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Complement analysis 2016: Clinical indications, laboratory diagnostics and quality control

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ABSTRACT

In recent years, complement analysis of body fluids and biopsies, going far beyond C3 and C4, has significantly enhanced our understanding of the disease process. Such expanded complement analysis allows for a more precise differential diagnosis and for critical monitoring of complement-targeted therapy. These changes are a result of the growing understanding of the involvement of complement in a diverse set of disorders. To appreciate the importance of proper complement analysis, it is important to understand the role it plays in disease. Historically, it was the absence of complement as manifested in severe infection that was noted. Since then complement has been connected to a variety of inflammatory disorders, such as autoimmune diseases and hereditary angioedema. While the role of complement in the rejection of renal grafts has been known longer, the significant impact of complement. In certain nephropathies has now led to the reclassification of some rare kidney diseases and an increased role for complement analysis in diagnosis. Even more unexpected is that complement has also been implicated in neural, ophtalmological and dermatological disorders. With this level of involvement in some varied and impactful health issues proper complement testing is clearly important; however, analysis of the complement system varies widely among laboratories. Except for a few proteins, such as C3 and C4, there are neither well-characterized standard preparations nor calibrated assays available. This is especially true for the inter-laboratory variation of tests which assess classical, alternative, or lectin pathway function. In addition, there is a need for the standardization of the measurement of complement activation products that are so critical in determining whether clinically relevant complement activation has occurred *in vivo*. Finally, autoantibodies to complement proteins (e.g. anti-C1q), C3 and C4 convertases (C3 and C4 nephritic factor) or to regulatory proteins (e.g. anti-C1inhibitor, anti-factor H) are important in defining autoimmune processes and diseases based on complement dysregulation. To improve the quality of complement laboratory analysis a standardization commmittee of the International Complement Society (ICS) and the International Union of Immunological Societies (IUIS) was formed to provide guidelines for modern complement analysis and standards for the development of international testing programs.

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1. Introduction

The complement system is a complex, evolutionarily well-conserved system. As a vital part of the body's innate immune system, complement provides a highly effective means for the elimination of 'waste' such as invading microorganisms, apoptotic and necrotic cells and immune complexes (Walport, 2001a,b). Furthermore, complement bridges the innate and adaptive immune response through modification of T- and B-cell responses by employing specific receptors on various immune cells (Carroll and Isenman, 2012). Today we know that complement also participates in hematopoiesis, reproduction, lipid metabolism and tissue remodeling (Ricklin et al., 2010).

Approximately 50 soluble and cell surface-attached proteins are currently recognized, including proteolytic components, cofactors, regulators and receptors of the entire complement cascade (Gros et al., 2008; Holers, 2014; Kemper et al., 2010; Ricklin et al., 2010). Complement genes are distributed across different chromosomes, with 19 genes comprising three significant complement gene clusters in the human genome (Mayilyan, 2012).

Complement can be activated via three major pathways, the classical, the alternative, and the lectin pathway, all of which merge in the activation of complement C3 and subsequently lead to the formation of the cytolytic membrane attack complex (MAC), C5b-9. However, complement activation may also occur by interaction of thrombin with complement C5, connecting complement to the coagulation system (Huber-Lang et al., 2002). Furthermore, properdin, known as the only positive regulator of the alternative pathway, has also been shown to specifically bind to pathogens and apoptotic cells, allowing the generation of C3 convertase on the target surface (Spitzer et al., 2007; Kemper et al., 2010) with subsequent opsonisation, i.e. covalent binding of C3b and iC3b. Following complement activation, the biologically active peptides C5a and C3a (anaphylatoxins) are released and elicit a number of proinflammatory effects, such as chemotactic recruitment of leukocytes, degranulation of phagocytic cells, mast cells and basophils, smooth muscle contraction and increase of vascular permeabiltity (Klos et al., 2009). Thereby, the inflammatory response is further amplified by subsequent generation of toxic oxygen radicals and the induction of synthesis and release of arachidonic acid metabolites and cytokines. Consequently, an (over-)activated complement system presents a considerable risk of harming the host by directly and indirectly mediating inflammatory tissue destruction (Ricklin and Lambris, 2013a).

Activation of complement is effectively controlled not only by limiting concentrations of proteins, such as factor D, and the rapid decay of the C3 and C5 convertases, but also by the coordinated action of soluble as well as membrane-associated regulatory proteins (Zipfel and Skerka, 2009). Soluble complement regulators, such as C1 inhibitor (C1-INH), C4b-binding protein (C4 bp), factors H (CFH) and I (CFI), clusterin and S-protein (vitronectin) control the action of complement in body fluids at multiple sites of the cascade. Equally important is the protection against complement attack on each tissue cell by membrane inhibitors, such as the complement receptor 1 (CR1/CD35), the membrane cofactor protein (MCP/CD46) as well as by the glycosylphosphatidylinositol (GPI)-anchored proteins, decay-accelerating factor (DAF/CD55), and CD59 (Zipfel and Skerka, 2009).

Modern diagnostic analysis now provides a comprehensive insight into the activation mode and state of the system allowing a better definition of disease genesis, severity, evolution and response to therapy (Mollnes et al., 2007; Tudoran and Kirschfink, 2012; Nilsson and Nilsson Ekdahl, 2012). With the introduction of complement targeted therapies such as eculizumab (Soliris ®) and more complement inhibitors in clinical studies (Ricklin and Lambris, 2013b; Morgan and Harris, 2015), high quality complement testing for primary diagnosis and for subsequent monitoring of the therapeutic outcome becomes indispensable. However, as is the case for all fields of immunodiagnostics the lack of standardization in complement analysis poses a major problem to disease recognition and follow-up.

Initiated by Prof. George Füst, Budapest, Hungary, in 2009, representatives of 18 international complement laboratories from 11 countries established the standardization committee of the International Complement Society (ICS) and the International Union of Immunological Societies (IUIS) to implement quality management in routine complement analysis. After preparation of standards for complement analytes the first external quality assessment began in 2010, now covering 39 complement diagnostic labs from 21 countries in 2015.

