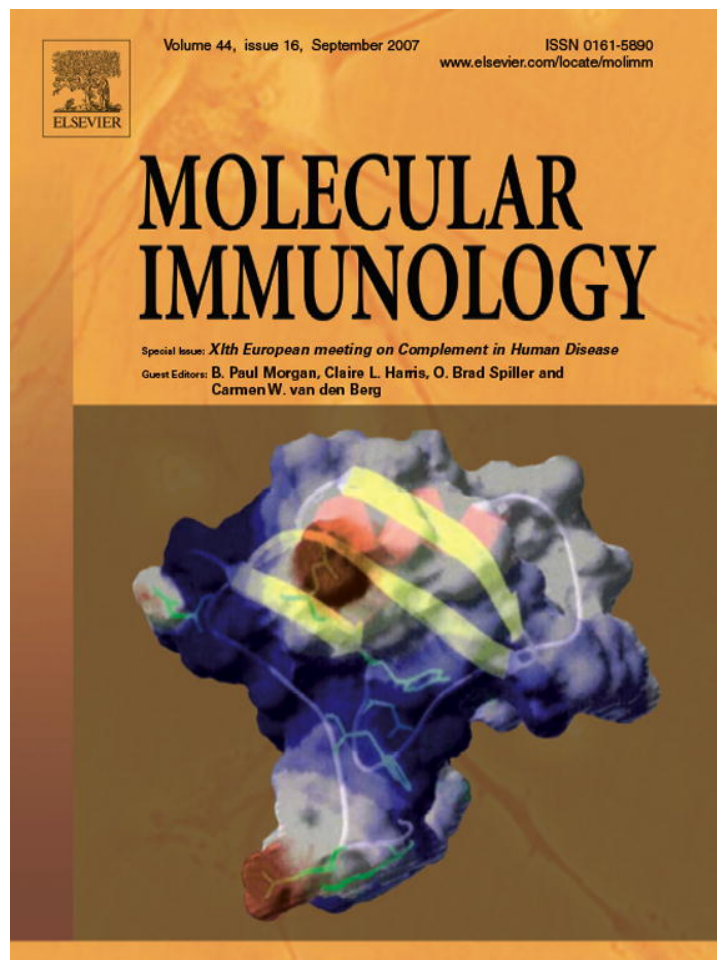


Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.



This article was published in an Elsevier journal. The attached copy is furnished to the author for non-commercial research and education use, including for instruction at the author's institution, sharing with colleagues and providing to institution administration.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

<http://www.elsevier.com/copyright>



Review

Complement analysis in the 21st century

Tom Eirik Mollnes^{a,b,*}, T. Sakari Jokiranta^c, Lennart Truedsson^d,
Bo Nilsson^e, Santiago Rodriguez de Cordoba^f, Michael Kirschfink^g

^a Institute of Immunology, University of Oslo, and Rikshospitalet, Oslo, Norway

^b Department for Laboratory Medicine, Nordland Hospital, Bodø, University of Tromsø, Norway

^c Department of Bacteriology and Immunology, Haartman Institute and Huslab, University of Helsinki, Finland

^d Institute of Laboratory Medicine, Section of Microbiology, Immunology and Glycobiology, Lund University, Sweden

^e Department of Radiology, Oncology and Clinical Immunology, Division of Clinical Immunology,

The Rudbeck Laboratory, University Hospital, Uppsala, Sweden

^f Centro de Investigaciones Biológicas (CSIC), Unidad de Immunología, Madrid, Spain

^g Institute of Immunology, University of Heidelberg, Germany

Received 31 May 2007

Abstract

Complement analysis in the clinic is usually associated with the quantification of C3 and C4, measurement of C1-inhibitor and screening for complement activity. These analyses have been available in routine diagnostic laboratories for decades. In recent years, however, the field of complement analysis has expanded considerably, with the introduction of novel assays to detect complement activation products, and spreading still further towards genetic analysis to reveal the basis of complement deficiencies and identify mutations and polymorphisms associated with defined diseases such as atypical haemolytic uraemic syndrome and age related macular degeneration. Here we review the current status of complement analysis, including assays for the quantification of complement activity and complement activation products, together with genetic methods for the detection of deficiencies, mutations and polymorphisms. This is an area where significant developments have been made recently, paralleling the research advances into the role of complement in human disease. It is clear, however, that there is a need for consensus and standardisation of analytical methods. This will be a major challenge for the complement society in the future.

© 2007 Elsevier Ltd. All rights reserved.

Keywords: Complement assay; Neoepitope; Proteomic analyses; Genetic analyses

1. Introduction

Over the last 3–4 decades, routine complement analyses in the clinical setting has relied upon the quantification of key complement components C3, C4 and C1-inhibitor. In some specialist laboratories, diagnostic tests extended to functional complement screening assays. Initially, complement was not perceived to play a key role in human disease; for example, the first two cases of C2 deficiency were discovered accidentally in otherwise healthy individuals. Additionally, representative *in vivo* experimental models of human inflammatory diseases were sparse so the role of complement in the initiation and

perpetuation of disease could not be studied. Gradually, it became clear that complement deficiency was frequently associated with severe diseases such as systemic lupus erythematosus (SLE), and it was recognised that such a complex biological system could not have evolved without being critically important for the host. Only recently has it been accepted that complement is not only important for protection against microbial infection, but also contributes to pathophysiology in a number of non-infectious diseases. Accumulating data on the decisive role of the complement system in various disorders drove the development of strategies to specifically inhibit complement in anti-inflammatory therapy. This, in turn, has stimulated a broad resurgence of interest in complement both in the clinic and in research. The future progress in this field, however, is critically dependent on further development of reliable methods to comprehensively analyse the complement system.

* Corresponding author at: Institute of Immunology, Rikshospitalet, N-0027 Oslo, Norway. Tel.: +47 90630015; fax: +47 23073510.

E-mail address: t.e.mollnes@medisin.uio.no (T.E. Mollnes).

