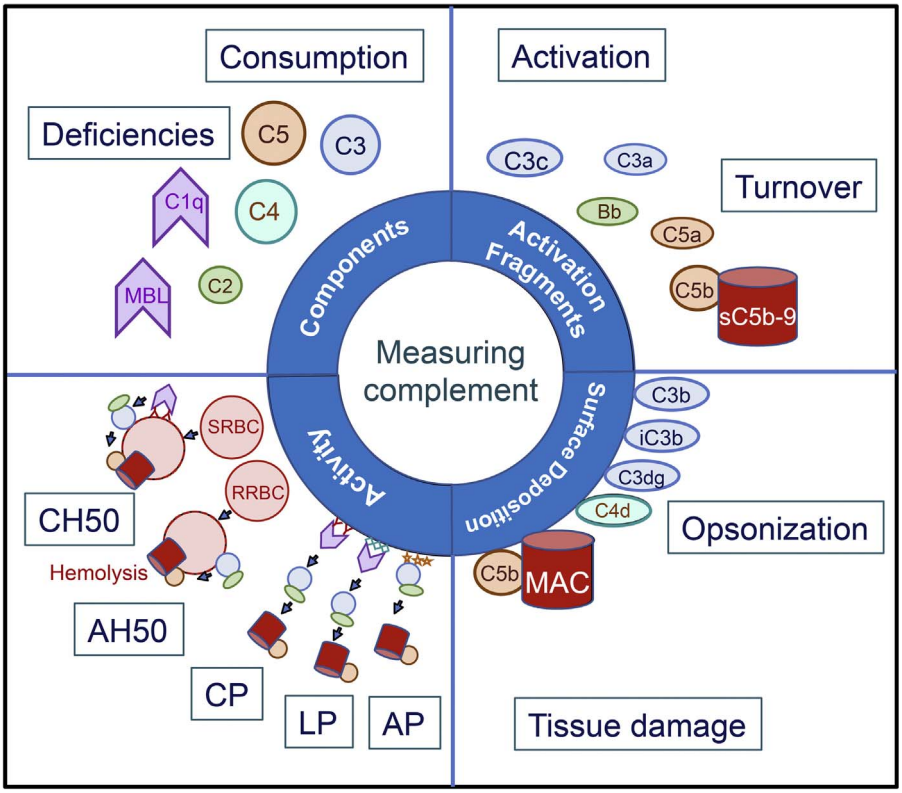

Fig. 2. *Overview of major classes of complement diagnostic approaches.* Whereas the traditional measurement of the plasma levels of intact complement components may provide evidence for complement deficiencies or high consumptive turnover, the overall information is rather limited. Including activation fragments in the analysis enables conclusions about the activation status and/or involvement of individual initiation or effector pathways. Similarly, the detection of complement deposition on cells and tissues by immunohistochemistry can provide information about the type and localization of complement activity. Finally, functional assays based on either hemolytic activity (i.e., CH50, AH50) or detection of membrane attack complex (MAC) formation by ELISA allow for the measurement of total complement activity in patient serum or plasma dependent on pathway-specific triggers. In addition, to the methods shown here, genetic analyses become increasingly prevalent in the complement diagnostics field. Abbreviations: MBL, mannose-binding lectin; RRBC, rabbit red blood cells; SRBC, sheep red blood cells.

iC3b, C3b and C3c), C3dg, Ba, Bb, C3bBbP, C5a and sC5b-9 (Fig. 3C). In contrast to measuring single components it is of critical importance that the sampling and storing conditions follow strict guidelines, *i.e.* by collecting EDTA plasma, immediately placed on ice, centrifuged within 30 min and stored at −80 °C (Bergseth et al., 2013).

The choice of individual activation product assays(s) largely depends on the purpose of the analysis. If it is of importance to document the initial activation mechanism, measuring early components can help to discriminate between CP/LP (C4 activation products) and AP activation (FB activation products). However, more than one pathway is involved in many conditions, and even if either the CP and/or LP are primarily activated, the AP will usually be engaged activated through the amplification loop (Harboe et al., 2009). Thus, it is often difficult to reveal an exact activation mechanism by just measuring the activation products. Specific inhibition of the recognition molecules may be required in such cases.