2. Indications for clinical complement analysis

Clinical and experimental evidence underlines the prominent role of complement in the pathogenesis of numerous inflammatory diseases including immune complex and autoimmune disorders (Figueroa and Densen, 1991; Botto et al., 2009; Ricklin and Lambris, 2013a). According to various national and international registries, complement deficiencies represent approximately 2–10% of all primary immunodeficiencies. In clinical practice, however, the consequences of an overactivated complement system with consumption of complement components as the cause of several inflammatory diseases and even life-threatening conditions are more apparent.

2.1. Clinical consequences of complement deficiencies

Genetic deficiencies of complement components are rare; their estimated prevalence is 0.03% (Figueroa and Denson, 1991; Grumach and Kirschfink, 2014). However, as an 'experiment of nature', these anomalies contribute significantly to our knowledge about the importance of this cascade system (Pettigrew et al., 2009; Skattum et al., 2011). Warning signs for complement deficiencies are (1) Meningococcal meningitis at > 5 years of age, (2) other recurrent bacterial infections, esp. with *Pneumococcus*, (3) autoimmune manifestations, (4) angioedema without urticaria, and (5) renal and ophthalmic inflammatory disorders (Grumach and Kirschfink, 2014). Defects of the early components of the classical pathway

(C1-C4) are often associated with autoimmune diseases, such as SLE, due to failure to mount a normal humoral response and to effectively eliminate immune complexes, apoptotic materials, and necrotic debris (Carroll, 2004; Karp, 2005). In active SLE, particularly with renal involvement, low total complement activity and low C4 due to complement consumption are more often the consequence of an increased *in vivo* activation, which can be verified by the detection of complement activation products. In patients with severe clinical outcome, such as lupus nephritis, autoantibodies to C1q are often found and considered to be of prognostic value for disease development (Bock et al., 2015). Deficiencies of early components of the lectin (MBL) and alternative (factor D, properdin) pathways increase susceptibility to infections and their recurrence due to an impaired recognition and opsonization of foreign invaders. Analysis of MASP-1 and MASP-2 is mandatory, if lack of lectin pathway function is not associated with MBL deficiency.

Recurrent *Neisseria* infections, particularly if systemic and with rare serotypes, are associated with complement defects, usually in the terminal pathway. The frequency of meningococcal disease reported in individuals deficient in C5, C6, C7, C8 and C9, reflects a 1000–10,000 fold higher risk in this population (Ross and Densen, 1984). Late component deficient patients, which are readily identified by assays of total complement activity and further identified by selective C5-C9 determination should be vaccinated with tetravalent vaccines against all four strains of *Neisseria meningitidis* (A, C, W135 and Y) (Morgan and Orren, 1998). There is also a high prevalence of properdin deficiency in patients with meningococcal disease, mainly caused by the uncommon serogroups W135 and Y, with a fatality rate of more than 25% (Linton and Morgan, 1999). The most common type 1 deficiency is characterized by the absence of properdin in plasma, whereas in type 2 deficiency properdin is low but detectable (<10% of normal).

Hereditary angioedema (HAE) is an autosomal dominant condition with reduced concentration (type 1) or function (type 2) of C1-INH (Cicardi et al., 2014; Lang et al., 2012). More than 200 point mutations in the C1-INH gene have been found to be associated with HAE. The recurrent angioedema is not induced by complement classical pathway activation, as was once thought, but mediated by bradykinin derived from activation of the contact system (Caccia et al., 2014). The patients present with acute swelling of the face and in the gastrointestinal tract (colic), but swelling is particularly dangerous if localized in the larynx and pharynx. Other organs can be affected causing intestinal obstruction, urinary retention and headache. Triggering factors are stress, trauma, infections and hormonal variations (menses, pregnancy, contraceptives, hormonal reposition). The attacks last usually 3–7 days and, if not treated, can lead to asphyxia. Considering the life threatening consequences of edema formation, early diagnosis of the C1-INH deficiency in these patients is extremely important. As spontaneous mutations can occur, a negative family history does not exclude the diagnosis. Furthermore, the penetrance differs significantly and minor symptoms or subclinical cases can be found. The diagnosis is based on C1-INH and C4 quantification. It is important to include both antigenic and functional assays for C1-INH, since about 15% of the patients have type 2 HAE with normal or even increased antigen concentration of the regulator. C4 is usually low in both cases, even between the attacks, and quantification serves as a valuable supplement to C1-INH analysis (Farkas et al., 2016). Acquired angioedema may occur in patients who develop autoantibodies to C1-INH, in some cases related to hematologic malignancies (Cicardi and Zanichelli, 2010). Here, C1-INH and C4 concentrations are often not affected, pointing to the importance of functional C1-INH testing. Acute attacks are treated with either a plasma-derived C1-INH concentrate, a recombinant C1-INH, a bradykinin B2 receptor antagonist or a plasma kallikrein inhibitor (Bork, 2016).

Reflecting the diversity in the underlying pathology of angioedema, a large group of patients with similar clinical manifestations to HAE but with normal C1-INH concentrations has been recognized (nlC1-INH-HAE, type III HAE) (Bork et al., 2015). Here, in about 25% of cases, so-called gain-of-function mutations in the FXII gene of the clotting system are found, ultimately leading to the increase of angioedema-inducing bradykinin (Zuraw et al., 2012; Germenis and Speletas, 2016).

In paroxysmal nocturnal hemoglobinuria (PNH) a somatic mutation in the gene coding for the phosphatidylinositol (PI) anchor (PIG-A) results in a decreased expression of membrane proteins linked to this structure, including decay accelerating factor (DAF, CD55) and CD59 (Brodsky, 2014). Decreased expression of DAF and/or CD59 renders the red cells susceptible to complement attack, which in combination with thrombocytopenia and thrombotic events is the hallmark of this condition. Diagnosis was traditionally made by Ham's test (acid lysis test), but is now specifically assessed by flow cytometric analysis of the respective cell surface proteins (Kwong et al., 1994). In addition to preventing hemolysis of red blood cells, CD59 protects neuronal cells from complement-mediated injury as deduced from animal experiments and manifest in non-PNH CD59 deficient patients who present with severe immune-mediated polyneuropathy (Nevo et al., 2013). Eculizumab, an antibody that inhibits C5 and thereby the terminal complement sequence, is the treatment of choice for patients with severe manifestations of PNH (Martí-Carvajal et al., 2014). Bone marrow transplantation as a cure for PNH should be reserved for those patients with suboptimal response to eculizumab (Nishimura et al., 2014).