2. Complement deficiency analysis

When complement deficiency is suspected, samples are screened by functional assays of each activation pathway to 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 [HAE]). When analysing complement function or protein levels, the way in which blood samples are collected and stored is, for some of the assays, critically important (see Section 3.1.3).

2.1. Functional assays of complement activation

Functional assays of the complement system assess the integrity of the individual activation pathways. Such assays are extremely useful in the detection of complement deficiencies and are needed to direct the subsequent component specific analyses.

2.1.1. Haemolytic assays

The commonly used assays for classical pathway activation are based on the use of antibody-coated sheep erythrocytes (EA), which are lysed in serum diluted in a buffer containing Ca^{2+} and Mg^{2+} ions. In the classical CH50 test the results are usually expressed as the reciprocal dilution of the sample needed to achieve 50% lysis of a fixed amount of EA. For the alternative pathway a similar assay, the AH50 test, can be done using rabbit (RbE) or guinea pig (GpE) erythrocytes, which specifically activate the alternative pathway of human complement when serum is diluted in a buffer containing Mg^{2+} ions only with Ca^{2+} chelated using EGTA to block the classical and lectin activation pathways.

Simplified and rapid haemolytic assays based on a fixed serum sample dilution and incubation time and an excess of target erythrocytes have been described using EA or RbE for the classical and alternative pathways, respectively (Nilsson and Nilsson, 1984). The proportion of lysed cells is linearly correlated to the amount of complement present in the serum sample. Another simple screening assay for complement deficiencies involves haemolysis-in-gel (Truedsson et al., 1981).

Here, EA in $\text{Ca}^{2+}/\text{Mg}^{2+}$ buffer or GpE in $\text{Mg}^{2+}/\text{EGTA}$ buffer for the classical and alternative pathways, respectively, are incorporated in agarose gels. These assays are very easy to perform and well suited for screening large numbers of samples. The use of such screening procedures has resulted in the identification of numerous complement deficient individuals. However, properdin deficiency is not always detected in the haemolytic alternative pathway assays. This may be explained by the action of properdin as a positive regulator, where deficiency changes the kinetics of the reaction but does not necessarily result in significant change in end-point haemolysis. Haemolytic assays are particularly suited to the detection of deficiencies in both the classical and terminal pathways. However, their value in monitoring complement activation in relation to disease status is questionable. Nevertheless, the CH50 and a simplified version of this assay can give quantitative results and can be used in routine analyses of classical pathway function, for example in SLE patients (Ekdahl et al., 2007).

2.1.2. Complement activation ELISA

Methods utilizing the enzyme-linked immunosorbent assay (ELISA) format have recently become available for general use. This methodology was first described by Zwirner et al. (1989), who reported that deposition of a series of complement components took place during activation on a suitable surface. They could detect deposition of C1q, C1s, C4, C3, factor B, C4b-binding protein, properdin and all terminal components on the plastic surface of a microtiter plate after serum activation. Based on these findings, ELISAs for screening of deficiencies of the classical and the alternative pathways were developed (Fredrikson et al., 1993). Plates were coated with IgM for activation of the classical pathway and lipopolysaccharide (LPS) from *Salmonella typhi* for the alternative pathway. To detect complement activation, either a monoclonal antibody against a C9 neoepitope or an antibody against properdin was used. These ELISAs clearly allowed detection of deficiencies within the classical, alternative and terminal pathways (Fig. 1).

Activation of the lectin pathway in a microtiter plate is easily achieved by coating the surface with mannan. However, to have a lectin pathway specific assay the other activation pathways have to be blocked. Use of a monoclonal anti-C1q antibody that

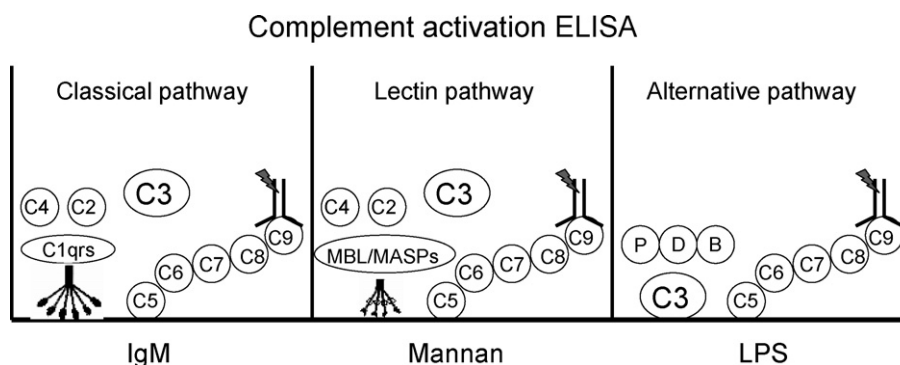


Fig. 1. ELISAs for measuring of complement activation (Seelen et al., 2005). Diluted serum is added to the well of a microtiter plate coated as shown to allow activation of the three pathways. The choice of buffer and the use of a classical pathway inhibiting antibody make the ELISAs specific for each pathway. After incubation, complement is activated and readout is the incorporation of C9 in the terminal complement complex, C5b-9.

Table 1
Complement deficiency and detection by complement activation ELISA

Impaired function/deficiency	Activity in ELISA		
	Classical	Lectin	Alternative
Component			
C1q, C1r, C1s	Low	Normal	Normal
C4, C2	Low	Low	Normal
MBL, MASP2	Normal	Low	Normal
B, D, P	Normal	Normal	Low
C3, C5, C6, C7, C8, C9	Low	Low	Low

inhibits classical pathway activation made this possible (Roos et al., 2003). A simple method comprising three ELISAs which could be used for screening for deficiencies of all three pathways was then developed (Seelen et al., 2005). This method is commercially available (Wielisa[®], Wieslab, Lund, Sweden), easy to perform and has several advantages compared with the haemolytic assays: it is not dependent on availability of erythrocytes, it covers all three activation pathways, and importantly, properdin deficiency is detected in the alternative pathway assay. The assay is performed with serum samples and detection of activation is at the final step, incorporation of C9 in the terminal complex (Fig. 1). In diagnosis, these assays are often used in combination with measurement of C3 and C4 and should be supplemented by measurement of an activation product to distinguish primary from secondary complement deficiency. The results from these ELISAs serve as a guide for further analysis if a deficiency is suspected (Table 1). In addition, deficiencies of some complement inhibitors (C1-inhibitor with corresponding C4 consumption, factor H or factor I with C3 consumption) result in secondary deficiency, which in some cases is revealed in these assays (Seelen et al., 2005).