Given their central role in fueling the amplification loop and effector pathways, the screening of C3 and C5 activation products is essential. Detection of the soluble form of the terminal complement complex (sC5b-9) is probably the best assay to use as a single screening assay. There are three main reasons for this: 1) It detects complement activation to the very final stage (activation of C9); 2) sC5b-9 has a relatively long *in vivo* half-life (60 min) (Deppisch et al., 1990; Mollnes 1985) as compared to C5a. Although C5a is the main inflammatory mediator of complement, it has a half-life of only approximately 1 min due to rapid clearance and binding to the C5a receptors (Oppermann and Gotze 1994; Wagner and Hugli 1984). sC5b-9 is by itself virtually biologically inert, yet acts as a surrogate marker for the highly proinflammatory C5a, since it hitherto has not been shown that sC5b-9 can be formed *in vivo* without release of C5a; and 3) sC5b-9 is more stable with respect to *in vitro* activation than the early component fragments. The addition measurement of a C3 activation product may be of importance as a supplement to sC5b-9 testing. This is of particular interest when testing patients with a complement activation mediated-disease that is treated with a C5 inhibitor like eculizumab, as for example in the case of; *e.g.* in aHUS where C3 activation is ongoing, whereas sC5b-9 formation is blocked by eculizumab.

It has to be considered that the degree of increased *in vivo* concentration of a complement activation product will directly depend on the present concentration of the native molecule. Thus, if C3 levels are low due to activation and subsequent consumption, the ratio between a C3 activation product and C3, *e.g.* C3dg/C3, might be a more sensitive and robust marker of the degree of C3 activation, as postulated more than 30 years ago (Nurnberger and Bhakdi 1984). This principle of ratio applies in principle to activation of any component, like in the case of C4 where the C4bc/C4 ratio was found to be substantially more sensitive than the C4d/C4 ratio, although both ratios were superior to the activation product or C4 alone (Nielsen et al., 1995).

5.5. Autoantibodies to complement components

Autoantibodies to complement components may appear alone or as part of an autoimmune or malignant disease, like anti-C1q antibodies in systemic lupus erythematosus (Mahler et al., 2013) or anti-C1-INH antibodies, mimicking genetic defect in C1-INH, in hematologic malignancies (Cicardi and Zanichelli 2010). The pathogenesis of the latter is, however, not related to complement, but to bradykinin release due to inefficient inhibition of the kallikrein-kinin system (Davis 2005). In contrast, anti-FH antibodies may result in serious complement-

Table 4
Complement tests in clinical diagnostics and treatment monitoring.

Type of assay	Examples	Indication and information	Sample treatment
Screen of total serum complement activity *in vitro*	Lytic CH50 and AH50. ELISA-based CP[1], LP and AP complement screen	Detect complement deficiency. Monitoring complement inhibitory therapy. Readout is lysis or C5b-9 formation. Normal activity indicates intact components of the actual pathway	Fresh serum without anticoagulant immediately stored at −80 °C
Single protein quantification	*E.g.* C1q, C1-Inhibitor, C3, C4.	Detect complement deficiency, *e.g.* HAE type I. General information on complement synthesis, activation, acute phase	Normal serum (most tests are not influenced by *in vitro* activation)
Single functional activity	*E.g.* C1-Inhibitor, FH	Normal protein concentration does not exclude functional defects, *e.g.* HAE type II, FH defects in several diseases	Fresh serum without anticoagulant immediately stored at −80 °C
Activation products	*E.g.* sC5b-9, C5a, C3a, C3bc, C3d, C4a, C4d, Ba, Bb	Detect activation of a certain component. *In vivo* useful for diagnostics, complement activity and monitoring complement inhibitory therapy	EDTA plasma. Sample kept at 4 °C, centrifuged within 30 min and stored at −80 °C
Autoantibodies	*E.g.* anti-FH, −C1q	Interference with function (*e.g.* aHUS for FH)	Normal serum or plasma
Cell and tissue expression of complement	*E.g.* CD55 and CD59 on red cells (FC), C4d and C5b-9 in kidneys (IF, IHC)	Diagnosis of PNH (CD55, CD59). Acute antibody-mediated kidney rejection (C4d in tubuli). C3G (C5b-9 in glomeruli	Fresh EDTA whole blood for FC. Frozen tissue section for IF and formalin tissue for IHC
Genetic assays	Screening for SNPs in complement genes, complotypes.	Detect risk of developing complement-mediated diseases including aHUS, C3G and AMD. “Disease packages”	Fresh EDTA whole blood or frozen EDTA blood sent on dry ice
Other assays with specific focus on monitoring treatment	Detection of eculizumab by ELISA; endothelial cell activation; Mass spectrometry	Level of eculizumab sufficient to block complement. ELISA coated with C5 (detecting binding of eculizumab), captured with anti-C5 detecting the level of eculizumab-C5 complexes. Mass spectrometry detection of bound and free eculizumab	Normal serum or plasma analyzed within a few days or stored at −20 °C. Independent on *in vitro* activation