2.2. Complement in nephropathies and renal graft rejection

The kidney appears particularly vulnerable to complement-mediated injury. Complement associated glomerular inflammation, associated with cell proliferation and influx of inflammatory cells, may lead to end-stage renal disease (ESRD) (Brown et al., 2007; Cook, 2013; Koscielska-Kasprzak et al., 2013). Intrarenal synthesis of complement proteins, often insufficiently counterbalanced by local expression of complement regulatory proteins, appears to contribute to disease progression (Zhou et al., 2001).

Complement activation may be the consequence of local e.g. atypical hemolytic uremic syndrome (aHUS) (Noris and Remuzzi, 2009) or systemic dysregulation of the alternative complement pathway as in C3 glomerulopathy(C3G)/dense deposit disease (DDD) (Lesher and Song, 2010; Sethi et al., 2012).

Hemolytic uremic syndrome (HUS) is characterized by microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure. In the diarrhea-negative aHUS more than 50% of all cases present with loss of or gain of function mutations of complement factors or regulators (CFH, CFI, MCP/CD46, THBD/CD141, C3 and CFB), deletions or autoantibodies (e.g. anti-factor H) which provide the molecular background for an unrestricted local complement activation on the vascular endothelium (Kavanagh et al., 2013). While the regulatory function of factor H in the fluid phase is usually not impaired by the known mutations, the majority of aHUS related mutations leads to an impairment of factor H binding to the endothelial surface or the glomerular basement membrane. Lack of recognition of "self" structures (glycosaminoglycans, anionic phospholipids or sialic acid) by factor H and subsequent targeted attack of the alternative pathway against endothelial and blood cells is the underlying cause of aHUS related to CFH mutations. Similarly, defects in the membrane anchored complement regulatory proteins (e.g. MCP/CD46, THBD/CD141) allow for unrestricted progression of the complement cascade on the vascular endothelium. The more common diarrhea-positive HUS, caused by Shiga toxin-producing *Escherichia coli* (STEC HUS) (Orth and Wurzner, 2010) as

well as thrombotic thrombocytopenic purpura (TTP) (Turner and Moake, 2013) has also been associated with complement alternative pathway activation via mechanisms involving P-selectin and platelet thrombi, respectively.

C3 glomerulopathy (C3G) is a recently reclassified group of kidney diseases (Pickering et al., 2013) which is based on dominant glomerular deposition of C3 within the mesangium and along the glomerular basement membrane (GBM) in the subendothelial area or within the GBM as the most common histological feature in these diseases. It includes dense deposit disease (DDD) and C3 glomerulonephritis (C3GN) (Sethi et al., 2012; Medjeral-Thomas et al., 2014; Pickering et al., 2013). Low serum C3, but normal C4 levels are a common finding in C3G. C3 nephritic factor (C3NeF), an autoantibody preventing regulation of the alternative pathway C3 convertase, is found in approximately 80% of patients with DDD and in 45% of patients with C3GN (Schwertz et al., 2001; Zhang et al., 2012), but investigation of C4 nephritic factor (C4NeF), a functional autoantibody stabilizing the classical pathway C3 convertase, also deserves attention (Miller et al., 2012). DDD, previously called MPGN type 2, has been the first nephropathy for which an excessive activation of the complement alternative pathway was identified (Appel et al., 2005; Licht and Fremeaux-Bacchi, 2009). Similar to aHUS, DDD and MPGN 1 patients can also carry mutations or polymorphisms in the CFH (Servais et al., 2012), CFHR3- CFHR1 (Malik et al., 2012), CFHR5 (Vernon et al., 2010), CFI (Servais et al., 2012), MCP/CD46 (Radhakrishnan et al., 2012) and C3 genes (Martinez-Barricarte et al., 2010).

IgA nephropathy is the most prevalent primary chronic glomerular disease worldwide (for review see: Wyatt and Julian, 2013). Since in biopsies C3 and properdin are most often associated with IgA deposits, a role for complement was assumed (Floege et al., 2014). The presence of C4d in glomeruli, indicative for disease progression (Espinosa et al., 2009), derives from the activation of the lectin pathway since MBL is found in the biopsies and IgA does not initiate the classical complement pathway.

While T-cell driven allograft rejection is largely controlled by immunosuppression, antibody-mediated renal injury has become increasingly implicated as a major cause of kidney allograft loss. It is strongly related to complement activation (Stegall et al., 2012). Sensitized individuals with pre-existing circulating antibodies (e.g. ABO) are at high risk to develop an immediate complement dependent reaction against the transplant organ. It appears that local synthesis of complement components as well as loss of regulatory mechanisms that limit the activation, especially of the pivotal component C3, significantly contribute to renal injury (Zhou et al., 2001). In acute renal graft rejection complement activation can be observed several days before the first clinical signs become obvious suggesting complement analysis to be of potential diagnostic value in posttransplant immune monitoring (Kirschfink et al., 1992). The presence of donor-specific antibodies (DSA) and C4d in peritubular capillaries, indicating a local complement activation, are often associated with interstitial fibrosis and tubular atrophy leading to transplant glomerulopathy (Stegall et al., 2012). Complement activation, potentially leading to tissue injury, may occur upon reperfusion of the organ and is most pronounced when the donor organ has undergone a significant period of ischaemia (Damman et al., 2011; Farrar et al., 2012).

2.3. Complement in autoimmune disorders

The prominent role of complement in the pathogenesis of immune complex and autoimmune disorders is well established (Chen et al., 2010). In SLE, impairment of complement-mediated elimination of immune complexes and apoptotic cells, most pronounced in patients with genetic deficiencies of C1q, C2 or C4, are the hallmark of this disease (Carroll, 2004). More recently, mutations in the complement receptors 2 and 3, as well as of complement inhibitors have also been associated with SLE, the latter related to earlier onset of nephritis (Leffler et al., 2014). More than 90% of individuals with hereditary C1q deficiency develop a SLE-like clinical syndrome. The impaired uptake of apoptotic cells by monocyte-derived macrophages in patients with C1q deficiency could be experimentally corrected by substitution with purified exogenous C1q (Taylor et al., 2000). The presence of anti-C1q autoantibodies, found in up to 30–60% of SLE patients, is strongly associated with hypocomplementaemia, disease activity and renal involvement (Yin et al., 2012; Mahler et al., 2013). In the absence of a deficiency, active disease in SLE is often accompanied by low levels of C1q, C4, C3 and decreased activity of the classical pathway, which indicate consumption of complement by inflammation.