2.1.3. Complement function in specific immune defense

The functional assays described above are developed to reveal deficiencies and are not necessarily optimal in the assessment of complement function as part of immune defense. Assays that use an antigen derived from a pathogen to initiate activation should in future be considered for assessing the activity of the complement system in human disease (Atkinson and Frank, 2006). With the introduction of more informative detection systems in routine diagnosis, the role of the various pathway(s) of complement activation in disease will be better defined allowing a better targeting of therapeutic agents.

2.2. Quantification of individual complement components

The standard immunochemical methods are valuable tools in diagnostic work and when necessary these are supplemented with functional assays. Correct interpretation requires validated reference intervals. 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 infants (Johnson et al., 1983; Roach et al., 1981; Sonntag et al., 1998).

2.2.1. Immunochemical assays

The concentration of many of the complement components can be measured by tests based on immune complex

formation, such as radial immunodiffusion, electroimmunoassay, nephelometry and turbidometry; for others Western blot, ELISA or time-resolved immunofluorometric assay (TRIFMA) (MollerKristensen et al., 2003) are necessary. The choice of specific antibodies is crucial in all immunoassays. Many of the assays currently used are based on polyclonal antibodies and measure different protein variants, irrespective of their functional activity. The commonly used methods for C3 and C4 not only measure the native proteins, but also their major soluble fragments C3c and C4c formed during activation. Thus, the results must be interpreted with this in mind. An advantage with such assays is that they are less sensitive to *in vitro* activation.

2.2.2. Functional assays of individual components

Functional activities of individual complement proteins need to be determined when a non-functional or dysfunctional variant is suspected. As mentioned before, *in vitro* activation due to improper handling has to be excluded (see Section 3.1.3). A simple way to detect the functional activity of a single complement component is to test the capacity of the sample to reconstitute the total complement activity of a serum that is deficient for a known component. This can be done either by haemolytic or ELISA assays (Sjoholm et al., 1991; Seelen et al., 2005). Special assays for MBL/MASPs functional activity have been described. One is based on the same principle as the activation ELISA but here C4b deposition is measured; serum samples are diluted in high salt buffers to block the classical pathway while permitting activation due to binding of the MBL/MASPs complex to mannan (Petersen et al., 2001). Alternatively, functional MBL levels can be assessed in serum based on the principle of yeast-induced bystander lysis of chicken erythrocytes (Kuipers et al., 2002).

In Type 1 HAE the C1-inhibitor concentration is low; however, normal or even increased C1-inhibitor concentration is common in Type 2 HAE, despite diminished function. Functional analysis of C1-inhibitor is thus mandatory for the diagnosis. Functional assay are commercially available and are typically based on the action of C1-inhibitor as an enzyme inhibitor and the use of chromogenic substrate (Munkvad et al., 1990). C4 concentration is consistently low in both Types 1 and 2 HAE and is usually measured as a supplementary test.

The assessment of factor H activity is of importance in diseases related to factor H dysfunction, i.e. atypical haemolytic uraemic syndrome (aHUS), membrano-proliferative glomerulonephritis (MPGN) type II, and age-related macular degeneration (AMD). A method for the determination of factor H-related regulation of complement activation has been described (Sanchez-Corral et al., 2004). Here, unsensitised sheep erythrocytes are incubated with serum; in normal serum, factor H binds the erythrocytes and protects from lysis, whereas sera lacking functional factor H cause erythrocyte lysis. For factor H in particular, but also for other regulators and components, genetic analyses are valuable in addition to protein and functional evaluation (see Sections 4.2.1 and 4.2.2).

2.2.3. The proteomic approach and future perspectives

Proteomic analysis of plasma and serum samples is now being used to identify biomarkers of human diseases. Antibody-based

microarrays have yielded promising results in multiplexed protein expression profiling (Wingren and Borrebaeck, 2006; Borrebaeck, 2006). It is now possible to design arrays covering virtually any specificity through generation of phage display libraries of human recombinant single-chain Fv antibody fragments (Soderlind et al., 2000). In such an array, numerous scFv antibodies specific for selected proteins are spotted on microarray slides. Serum or plasma samples are biotinylated, incubated on the arrays and dye-labeled streptavidin is added. Scanning of the microarrays can give a semi-quantitative measure of concentration. This technology has now been applied to simultaneously screen several complement proteins with promising results (Ingvarsson et al., submitted for publication). This approach offers considerable potential as an analytical tool in complement diagnostics and research. By selecting antibodies against different polymorphic complement protein variants and fragments it will be possible to measure not only complement protein concentrations but also activation and protein variants in samples of 1 μ l volume.

Another antibody-based proteomic approach involving a reverse array format has been described, where serum samples are spotted on a microarray slide (Janzi et al., 2005). A large number of samples can then be screened for their content of a certain serum protein in a single experiment using target-recognising antibodies and fluorochrome-labelled secondary antibodies. This procedure has been used for analysis of serum IgA and C3 (Janzi et al., 2005). The results suggest that it is possible to simultaneously screen thousands of complex clinical serum samples for specific serum proteins of clinical relevance.

3. Analysis of complement activation products

3.1. Analysis of activation in plasma

Reduced total haemolytic activity and levels of individual complement components either reflect a deficiency state, or indicate ongoing, consuming complement activation. Since most complement components are acute phase reactants and *in vivo* complement activation is often associated with an acute phase reaction, levels of individual components may stay within the normal range despite ongoing consumption. In general, total haemolytic activity and individual component measurements are useful as first level screening techniques but are not sufficiently sensitive to detect pathologically increased complement activation *in vivo*. The need to measure complement activation products in this context is illustrated in Table 2.