[1]Abbreviations: CP (classical pathway), LP (lectin pathway), AP (alternative pathway), HAE (hereditary angioedema), FC (flow cytometry), IF (immunofluorescence), IHC (immunohistochemistry), aHUS (atypical hemolytic uremic syndrome), C3G (C3 glomerulopathies), AMD (age-related macular degeneration).

mediated diseases due to uncontrolled activation of the AP, similar to genetic FH dysfunction leading to aHUS or C3G (Durey et al., 2016). It is important to distinguish the genetic FH deficiency from autoantibodies in aHUS due to different treatment approaches. The genetic form usually responds efficiently to eculizumab, but not to plasma exchange and immunosuppression, which are alternative options to eculizumab in the treatment of antibody-induced aHUS to reduce the level of autoantibodies (Chiodini et al., 2014; Loirat et al., 2015).

5.6. Cell surface expression and tissue deposition of complement

Cell surfaces are the focal area of complement activation disorders. The absence or reduced expression of membrane-bound complement regulators can contribute to increased complement activation, which leads to the deposition of opsonins (*e.g.*, C3b, C4b) and effectors (*e.g.*, C5b-9n). Measuring complement components and activation products directly on a cell surface can therefore provide valuable information. Flow cytometry is the standard technique for the diagnosis of PNH, by detecting reduced levels of CD55 and CD59 on blood cells. Detection of complement activation products like C3d and C4d on red cells using flow cytometry has also been used to evaluate *in vivo* complement activation in *e.g.* autoimmune diseases and trauma (Kao et al., 2010; Muroya et al., 2014). Deposition of various complement components in the glomeruli may help in the diagnosis of glomerulopathies (Cook and Pickering 2015) and peritubular capillary deposition of C4d, as measured by immunofluorescence in frozen sections, is one of the Banff criteria for diagnosis of acute antibody-mediated renal rejection (Solez et al., 2008; Stites et al., 2015).

5.7. Genetic studies

During the past two decades genetic studies have substantially improved our understanding of etiology and pathogenesis of complement-mediated diseases and genetic tests have meanwhile been established in routine diagnostics. Diseases such as aHUS, C3G and AMD are closely associated with genetic variants or defects leading to loss-of-function of the regulatory proteins like FH, FI, MCP or gain-of-function of C3 or FB (de Cordoba 2015; De Vriese et al., 2015; Harris et al., 2012; Heurich et al., 2011; Le et al., 2010; Roumenina et al., 2011; Servais et al., 2012). Of particular importance is a rapid diagnosis of pediatric aHUS in order to distinguish it from other thrombotic microangiopathies and initiate the correct therapy. Many laboratories now offer both single gene sequencing as well as ‘diagnostic packages’.