Autoantibodies or immune complexes induce complement activation not only in SLE (Lech and Anders, 2013) but also in urticarial vasculitits, anti-glomerular basement membrane (anti-GBM) disease (Ma et al., 2013), in anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides (Kallenberg and Heeringa, 2013) and in Henoch-Schoenlein purpura (Lin et al., 2012).

2.4. Complement in trauma, shock and sepsis

Complement activation after polytrauma substantially contributes to the development of systemic inflammatory response syndrome (SIRS) and multiple organ failure (Zilow et al., 1990; Rittirsch et al., 2012) and is a critical event in the pathogenesis of sepsis and septic shock (Ward, 2010). By interaction with its receptors (C5aR-CD88, C5aR2) C5a appears to impair phagocyte function and *in vivo* inhibition of C5a or of the C5aR is protective and substantially improved survival of septic mice (Bosmann and Ward, 2013). Complement has also been recognized as a major effector mechanism of reperfusion injury (Arumugam et al., 2004). The inflammatory response induced by artificial surfaces in hemodialysis and cardiopulmonary bypass may lead to organ dysfunction. Here, complement activation has been shown to be associated with transient neutropenia, pulmonary vascular leukostasis and occasionally with anaphylactic shock of variable severity (Nilsson et al., 2007). Following traumatic brain injury involvement of complement in the local disease process is reflected by findings of C1q, C3b, C3d and C5b-9 in the immediate vicinity of the cerebral contusion (Bellander et al., 2001).

2.5. Complement in neural, ophtalmological and dermatological disorders

In recent years the role of complement in processes of neuroinflammation and neurodegeneration has been investigated. Data from human studies and animal models indicate a complex role of complement in Alzheimer disease (AD) with evidence for both detrimental and beneficial functions (Veerhuis et al., 2011). Complement proteins of the alternative pathway were found associated with amyloid plaques in the AD patients' frontal cortex at various neuropathological stages of disease progression (Lettiero et al., 2012). Local generation of C3a and C5a via the production of secondary mediators by activated microglia may then create an inflammatory environment. Formation and deposition of C5b-9 (MAC) on neuronal cells could be responsible for much of the neuritic loss and goes along with the observation that the membrane-bound regulator CD59 is significantly decreased in the affected tissue of AD patients (Yang et al., 2000). Therefore, limited or impaired activity of complement inhibitors might partly explain the activation of the whole complement cascade. Complement appears also to be involved in other neurological disorders such as Parkinson's disease (Yamada et al., 1992), Pick's disease (Singhrao et al., 1996), multiple sclerosis (Lindblom et al., 2016), in

Table 1
Complement mediated diseases and recommended complement analysis.

Disorders	*Recommended Analysis*
Infectious disorders	CH50, AH50, Functional ELISA for CP, AP, LP, C3, C4, C1q C3a/C3d, sC5b-9 C5–C9 (especially in *Neisserial* infections) Properdin (especially in *Neisserial* infections) MBL (especially in *Neisserial* infections)
Systemic Lupus Erythematosus, Hypocomplementaemic urticarial vasculitis syndrome	CH50/Functional ELISA for CP C4 (C4A/B), C1q, C3 C3a/C3d, sC5b-9 Anti-C1q autoantibodies
Angioedema	C1-INH (antigenic and functional assay) C4 C1q Anti-C1-INH autoantibodies (acquired autoimmune form)
Atypical Haemolytic Uremic Syndrome	CH50, AH50/Functional ELISA for CP, AP C3, Factor B C3a/C3d, sC5b-9 Factor H, Factor I anti-factor H autoantibodies molecular analysis of *CFH, CFI, C3, FB, CD46,*
C3 Glomerulopathy	CH50, AH50//Functional ELISA for CP, AP C4, C3 C3a/C3d, sC5b-9 Factor H, Factor I C3 (and C4) nephritic factor autoantibodies to factor H, factor B, C3 convertase molecular analysis of *CFH, CFI, C3, FB, CD46*
Paroxysmal nocturnal hemoglobinuria	CD55, CD59 (FACSCAN) Acid lysis test, FLAER (fluorescently labeled aerolysine)

Huntington's disease (Woodruff et al., 2006) and amyotrophic lateral sclerosis (Woodruff et al., 2008). Treatment with a C5a receptor antagonist has led to a decrease in pathology, indicating a detrimental role for C5a in these disorders.

Pathogens such as *Neisseria meningitidis* and Epstein-Barr virus (EBV) misuse the complement system to their advantage by employing the complement regulatory protein CD46 and the complement receptor 2 (CD21), respectively, to cross the blood brain barrier (Carel et al., 1989). HIV-1 escapes complement not only by binding the complement-regulatory membrane proteins CD46, CD55 and CD59, acquired upon budding from its host cell (Dierich et al., 1996), but also by binding of factor H to gp41 (Stoiber et al., 1995).

Age-related macular degeneration (AMD) is a genetically complex, multifactorial disease of the retina and adjacent structures and considered the leading cause of blindness in old age in many countries. The formation of so-called drusen leads to a pronounced central visual loss. Replacement of thymine (T) by a cytosine (C) in the CFH gene (Y402H polymorphism) with subsequent impairment of local complement regulation poses a significantly increased risk of disease to the affected individual (Fritsche et al., 2014). Various complement proteins, their activation products, and regulators have been identified in retinal deposits of AMD patients. Several genes that are associated with AMD code for proteins of the complement system, including C3, C2, and factor B, and the regulators factor H and factor I (McHarg et al., 2015).