3.1.1. The neoepitope analysis principle

During the last two decades, highly specific monoclonal antibodies have been produced which recognise neoepitopes exclusively exposed upon activation-induced conformational changes in complement proteins (Mollnes and Harboe, 1993). This enables direct capture of the activation fragment without interference from non-activated components. Quantification can be achieved using traditional ELISA (Mollnes, 1997), by binding to and elution from high-capacity immunosorbents (Hartmann et al., 1993), or on microbeads in multiplex flow cytometric methods. Today, neoepitope based assays have replaced the older generation of tests for activation products. Furthermore, neoepitope-specific antibodies are particularly suitable to detect *in situ* complement activation in tissues since they discriminate between activation products and passively trapped native components.

3.1.2. Panel of assays

Complement activation products are either split fragments, generated after enzymatic cleavage of certain components, e.g. C4 (C4a, C4b/c, C4d), C3 (C3a, C3b/c, iC3b, C3d), factor B (Ba, Bb) and C5 (C5a), or protein complexes where activated components are bound to their respective regulators, like C1rs-C1-inhibitor, the properdin-containing alternative pathway convertase, C3bBbP, and SC5b-9 (soluble terminal complement complex). A number of assays, many of them neoepitope-based and commercially available, have been described for detection of activation at the various steps in the cascade. Thus, C1rs-C1-inhibitor (Fure et al., 1997) or C1q-C4 (Wouters et al., 2005) assays measure classical pathway activation, C3bBbP (Mollnes et al., 2002) assay reflects alternative pathway activation, and SC5b-9 assay shows activation of the terminal pathway to completion (Mollnes et al., 1985; Deppisch et al., 1990). Specific assays for activation products of the lectin pathway in the fluid-phase have not yet been described. However, lectin pathway activation should be suspected if C4 activation products are detected without evidence of classical pathway activation.

3.1.3. Collection of samples

Complement activation products are present only in trace amounts in normal plasma *in vivo* but they are rapidly generated *in vitro* (Mollnes et al., 1988). Therefore, it is crucial that samples are collected and stored properly in order to avoid *in vitro* activation. Blood must be drawn directly into tubes containing EDTA at a final concentration of at least 10 mM. The

Table 2
Plasma concentration of C3, C4 and complement activation products (C3dg and TCC) in two patients with liver disease

Gender	Age (years)	C3 (g/L)	C4 (g/L)	C3dg (AU ^a /mL)	TCC ^b (AU/mL)
Female ^c	24	0.20	0.09	25	4
Female ^d	35	0.30	0.06	126	16
Reference range		0.50–1.00	0.10–0.50	20–45	2.2–6.6

^a AU, arbitrary units.

^b TCC, terminal SC5b-9 complement complex.

^c Patient with terminal liver failure on the waiting list for transplantation. Low C3 and C4 due to decreased synthesis. Low concentration of activation products.

^d Patient with chronic active hepatitis without liver failure. Low C3 and C4 due to consumption with correspondingly increased activation products.

addition of nafamostat mesilate (Futhan, FUT-175) to EDTA further reduces *ex vivo* complement activation (Pfeifer et al., 1999). Citrate and heparin do not block complement activation efficiently and should be avoided. The sample must then be cooled, plasma separated promptly and stored at -70°C . Importantly, short-term storage of EDTA-blood at room temperature, which often cannot be avoided in the clinic, does not significantly alter C3a/C5a anaphylatoxin levels (Stove et al., 1995).

3.1.4. Biological considerations

The various complement activation products have different half-lives *in vivo*. This is important in deciding the parameter(s) to be measured. Due to rapid receptor binding, the biologically highly active and important C5a fragment has a half-life of approximately 1 min (Wagner and Hugli, 1984; 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 and are readily detectable (Teisner et al., 1983). The half-life of SC5b-9 is 50–60 min (Mollnes, 1985; Deppisch et al., 1990). SC5b-9 levels, in contrast to C3 activation products, are relatively stable *in vitro* and, since it reflects activation to the end of the terminal sequence irrespective of the initial pathway involved, it is a particularly good candidate for general evaluation of complement activation. When measuring an activation product from a single component (e.g. C3a or C3c), the amount should be related to the concentration of the native component, since a low level of native component would yield smaller amounts of activation products during *in vivo* activation. The ratio between activation product and native component is therefore a more sensitive indicator of *in vivo* activation than the product alone (Nurnberger and Bhakdi, 1984).

3.1.5. Measuring complement activation products in animals

Whereas a number of neoepitope specific assays have been described for human complement activation, only a few are available for animal studies. These are either based on human assay reagents that cross-react with other species, or on reagents specifically designed for animal studies. The latter include assays based on monoclonal antibodies to neoepitopes of guinea pig C5a (Link et al., 1999) and C3 activation product (Hawlich et al., 2000), rabbit C5a (Bergh and Iversen, 1989), rat TCC (Schulze et al., 1989), and mouse C3a and C3b/iC3b/C3c (Mastellos et al., 2004). A rat/mouse C3a-desArg ELISA is commercially available (Bachem, Bubendorf, Switzerland). Several of the human neoepitope-specific assays cross-react sufficiently well for use in other species, for example, in the baboon C3b/c (Hiramatsu et al., 1997), C5a and SC5b-9 (Mollnes et al., 1993), C4d, C3a, Bb and C5b-9 (Fung et al., 2001) can all be measured using human assays. Human SC5b-9 assays measure porcine SC5b-9 (Jansen et al., 1993). The species cross-reactivity of a particular assay made for human complement activation can easily be checked by activating the serum from the respective species using heat aggregated immunoglobulin (classical pathway) or zymosan (alternative and lectin pathway), and comparing with the non-activated control. If the signal in activated serum is stronger than in the non-activated, the assay cross-

reacts with this species and the degree of cross-reactivity can be estimated.