5.8. Assays to monitor treatment with eculizumab

The availability of eculizumab has changed the therapeutic landscape for both PNH and aHUS, and standard treatment plans have meanwhile been established in many cases. However, there are several reasons why treatment with eculizumab would largely benefit from an individualized therapy regimen for each patient. First of all, excessive dosages should be avoided due to the high cost of the drug (sometimes exceeding $500,000 per year and patient). The pharmacokinetics of eculizumab may differ between individuals and between different disease conditions, and the concentration of C5 varies substantially between individuals as does the blood volume. Thus, the dose required for a full blockade of C5 activity, which is the primary aim of the therapy, will vary considerably between patients. The doses administrated are based on generalized guidelines for all adults adapted guidelines and similarly generalized reduced doses for children (< 40 kg). Unfortunately, there is no consensus yet on the best option to follow these patients with analyses that can guide an individualized follow-up therapy.

Tests for total complement activity, including lytic CH50 and ELISA-based C5b-9 formation (total complement screen Wieslab®), have been used to follow patients and revealed a substantial difference in the

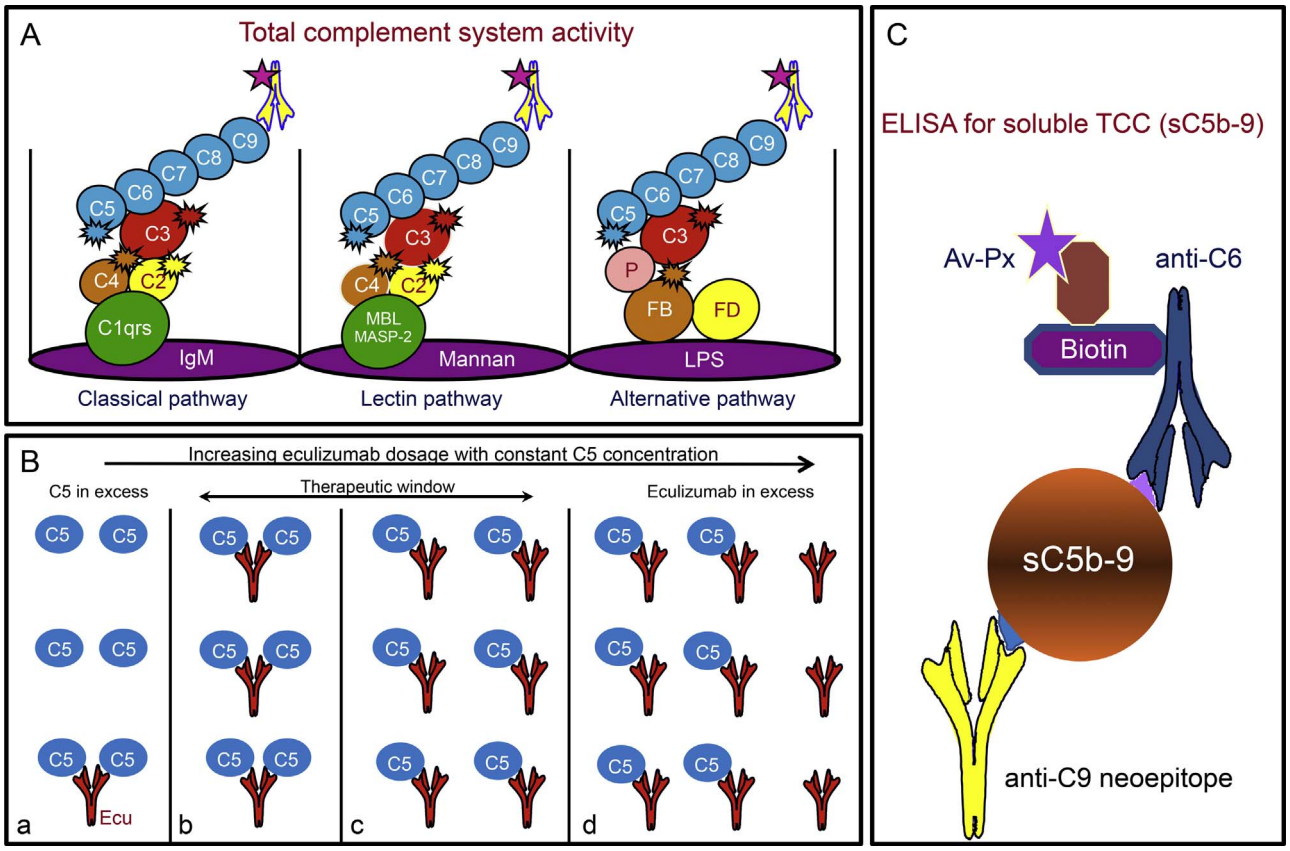

Fig. 3. *Illustration of different approaches to test terminal complement activation by TCC formation (MAC or sC5b-9).* (A) The total complement activity present in serum tested in the classical, lectin and alternative pathways using ELISA. This screening assay determines the functional activity of all complement components by *in vitro* generation of C5b-9 deposited on the solid-phase after incubation of serum in the wells. Read-out is detection of a C9 neoepitope exposed in C9 only after activation. This test is indicated for screening of complement deficiencies. (B) Samples from patients treated with the C5 inhibitor eculizumab can be tested in the screening assay described above. If C5 is in excess (a) it will be detected as activity in the screening test, if all C5 molecules are bound either uni- (b) or bi-valently (c) there will be no activity in the screening test. If eculizumab is present in excess (d) the result will be the same as for (b) and (c). (C) ELISA assay for detection of the fluid-phase generated C5b-9 (sC5b-9). This complex can be detected in increased amounts in plasma from patients with *in vivo* activation of complement and should be low or undetectable in patients treated with eculizumab (similar as for a homozygous C5 deficiency).