Overactivation, but also dysregulation of complement are observed in various skin diseases (for review see: Panelius and Meri, 2015). This may be the consequence of autoantibody-induced cytotoxic effects of the complement membrane attack complex on epidermal or vascular cells causing inflammation and tissue destruction. Complement activation products are seen in skin manifestation of SLE, of the phospholipid antibody syndrome, and bullous skin diseases like pemphigus, pemphigoid, epidermolysis bullosa acquisita and dermatitis herpetiformis. By evading complement attack, some microbes like *Borrelia* spirochetes, *Streptococcus pyogenes* and staphylococci can persist in the skin and cause prolonged symptoms.

3. Pre-analytical phase considerations

Precise preanalytical work is a prerequisite for accurate and conclusive laboratory complement diagnostics. Proper collection and processing of all body fluid samples for complement analysis is essential but challenging. It should aim at capturing the complement activation status of the patient at the time of blood draw. Without inhibition, physiological and pathological complement activation continues *ex vivo* obscuring the actual complement activation status and preventing meaningful data interpretation. Serum is the appropriate sample to measure complement activity, components, regulators and autoantibodies. It should be separated by centrifugation after full clotting, but serum separating tubes are also useful. Separated serum samples should be used immediately or can be stored at −70 °C for short-term. Since coagulation enzymes can also cleave complement components with subsequent generation of activation products, EDTA at ≥10 mM final concentration is used as standard anticoagulant since it blocks the *in vitro* activation of the complement system by Mg^{2+} and Ca^{2+} chelation. Heparin and citrate based anticoagulants are less useful (Mollnes et al., 1988). For most complement activation products, centrifuged EDTA-plasma is stable for up to 4 h at room temperature (Yang et al., 2015) but should better be kept on ice or in a refrigerator if analyzed on the same day. For later processing, the sample should be aliquoted, frozen and stored at −70 °C (−20 °C even for short-term storage may be problematic for testing complement function and activation products). Frozen samples should be thawed at room temperature or on ice, but not in a water bath at 37 °C. Repeated freezing and thawing of aliquots should be avoided because of the risk of *in vitro* activation and degradation (see above). Frozen samples should be shipped on dry ice by courier, if transport is necessary. If patients are treated with plasma infusion or exchange, samples should be collected prior to such treatment.

In urine, the measurement of complement activation products can be affected by high amounts of urea and urine proteases, so that the addition of protease inhibitors and EDTA is required. However, as recently shown by van der Pol et al. (2012), appearance of complement activation products in proteinuria may also be the consequence of extrarenal (artificial) rather than intrarenal complement activation.

4. Methods of modern complement analysis

In recent years, great progress has been made in complement analysis to better define disease severity, evolution and response to therapy (Mollnes et al., 2007; Nilsson and Nilsson Ekdahl, 2012; Tudoran and Kirschfink, 2012). However, a professional complement analysis going far beyond C3 and C4 requires the involvement of specialized accredited laboratories. The recommended analysis for the most important complement mediated disorders is outlined in Table 1.

When complement deficiency is suspected, samples are screened either by functional assays of each activation pathway to

Table 2
Methods of clinical complement laboratory diagnostics.

Functional assays	Functional activity of single components: Total complement activity (screening for complement deficiency): • CH50 and AH50 hemolytic assays for CP and AP activity, Lipolytic assay (CP) • Functional ELISA for CP, LP and AP activity Functional activity of single components: • Hemolytic assays for single components (e.g. C3) using corresponding deficient sera as test system • Hemolytic assays for determination of Factor H function • C1 inhibitor assay (chromogenic substrate) or ELISA
Protein quantification	Immunoassays, measuring the concentration of single components by immunoprecipitation (RID, nephelometry), ELISA or Western Blot
Activation products	Immunoassays preferentially based on antibodies to neoepitopes expressed on the activation products: • Split products from components after proteolytic cleavage (e.g. C3a, C3d, C4a, C4d, C5a, Bb) • Complexes between the activated component and its inhibitor (e.g. C1rs-C1 inhibitor) • Macromolecular complexes (e.g. the AP convertase C3bBbP and the terminal sC5b-9 complex)
Autoantibody analysis	ELISA (anti-C1q – SLE, HUVS; anti-C1INH – angioedema; anti-fH – aHUS; anti-fB – C3G) or functional assay (C3/C4 nephritic factors – C3G)
Surface proteins	Flowcytometric quantification of CD55/DAF and CD59 (PNH,) of CD46/MCP (aHUS), of CD11b/CD18 (LAD1), of CD21 (B cell deficiency)
Genetic analsyis	Detection of complement deficiencies and of disease associated genetic variants (SNPs, CNVs) e.g. factor H, MCP/CD46, factor I, factor B, C3 (aHUS or C3G) including family analysis

CP–classical pathway, AP–alternative pathway, LP–lectin pathway, aHUS–atypical haemolytic syndrome, C3G–C3 glomerulopathy, HUVS–Hypocomplementaemic urtucaria vasculitis syndrome, RID– radial immunodifusion, SNPs–Single Nucleotide Polymorphisms; CNVs- Copy Number Variants.

reveal the deficiency in any particular system (e.g. in patients with recurrent infections), by measuring individual proteins directly, or by measuring the functional activity of a single component in the pathway in question (e.g. C1-inhibitor in hereditary angioedema).

The standard immunochemical methods are valuable tools in diagnostic work and when necessary these are supplemented with functional assays. Correct interpretation requires validated reference ranges. Here it should be emphasised that the reference intervals for several components are age-related, and this must be taken into account when analysing samples from newborns and infants (Johnson et al., 1983; Roach et al., 1981; Sonntag et al., 1998; Grumach et al., 2014). Table 2 outlines the most common assay systems/methods currently used in complement analysis.

The diagnostic work up of a patient with a suspected complement-associated disease should start with the assessment by functional titration of the total hemolytic activity of the classical (CH50) and alternative (AH50) pathways. These assays can be simplified by a fixed serum sample dilution and incubation time in the presence of excess of target erythrocytes (Nilsson and Nilsson, 1984). Function of the classical pathway can also be assessed by a liposome enzymatic assay.

A functional ELISA with either IgM, mannose or lipopolysaccharides coated on the plate has been developed that can examine all three activation pathways in parallel. In the presence of appropriate buffers to block the other two pathways, complement is activated in patient's serum and can be visualized by detection of C5b-9 with a neoepitope-specific conjugate antibody (Seelen et al., 2005). Although properdin deficiency is usually not detected in the hemolytic alternative pathway assays it may be recognized by the functional ELISA.