3.2. Analysis of activation on cells and in tissue

Detection of C4 and C3 activation products on red blood cells has proven useful in differential diagnosis of (auto)immune haemolytic anaemia (Garratty, 1984). A recent study (Navratil et al., 2006), suggests that platelet-bound C4d is a useful biomarker for SLE disease activity that may be linked to thrombotic and vascular complications. In comparison with plasma detection, immunohistochemical proof of complement activation better reflects pathophysiologic relevance, and has provided valuable insight into the roles of complement in renal disorders (Couser, 1993) and ischemia reperfusion injury (Arumugam et al., 2004). Endothelial C4d deposition in peritubular capillaries of renal grafts is a reliable indicator of antibody-mediated alloreactivity, predictive of impending organ rejection (Lederer et al., 2001; Vargha et al., 2006). Analysis of the binding of complement activation products on antigen microarrays, either directly or via antibody, opens new perspectives to understanding the complexity of the immune response (Papp et al., 2007).

4. Genetic analyses of complement

Genetic complement deficiencies are responsible for 1–6% of primary immunodeficiencies (Notarangelo et al., 2004). Due to lack of specialist diagnostic laboratories, this number is likely an underestimate. Sequencing of the genes coding for complement components has long been used to identify the mechanism behind an observed deficiency in activation of one or more pathways of complement. Lately, point mutations and polymorphic variants of certain complement components have also been found in diseases where complement activation itself is normal or only slightly impaired.

4.1. Genetic analyses in defective complement activation

Different types of complement deficiencies are associated with different diseases and clinical outcomes. When deficiency of one pathway is observed, it is usually enough to know which complement component is missing or non-functional (see Section 2.2). Genetic analyses are more expensive and harder to use for screening purposes, and therefore few complement component deficiencies are primarily analysed using genetic tools.

If a complement component is absent or non-functional, the next decision is whether or not to explore the genetic background of the defect. The advantages in revealing the mutation are verification of the hereditary nature of the defect, a deeper understanding of the characteristics of the deficiency for selecting the best treatment options, and enabling screening for carriers of the mutations in the family. The genetic approaches for different components are fairly similar and straightforward, and aim to find the mutation(s) in the gene encoding the defective protein. For mutation screening, each exon of the candidate gene is amplified from genomic DNA of the patient (and close rela-

tives) using specific primers derived from the 5' and 3' intronic sequences, and sequenced in both strands. Genetic analyses for deficiency of MBL and C4 are here reviewed as examples.

4.1.1. Genetic analysis of MBL deficiency

Deficiency of MBL is the most frequent genetic complement deficiency, varying between 5 and 20% of population depending on the region studied and the definition of the deficiency. The clinical relevance of lectin pathway dysfunction, i.e. MBL or MASP-2 deficiency, has not been generally accepted yet, but it seems that in the presence of other factors that impair immunity, these deficiencies increase susceptibility to certain infections, especially in children (Thiel et al., 2006). The background for the observed impaired activity is usually the presence of one or two alleles of the MBL-coding gene MBL2 that contain a mutation either in the promoter region (L/H or X/Y alleles) or in the exons (alleles B, C, or D instead of the normal allele A), as recently reviewed (Garred et al., 2006). The issue of MBL-deficiency is complex since alleles leading to a non-functional chain cause expression of MBL antigenic material in plasma. Therefore, from a clinical point of view genetic analyses are not required for diagnosis, but for research purposes and assessment of disease associations, they are necessary.

4.1.2. Genetic analysis of C4 genes

Less than 40 cases of total deficiency of C4 have been reported to date. C4 deficiency is strongly associated with SLE, and in some cases with glomerulonephritis and bacterial infections (Yang et al., 2004). There are usually two genes coding for C4, termed C4A and C4B, located in the class III MHC region on chromosome 6. C4 is the most polymorphic protein of the complement system, possibly due to the human endogenous retrovirus HERV-K(C4) located within the C4A gene. This is the reason why each individual has up to six alleles coding for C4. The proteins C4A and C4B coded by the corresponding genes have different biochemical properties in binding covalently to the target. Therefore, biochemical verification that C4 is present in plasma and functional *in vitro* fails to provide all the information about C4 function *in vivo*. Associations between partial C4 deficiency, i.e. either C4A or C4B deficiency, and a number of diseases have been searched for and several interesting associations found (Samano et al., 2004).

4.2. Genetic analyses when complement activity in plasma is normal

A number of diseases have been associated with mutations or variations of complement proteins, especially the regulators of complement activation that do not cause altered complement activity in plasma. Here we review genetic analyses in two different clinical situations, aHUS and AMD. In each, complement activation in plasma is usually not increased, while mutation or variation in one or more complement proteins is associated with abnormal complement activation in a particular body compartment.

4.2.1. aHUS

HUS is characterized by thrombocytopenia, Coomb's test negative microangiopathic haemolytic anaemia and acute renal failure. The typical form of the disease follows a diarrhoeal prodrome and is associated with O157:H7 *E.coli* infections. However, 5–10% of HUS patients lack an association with infection and have the poorest long-term prognosis. This disease is called atypical HUS (aHUS). The disease involves alternative pathway dysregulation and likely develops as a consequence of defective protection of cellular surfaces from complement activation due to an improper function of complement regulatory proteins. Multiple hits, involving plasma and membrane-associated complement regulatory proteins, as well as complement activators, are likely required to cause dysregulation and impair protection to host tissues. Pathogenesis of aHUS is discussed in more detail elsewhere (Jokiranta et al., this issue).

aHUS is associated with mutations or polymorphisms in the genes encoding the complement regulatory proteins factor H (Warwicker et al., 1998; Perez-Caballero et al., 2001; Caprioli et al., 2001; Richards et al., 2001; Sanchez-Corral et al., 2004; Manuelian et al., 2003; Caprioli et al., 2003; Venables et al., 2006), membrane cofactor protein (MCP, CD46) (Noris et al., 2003; Richards et al., 2003; Esparza-Gordillo et al., 2005; Fremaux-Bacchi et al., 2005) and factor I (Fremaux-Bacchi et al., 2004; Kavanagh et al., 2005). More recently, aHUS has also been linked with mutations in factor B (Goicoechea de Jorge et al., 2007). Mutation screening in these genes is done by exon amplification from genomic DNA and sequencing both strands of the generated amplicons. Rearrangements in the factor H gene region, involving deletion of the *FHR1* and *FHR3* genes and generation of hybrid *factor H:FHR1* genes have been described in aHUS. These are revealed by multiple ligation probe amplification (MLPA) methodologies (Venables et al., 2006).