interval before complement activity starts to increase after an infusion. This varies from days to several weeks, and these tests should be useful as indicators of sufficient dose given and when it is time for a new dose (Cugno et al., 2014; Gustavsen et al., 2017; Peffault de Latour et al., 2014; Volokhina et al., 2015b; Wehling et al., 2016). An alternative "functional" assay tested the residual activity of C5 present in a serum by activation the serum *in vitro* with subsequent quantification of soluble TCC (sC5b-9) (Riedl et al., 2016). This is a more laborious test than the screening ELISA and it remains to be shown whether it will have a place in routine diagnostics.

Other tests have been developed for specific investigation of the effects of eculizumab, including an elegant study using deposition of C5b-9 on endothelial cells as readout (Noris et al., 2014). Furthermore, an ELISA assay for measuring eculizumab-C5 complexes has been developed by capturing C5 by anti-C5 on the plate and detecting the complex by anti-IgG4 (Hallstensen et al., 2014). By this assay it was shown that eculizumab hardly passed the placenta from the mother to the child since only trace amounts of the complexes was found in the umbilical cord and plasma of the newborn and the newborn's serum had normal complement activity. This observation was confirmed in a later study (Gustavsen et al., 2017). This assay can also be used to test whether eculizumab or C5 is in excess in a sample, by *in vitro* adding purified C5 or eculizumab, respectively, and determine whether the complexes increases. These assays are, however, relatively demanding and thus less suitable for rapid routine testing.

Recently an ELISA has been developed for the detection of eculizumab in patient samples based on eculizumab binding to C5-coated microtiter-wells and detected by anti-Ig antibodies (Gatault et al., 2015; Peffault de Latour et al., 2014; Volokhina et al., 2017). These are in-house assays and it is uncertain how much of the total eculizumab in the sample is detected. Excess of free eculizumab will certainly bind, but the equilibrium of antigen-antibody reaction will imply a continuous shift of eculizumab molecules between the C5 on the surface and in fluid phase, and the binding to surface-bound C5 will finally depend on the molar ratio between the molecules. An assay based on mass spectrometry was, however, recently published and documented to detect the total amount of eculizumab present in the sample as measured by the light chain of the IgG2/4 chimera (Ladwig et al., 2016). This assay is probably the most exact assay published so far for measuring the total amount of eculizumab in the sample, but it does not discriminate between the bound and the free antibody in the current version of the assay; however, discrimination will probably be possible upon modification of the assay.