Analysis of the plasma concentrations of individual complement components, such as C3 and C4, is still performed by radial immunodiffusion or nephelometric tests in most clinical laboratories. However, these tests detect both native and activated complement proteins. They are strongly influenced by fluctuations in protein synthesis, in particular during the early acute-phase reaction and in obesity. Other complement components and regulators are assessed by ELISA or time-resolved immunofluorometric assay (TRIFMA) (Frederiksen et al., 2006).

Modern complement analysis relies on the quantification of complement-derived split products (e.g. C4d, C3a, C3d or Bb) or protein–protein complexes (sC5b-9, C3bBbP), reflecting the actual activation state of the complement system. This analysis requires EDTA-plasma to avoid high levels due to the clotting process. Activation products like C3a or C3d, Bb or sC5b-9 should be utilized as global markers of complement activation; they appear to be particularly useful in monitoring patients under eculizumab therapy. By choosing the appropriate parameters, it is possible to determine exactly which pathway is activated. However, as previously outlined (Mollnes and Kirschfink, 2006), since the various complement activation products have different half-lives *in vivo* it is important to decide which parameters(s) should be measured. Due to rapid receptor binding, the biologically highly active and important C5a fragment has a half-life of approximately one minute (Oppermann and Gotze, 1994) and is difficult to detect in samples obtained *in vivo*. In contrast, the various C3 activation products have half-lives of a few hours (Teisner et al., 1983; Norda et al., 2012). The half-life of sC5b-9 is about 50–60 min (Mollnes, 1985). Being relatively stable *in vitro*, sC5b-9 reflects activation of the entire cascade irrespective of the initial pathway involved. When measuring an activation product from a single component (e.g. C3a or C3d), the amount should be related to the concentration of the native component, since a low level of native component (upon consumption) yields smaller amounts of activation products during *in vivo* activation. Thus, the ratio between activation product and native component appears to be a more sensitive indicator of *in vivo* activation than the activation product alone (Nurnberger and Bhakdi, 1984).

Monitoring of complement targeted drugs such as eculizumab is usually done by using CH50 or AH50 to monitor inhibitory efficacy (Peffault de Latour et al., 2015; Volokhina et al., 2015; Andreguetto et al., 2015). In patients receiving eculizumab, sufficient levels of the anti-C5 antibody are reflected by an abolished function of the classical and alternative pathways as well was of C5. It is important to include complement activation markers (C3 and factor B split products as well as sC5b-9) to estimate still ongoing complement activation in vivo (Wehling and Kirschfink, 2014). Measurement of the serum (and urine) concentration of eculizumab is performed as in-house assays by some laboratories (Gatault et al., 2015).

Similar to loss- or gain of function mutations in complement regulators or activating components, an overactivation of complement can also be caused by autoantibodies. Autoantibodies to CFH (DEAP HUS) (Jozsi et al., 2008), CFB (DDD) (Strobel et al., 2010), C1q (SLE) (Mahler et al., 2013), or to the C1-INH (hereditary angioedema) (Cugno et al., 2008) can be detected by ELISA with the respective purified complement proteins immobilized on a microtiter plate. A standardized ELISA for Factor H autoantibodies, tested in an international multi-center study, is available (Watson et al., 2014), but ELISAs for anti-Factor B and other complement

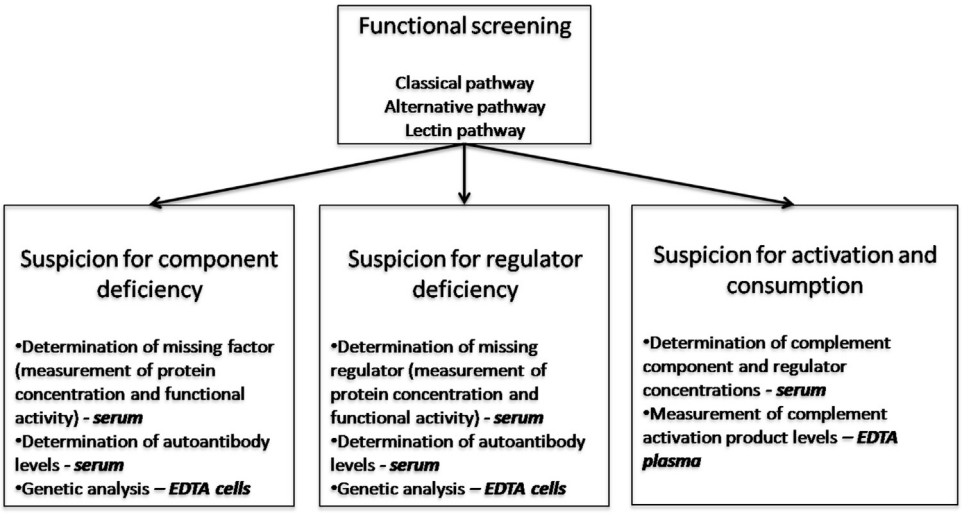

Fig. 1. Algorithm of complement analysis. If functional activity of one or more of the three pathways is undetectable or very low, further analysis of individual complement components, regulators and activation products is required to identify either a primary deficiency (lack of individual component (as protein and/or function), but usually normal levels of activation products) or as a consequence of strong activation with consumption (high levels of activation products).

proteins are still only used in research projects. C3 nephritic factors (C3Nef) are a heterogenous group of C3/C5 convertase stabilizing autoantibdies, found in different nephropathies (Schwertz et al., 2001; Paixão-Cavalcante et al., 2012). They are often measured in a semi-quantitative hemolytic assay (Rother, 1982). Fluid-phase conversion of C3 to C3b and C3c upon mixture of normal serum and C3NeF containing patient's serum can also be visualized using an immunofixation assay (Koch et al., 1981).

The diagnosis of many immune-mediated inflammatory diseases often relies on the detection of immunoglobulins and complement tissue deposition. For immunohistochemical diagnosis, especially in nephropathies but also in autoimmune disorders, antibodies against C1q, C3b, C4b, C4d, and C5b-9 are used for frozen tissue and also in part for paraffin-fixed tissue. Positive staining identifies a direct impact of complement in the disease process, indicates disease activity and allows for the differentiation of the complement activation pathways involved. The presence of C3b suggests ongoing inflammation, while C3d deposits in the absence of C3b point to a non-active disease process. C4d has been accepted as biomarker of humoral renal graft rejection (Feucht et al., 1993; Cohen et al., 2012).