Miss-sense mutations in the carboxy-terminal region of factor H are the most prevalent genetic alteration among aHUS patients (15–20%), followed by mutations in MCP (10–15%). Factor I mutations are found in 5% of aHUS cases and *factor H:FHR1* hybrid genes in 2–4%. In addition, two common haplotypes in factor H and MCP confer significant risk of aHUS (Caprioli et al., 2003; Esparza-Gordillo et al., 2005). An updated record of all aHUS mutations can be found at the FH-HUS Database (<http://www.fh-hus.org/>). The genetic analyses described here identify the molecular defect in approximately 50% of aHUS patients. Research in this area is progressing very fast and it is expected that additional genetic factors conferring aHUS susceptibility will be added to the candidate gene list.

In family studies with multiple carriers of aHUS-associated mutations and low penetrance of the disease among mutation carriers, concurrence of mutations in different complement regulatory and activating genes should be considered (Esparza-Gordillo et al., 2005; Esparza-Gordillo et al., 2006; Goicoechea de Jorge et al., 2007). Mutation screening to identify patients carrying mutations in MCP is useful, as these have a better renal transplant outcome (Richards et al., 2007). If no mutations in complement genes are found, serum from the aHUS patient should be tested in ELISA for the presence of anti-factor

H antibodies (Dragon-Durey et al., 2005; Zipfel and Skerka, 2006).

4.2.2. AMD

AMD is the most common cause of blindness in the elderly in developed countries. The hallmark of early-stage disease is drusen, lipoproteinaceous deposits localised between the retinal pigment epithelium and Bruch's membrane. Later, an extensive atrophy of the epithelium and overlying photoreceptor cells (geographic atrophy) or aberrant choroidal angiogenesis is observed; the latter, affecting the macular area, causes blindness.

AMD is a multifactorial disease, influenced by age, ethnicity and a combination of environmental and genetic risk factors. Genetic predisposition has been suggested based on familial segregation and twin studies, involving several candidate genes such as ABCA4, APOE, FBLN5, ELOVL4, and TLR4. However, the individual contribution of these genes to overall AMD prevalence appears relatively minor. Two major AMD susceptibility loci (1q31 and 10q26) have been recently identified by candidate region linkage studies (Majewski et al., 2003). The most studied single nucleotide polymorphism at the 1q31 locus is rs1061170 which causes a Tyr402His amino acid substitution in complement factor H, increasing the risk for AMD with an odds ratio between 2.1 and 7.4 (Edwards et al., 2005; Haines et al., 2005; Hageman et al., 2005; Klein et al., 2005). Interestingly, a number of other complement polymorphisms have been found to protect from AMD. These include common haplotypes in the factor H gene (Maller et al., 2006), the factor B gene and the C2 gene (Gold et al., 2006), and the deletion of the FHR1 and FHR3 genes within the RCA gene cluster (Hughes et al., 2006). It is expected that a precise definition of the AMD risk (or protective) factors associated with the factor H, factor B and FHR1/FHR3 genes will provide insights into the pathogenesis of AMD and that genotyping will help to delineate the individual risk to develop AMD.

4.3. Genetic analysis in HAE

HAE is caused by mutations leading to lack of C1-inhibitor activity but clinically it is indistinguishable from acquired angioedema (AAE) (see Section 5.2.3). The first tests for diagnosing HAE are antigenic and functional assays of C1-inhibitor (see Section 2.2.2). Genetic analysis is indicated in those patients who have no family history of angioedema or whose C1q concentration is borderline. Most HAE-associated mutations are in exon VIII of the C1-inhibitor gene which codes the reactive center. An updated record of mutations found in HAE can be found at the C1-inhibitor gene mutation database (<http://hae.enzim.hu/index.php>). Differentiation between HAE and AAE is important not only for academic reasons but also to guide treatment (Weiler and van Dellen, 2006).

5. Clinical indications for complement analysis

Clinical indications for complement analyses can be divided into two major categories: autoimmune immune complex disease and complement deficiencies.

5.1. Autoimmune diseases

5.1.1. Autoimmune immune complex diseases

SLE, the phospholipid syndrome and leukocytoclastic vasculitides are immune complex diseases often of autoimmune origin. The latter group includes cryoglobulinemia types I–III, and systemic forms of rheumatoid arthritis and other connective tissue diseases (Crowson et al., 2003; Gertz, 2005). Complement analyses can be used both for differential diagnosis and to follow the disease activity, in particular in patients with renal involvement (Ekdahl et al., 2007; Sturfelt and Sjöholm, 1984). This group of diseases is accompanied by *in vivo* classical pathway activation leading to consumption of components. Activation reflects the activity of the disease and often precedes clinical exacerbation. The commonly used combination of C3 and C4 concentrations should be interpreted with caution since both sensitivity and specificity is low (Ekdahl et al., 2007). In order to determine the complement status of the patient when admitted for the first time, a more thorough examination is useful. A functional assay, either haemolytic (CH50) or ELISA based (Seelen et al., 2005), combined with assays to determine the concentrations of C1q, C4, C3 and one or more complement activation products, e.g. C3dg or SC5b-9, is recommended. For long-term monitoring of patients, a single test can be used, either a functional test or measurement of C1q, C4 or C3dg. Measurement of C3dg should always be accompanied by determination of the C3 concentration so that the ratio C3dg/C3 can be calculated (see Table 2) (Nurnberger and Bhakdi, 1984). Cryoglobulins activate the classical pathway *in vivo*, but activation is often amplified *in vitro* due to presence of the cryoglobulin and sub-optimal handling of the sample (Pascual et al., 1997). This *in vitro* activation causes a very low classical pathway function in serum, without the corresponding consumption of classical pathway components as determined by immunochemical assays. The combination may be misdiagnosed as a complement component deficiency. Identification of cryoglobulins will resolve the problem.