A couple of issues have been raised with respect to blockage of C5 by eculizumab. First, it is known that a C5 mutant predominantly observed in Asia confers resistance to eculizumab (Nishimura et al., 2014). Second, it has been shown that eculizumab prevent convertase-mediated C5 activation but not direct cleavage by thrombin (Riedemann et al., 2017) and that there is a residual activity in the alternative hemolytic assay when the complement activation is particularly strong (Harder et al., 2017). These observations illustrate potential challenges in the design and monitoring of eculizumab therapy yet the clinical relevance remains to be further explored.

It is evident that immunological assays are prone to pitfalls, and the testing of samples from patients treated with eculizumab is no exception. It has been previously claimed in a letter in *Blood* that treatment

with eculizumab would not prevent formation of C5a *in vivo* when the authors observed that C5a, but not sC5b-9, increased after infusion of eculizumab (Burwick et al., 2014). This was, in our opinion, intuitively contradictory and could not be explained by established molecular mechanisms concerning the cleavage of C5. We therefore investigated this phenomenon in detail and showed that the unexpected finding was indeed caused by a false positive reaction with the specific C5a assay the authors used (provided by BD). We confirmed the reported results using the same assay, whereas we found no detectable C5a in two other well established C5a assays (from Hycult and RND) (Volokhina et al., 2015a). Binding of eculizumab to C5 induces a C5a neoepitope which is falsely detected as free C5a in the BD C5a assay. Thus, assays based on monoclonal antibodies to detect activation of complement should be carefully investigated, in particular with respect to C5 in the case of eculizumab, but also as a general principle to avoid misinterpretations of important tests used to follow-up of patients. Complement activation products are indeed important not only in diagnostics, but also in therapy monitoring. Thus, in the case of inhibiting C5, both the functional tests and the detection of activation products beyond C5 (*i.e.* C5a and sC5b-9) should be negative.

In conclusion, prospective studies comparing different assays in the evaluation of patients treated with eculizumab, and with other complement inhibitors in the future, are necessary to provide robust guidelines for optimal and individualized therapies in complement-mediated diseases.

5.9. Quality and standardization

Since many of the complement tests have not yet been officially approved and various laboratories use modifications of previously described tests or use their own in-house assays, there is a definite need for improvement of test quality and for standardization of the assays. Therefore, the International Complement Society (ICS) has established a committee working on standardization and external quality assessment (EQA) of complement diagnostics. This entity is an official sub-committee for the Standardization and Quality Assessment of Complement Measurements, belonging to the IUIS Quality Assessment and Standardization Committee (http://www.iuisonline.org/index.php?option=com_content&view=article&id=64&Itemid=69). This committee is active in EQA with a number of international complement laboratories being involved, and it will become even more important in the future to guide recommendations of complement tests both for diagnostics and for treatment monitoring. Furthermore the European Complement Network has established a link on their website to laboratories offering routine complement diagnostics across country borders (http://www.ecomplement.org/).

6. Conclusions and perspectives

The status of complement in the clinical arena has profoundly changed, thanks to new biomedical and molecular insight in its disease involvement and the availability of the first complement-targeted drugs in the clinic or in clinical trials. Many of the initial challenges and reservations could be diminished, giving rise to a new surge in the development of new drug candidates and the identification of interesting potential indications. Owing to the vast and constantly growing diversity in disease processes, potential indications and emerging treatment options in complement-mediated disorders, knowing a patient's distinct complement profile will become ever more important. The recent improvements and diversification in diagnostic tools to determine the concentration, function, activity and alteration of complement cascade components are important steps in this context. Genetic tools in particular have changed the field substantially and may help narrowing down disease spectra, choosing appropriate treatment options and selecting the best patient population to be enrolled in clinical trials. This will be critical for advancing complement even further to the clinic. Diagnostic centers offering complement tests are already emerging in many regions. However, even the best diagnostic tool is a blunt instrument without the capability of correctly interpreting the test results and tailoring the treatment strategy accordingly. Careful standardization efforts as well as close collaboration and scientific exchange between academic researchers, industrial scientists, diagnostic and clinical experts will therefore be critical for achieving the next stage in complement-targeted therapies.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2017.05.013.

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