A general algorithm of complement analysis is given in Fig. 1.

5. Quality assessment

It was in 2008, during the XXII[nd] International Complement Workshop, when the idea of establishing a working group to develop external quality assessment and standardization of diagnostic complement testing was proposed by George Füst, Semmelweis University, Third Department of Internal Medicine, Budapest (Hungary). The first meeting of the Complement EQA working group was held in 2009 in Budapest. Organizational questions were discussed and the participants decided on electing chairs, vice-chairs and a secretary for the group. The elections took place in Visegrád (Hungary) in 2009, during the 12th European Meeting on Complement in Human Disease, where the Sub-Committee for the Standardization and Quality Assessment of Complement Measurements, belonging to the IUIS Quality Assessment and Standardization Committee (http://www.iuisonline.org), was formally established (the 'Complement EQA Group'). Five external quality assessment rounds were organized by the group between 2010 and 2015 with the logistic and financial help of INSTAND e.V, a Düsseldorf (Germany) based institute for promoting quality assurance in medical laboratories.

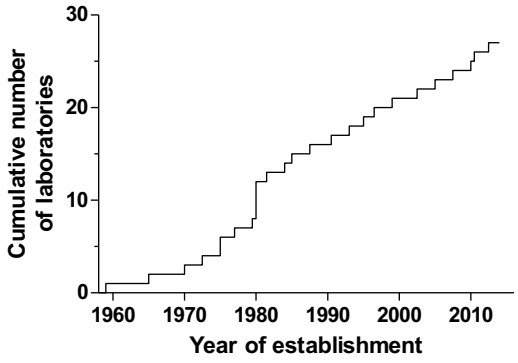

Fig. 2. Cumulative number of complement diagnostic laboratories operating in 2015, according to the year of establishment. Data based on 27 responding laboratories of the EQA5 round (2015).

5.1. Complement EQA concept, parameters and evaluation

The purposes of these EQA rounds were to test the performance of participating complement diagnostic laboratories and to provide training and advice for their quality management processes by making available coded samples for determinations of various parameters. As in many fields of clinical laboratory analysis, achievement of these goals are limited by the fact that participating laboratories use various methods, calibrators and reagents to measure the same analytes or parameters. Therefore an additional purpose of the EQA program was to facilitate standardization of diagnostic complement testing by developing calibrator materials and test recommendations. Members of the Complement EQA Group prepared and evaluated a serum standard for quantitative and functional complement analysis in normal (non-activated) human serum. This standard was named "Standard 1" (ICS#1). An activated standard was also prepared for assays to quantify complement activation products, "Standard 2" (ICS#2) (Bergseth et al., 2013). These samples, together with single patient pathological samples e.g. for determination of anti-complement autoantibodies, were used in the EQA rounds.

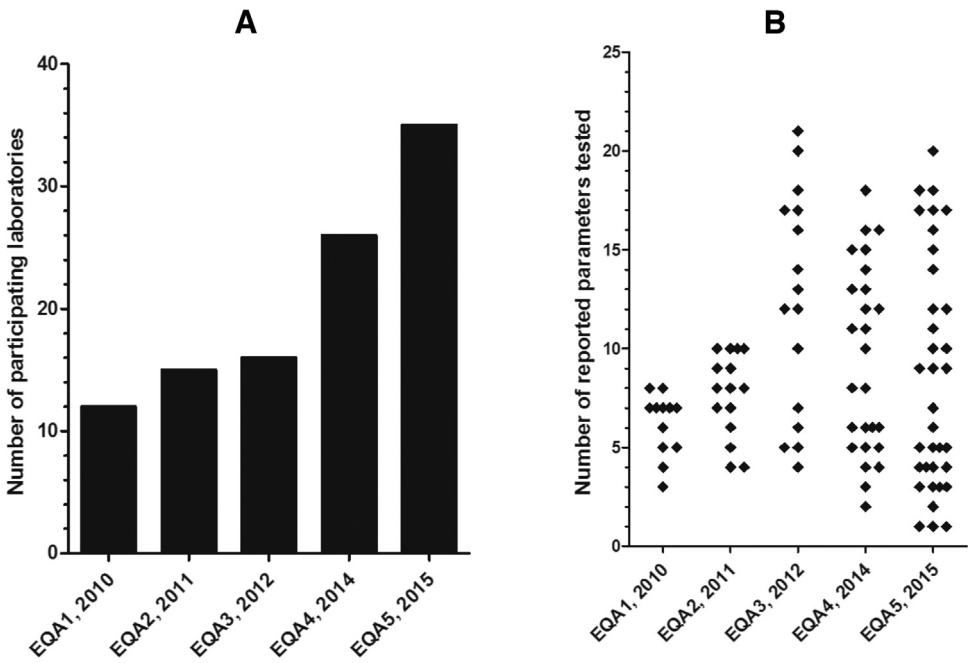

Fig. 3. Number of participating laboratories (A), and number of parameters finally reported by the participating laboratories (B) in the various Complement EQA rounds.

Table 3
Results of the 5th External Quality Assessment round (2015): assays with quantitative results.

Assay	No. of responses	Sample	Median of normalized results	25th Percentile of normalized results	25th Percentile of normalized results	Between-laboratory coefficient of variation***
Classical pathway activity	27	A2*	0.68	0.60	0.79	0.44
		A3**	0.63	0.54	0.77	0.35
Alternative pathway activtiy	22	A2	0.55	0.36	0.69	0.40
		A3	0.54	0.37	0.71	0.44
Lectin pathway activity	11	A2	0.21	0.11	0.59	0.99
		A3	0.53	0.31	0.75	0.52
C3	26	A2	1.12	1.06	1.15	0.07
		A3	0.76	0.73	0.78	0.16
C4	26	A2	1.25	1.17	1.32	0.10
		A3	0.98	0.92	1.02	0.10
C1-inhibitor antigen	20	A2	0.78	0.73	0.89	0.19
		A3	0.61	0.59	0.65	0.16
C1-inhibitor function	18	A2	0.64	0.45	0.82	0.43
		A3	0.62	0.49	0.80	0.28
C1q	18	A2	0.60	0.52	0.73	0.40
		A3	0.66	0.58	0.71	0.16
Factor H	15	A2	0.99	0.86	1.10	0.17
		A3	0.81	0.68	0.87	0.14
Factor I	12	A2	0.90	0.84	1.03	0.16
		A3	0.74	0.68	0.83	0.13

*A2, **A3–NHS, spiked with various amounts of heat inactivated NHS, ***Variation coefficient of the normalized assay results (assay result for samples A2 and A3 were divided by the result of the pooled serum) across laboratories.