5.1.2. SLE

SLE is the archetypal autoimmune immune complex disease. Most organ systems are involved and the syndrome is defined by set criteria (Hay, 1995). In SLE patients, either the C1q concentration or haemolytic CH50 assays can be used both for diagnosis and long-term monitoring (Sturfelt and Sjöholm, 1984; Nilsson and Nilsson, 1984). Anti-C1q antibodies in plasma are common in SLE, often associated with kidney involvement (Trendelenburg, 2005). The lupus band test performed in skin biopsies showing granular deposits of immunoglobulins and complement along the basal membrane (Fig. 2A) may be a useful supplement to other laboratory tests (Provost and Reichlin, 1988; Zecevic et al., 2006). Kidney biopsies may also help in mapping the distribution of disease in SLE patients (West, 1989).

5.1.3. The anti-phospholipid syndrome

The anti-phospholipid syndrome is characterized by thrombosis, thrombocytopenia and recurrent spontaneous abortions.

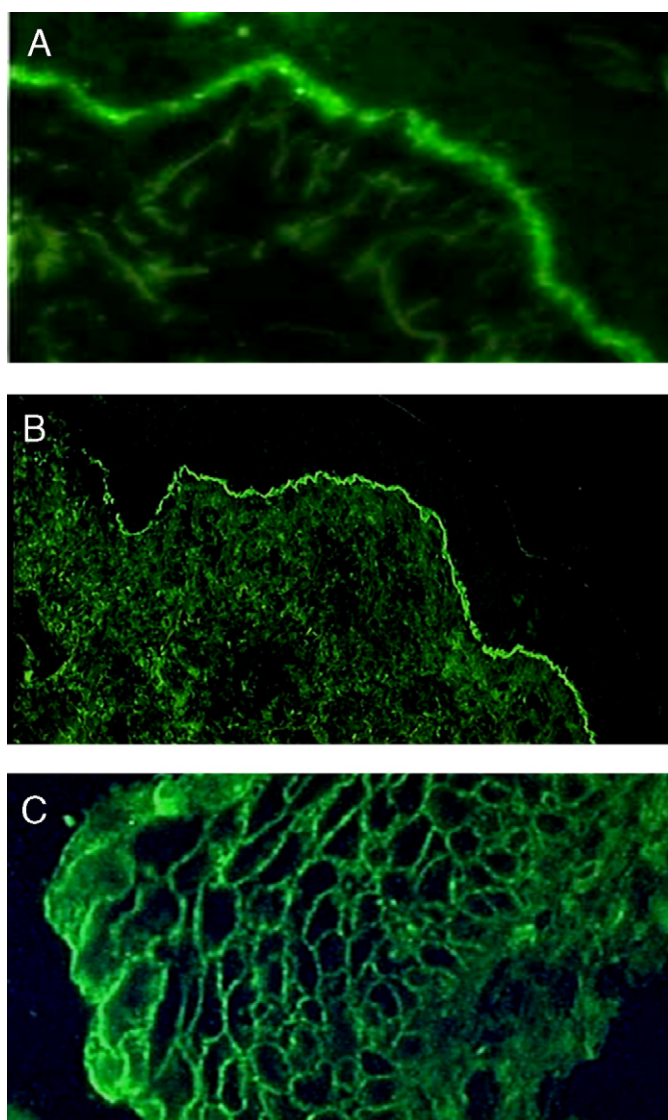


Fig. 2. Immunofluorescence staining showing immunoglobulins and complement deposits in skin biopsies. (A) Granular deposition reflecting immune complexes along the basal membrane in systemic lupus erythematosus (“lupus band”), (B) linear staining along the basal membrane in pemphigoid, (C) intracellular staining in epidermis in pemphigus vulgaris. We acknowledge Dr. Johan Rönnelid, Clinical Immunology, Uppsala University, Sweden, for providing the immunofluorescence pictures.

The syndrome may either appear alone (primary) or secondary to SLE. It is caused by autoantibodies to β 2-glycoprotein 1 (B2GP1) or other coagulation factors such as prothrombin (Pengo et al., 2006). The primary antigen B2GP1 binds to phospholipids on platelets and endothelial cells, and immune complexes are formed when autoantibodies bind. These soluble and membrane bound immune complexes activate complement through the classical pathway (Bever et al., 2004; Odorcuk et al., 1999; Ramos-Casals et al., 2004). It has become evident that complement is an important pathogenic factor in the recurrent spontaneous abortions seen in this condition (Salmon and Girardi, 2004).

5.1.4. Leukocytoclastic vasculitis

This group of diseases present with skin lesions similar to those found in allergic urticaria. In addition to urticarial vasculitis, cryoglobulinemia types I–III, systemic forms of rheumatoid arthritis, and other connective tissue diseases also present with leukocytoclastic vasculitis (Crowson et al., 2003; Gertz, 2005). Diagnosis is by immunochemical detection of immunoglobulins (IgG, IgM, IgA) and complement in biopsies from affected skin. Complement is activated via the classical pathway. In the more serious hypocomplementaemic urticarial vasculitis syndrome, vasculitis is combined with other systemic manifestations, e.g. pulmonary fibrosis and glomerulonephritis, and complement consumption is pronounced and combined with appearance of anti-C1q antibodies (Davis and Brewer, 2004). These antibodies are distinct from those in SLE since they recognize reduced and denatured C1q by Western blotting (Martensson et al., 1992).