Interest in diagnostic complement analysis (Fig. 2), hence, participation in the Complement EQA program has increased constantly over time (Fig. 3). Fig. 3A shows the number of participating laboratories in the different EQA rounds and Fig. 3B the total number of parameters reported finally by the participating labs in the various EQA rounds. It became evident that about half of the complement laboratories are determining most of the parameters, whereas the other half restricts analysis to only a few parameters. In Fig. 4 detailed results of the last round (EQA5, 2015) are summarized. Most complement laboratories are determining C3, C4, classical and alternative pathway activities, whereas the remaining parameters (AP factors and regulators, C1-INH, anti-complement autoantibodies and activation products) are less frequently analyzed even in specialized complement laboratories.

5.2. Outcomes of the complement EQA5 (2015) round

Since participating laboratories may use different methods and calibrators, aggregate evaluation of EQA5 results was done in the following way: Three samples (ICS#1 = A1, the reference material, and two 'pathological' samples, A2 and A3, respectively) were sent to the laboratories. Due to the lack of target value for these determinations, results of ICS#1/A1 measurement were used to normalize assay readouts of A2 and A3 samples. Aggregate results (median and interquartile ranges of normalized values) with between-laboratory coefficients of variations are listed in Table 3. The median values ranged from 0.21 to 1.25, whereas the CV% ranged between 0.07 and 0.99. There was a clear relationship between the median values and CV%: the lower the median value was (i.e. large devia-

Table 4
Results of the 5th External Quality Assessment round (2015): assays with semi-quantitative results.

Assay	Sample target value	Positive/borderline/negative responses	Success rate*
C3 Nephritic Factor	Negative/borderline	1/0/11	0.50
	Positive	7/1/4	
Anti-C1q IgG	Positive	13/0/0	0.62
	Positive/borderline	8/0/5	
Anti-Factor H IgG	Negative	1/0/15	0.89
	Positive	14/0/2	
Anti-C1-inhibitor IgG	Negative	2/0/8	0.60
	Positive	8/1/1	
	Negative	2/0/8	
	Negative	2/0/8	
Anti-C1-inhibitor IgA	Negative	0/1/8	0.66
	Negative	0/0/9	
	Negative	1/0/8	
	Positive	8/0/1	
Anti-C1-inhibitor IgM	Positive	9/0/0	0.66
	Negative	1/0/8	
	Positive	6/1/2	
	Negative	0/0/9	

*Success rate: number of laboratories reporting consistently appropriate results, divided by the number of participating laboratories.

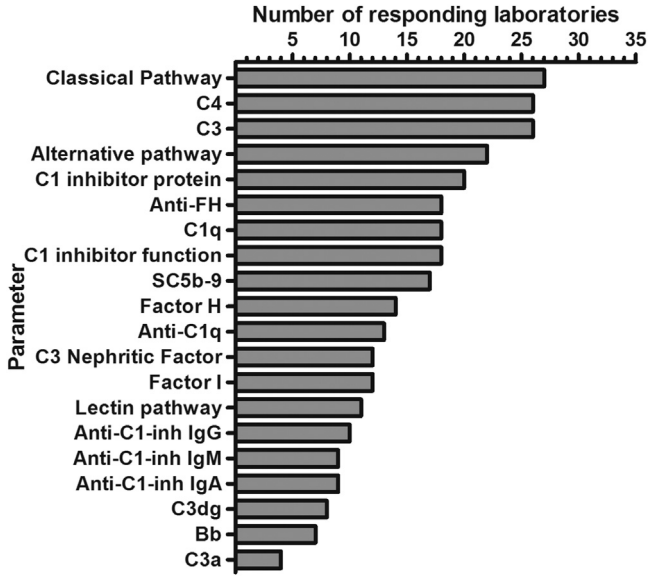

Fig. 4. Number of laboratories that reported results for the parameters listed on the Y-axis in EQA5 round (2015).

tion from normal sample), the higher CV% was observed (Spearman correlation coefficient $r = -0.81$, $p < 0.0001$). In addition, CV% values were generally higher for functional assay (CP, AP, LP and C1-INH activity) than those of protein measurements.

For evaluation of anti-complement autoantibody determinations single patient based positive or negative samples were provided for the laboratories. Results were compared to the target value (determined by the laboratory that provided the samples), here presented in aggregate form in Table 4. Success rates were calculated for consistently appropriate results, i.e. the laboratory succeeded if all of the tested samples yielded accurate results for the given autoantibody. The best success rate was observed for anti-FH IgG determination. Anti-C1q and anti-C1-INH results were similar, however, the measurement of C3Nef was correct in only half of the participating laboratories. It has to be noted that the C3Nef determination relies on somehow more complex functional complement measurements, whereas the remaining anti-complement autoantibodies are measured by ELISA.

5.3. Future plans

The Complement EQA group met in Budapest on January 22–23, 2016, with 31 participants representing 21 complement laboratories. The group reviewed the accomplishments of the first five years of the program and discussed the path forward. Participants critically assessed their achievements and defined the next steps for improvement. The focus was to develop recommendations on assay preference, calibration, presentation and interpretation of results. Small working groups will be established for various assays, wet lab experimentation will be done for assay evaluation and a final document will be released by the group documenting guidelines and recommendations for diagnostic complement analysis. As illustrated by the aggregate results presented in Tables 3 and 4, special attention has to be paid to functional measurements of complement; these assays require careful evaluation, harmonization and standardization.

Conflict of interest

Michael Kirschfink received fees as consultant (Eurodiagnostica) and for lectures (Alexion), Zoltán Prohászka received fees for lectures (Alexion).

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