5.2. Complement component deficiencies

Complement component deficiencies are associated with an increased risk of infections and, when affecting the classical pathway, autoimmune immune complex disease. Functional assays can be used to screen patients for homozygous complement deficiencies (see Section 2.1). The location of the deficient component in the complement sequence determines the type of infections that the individual is prone to. Lack of membrane complement receptors and regulators lead to other types of problems, often associated with uncontrolled complement activation.

5.2.1. Deficiencies of classical, alternative and terminal pathways

Classical pathway deficiencies predispose to SLE. C1q deficient individuals invariably develop classical SLE. In contrast, SLE is rare in C2 deficient individuals and not associated with C3 deficiency (Sjoholm et al., 2006). Deficiencies of C3 and alternative pathway components increase the risk of invasive bacterial infections with encapsulated bacteria such as *Pneumococcus*, *Streptococcus* or *Hemophilus*, which might also affect persons with classical pathway component deficiencies (Figueroa and Densen, 1991). Deficiencies of terminal pathway components are associated with systemic neisserial infection, typically recurrent, relatively low grade, with rare serotypes. Properdin deficiencies are also associated with neisserial infections.

5.2.2. Lectin pathway deficiencies

Deficiency of mannose binding lectin (MBL) has been associated with microbial infections in childhood (typically in the 6–18 month “susceptibility window”), and in adults, secondary to other immune deficiencies such as immunosuppression, AIDS and certain autoimmune diseases (Turner, 2003; Bouwman et al., 2006). MBL deficiency is common and most deficient individuals do not suffer from increased susceptibility to infection. Whether deficiencies of other components of the lectin pathway, like MASP-2 or ficolins, predispose to infections is not known.

5.2.3. C1-inhibitor deficiency

C1-inhibitor deficiency is found with a frequency of approximately 2/100,000. It causes HAE, characterized by recurrent edema which may lead to death from suffocation if the larynx is involved. Edema is commonly located in the head and neck region, lasts for 2–5 days and does not respond to anti-allergic treatment. HAE presents in either of two forms: Type 1 has reduced concentration of C1-inhibitor, whereas in Type 2 there is a dysfunctional protein with normal or even increased plasma concentration. For the diagnosis it is therefore important to include both protein quantification and a functional assay for C1-inhibitor. The concentration of C4 is constantly low in both types. An acquired form of the disease (AAE) is frequently caused by autoantibodies to C1-inhibitor and an accompanying haematologic malignancy. In contrast to HAE, AAE is associated with a low concentration of C1q. The symptoms in HAE are caused by a failure of C1-inhibitor to control the contact activation system, leading to an increase in bradykinin, which causes the capillary leakage. Thus, the disease is not a true “complement disease”, but the diagnosis is usually performed in complement laboratories. It is crucially important to diagnose HAE patients since it is a potentially life-threatening condition and efficient treatment is available (see Cicardi et al., this issue).

5.2.4. Paroxysmal nocturnal hemoglobinuria

Paroxysmal nocturnal hemoglobinuria (PNH) is rare disease caused by a clonal somatic mutation affecting hematopoietic stem cells (Luzzatto and Gianfaldoni, 2006). The mutation affects the PIG-A gene which codes for the phosphoinositol glycosyltransferase that couples the first inositol to the phosphatidylinositol anchor that links numerous membrane proteins to the cell surface (Brodsky and Hu, 2006). Two of these proteins, DAF (CD55) and CD59, are important complement regulators and deficiency causes spontaneous haemolytic attacks, thrombocytopenia and platelet activation leading to thrombosis. Several diagnostic tools are available, but flow cytometry to demonstrate the absence of CD55 and CD59 on haematopoietic cells such as erythrocytes is currently the method of choice. Recently, a novel therapeutic treatment was introduced for these patients using an anti-C5 monoclonal antibody which blocks formation of C5b-9 and therefore protects the red cells against complement mediated lysis (Hillmen et al., 2004).

5.2.5. aHUS

aHUS is a disease associated with microangiopathic haemolytic anemia, thrombocytopenia and acute renal failure, most probably due to an inefficient regulation of complement at the surface of the endothelial cell. The disease is frequently associated with genetic variants of factor H and several other complement components and regulatory proteins (see Section 4.2.1 and a separate article by Jokiranta et al. (this issue)).

5.3. Other complement-associated conditions

5.3.1. Membranoproliferative glomerulonephritis

Membranoproliferative glomerulonephritis (MPGN) type II and III may be associated with C3 nephritic factors (C3Nef),

autoantibodies directed against a complement convertase. In MPGN type II the antibody is directed against the alternative pathway C3 convertase and prolongs the half-life of the convertase leading to uncontrolled complement activation (West, 1994). In type III MPGN, the stabilized convertase also cleaves C5 (West, 1994). C4Nef (Seino et al., 1990), directed against the classical pathway C3 convertase, has also been described in MPGN.

5.3.2. Post-streptococcal glomerulonephritis

In children suffering from streptococcal glomerulonephritis C3 may be consumed in the rehabilitation period up to 6 months after the infection (West, 1989). Complement levels may be very low and C3dg levels are elevated relative to the C3 concentration. In contrast to C3Nef, post-streptococcal glomerulonephritis is associated with a concomitant consumption of properdin.

5.3.3. Pemphigoid and pemphigus vulgaris

These conditions are blistering diseases affecting the skin and mucous membranes caused by autoantibodies directed against desmosomes and hemidesmosomes of the epidermis (Liu and Diaz, 2001). The pemphigoid antibodies activate complement leading to C5a generation which attracts leukocytes to the basal epidermis where inflammation causes the cells to disconnect from the basal membrane. In pemphigus vulgaris complement is also activated by autoantibodies primarily directed against desmosome but also against non-desmosome autoantigens between the keratinocytes of epidermis (Kurzen and Brenner, 2006). The inflammation caused by these antibodies leads to detachment of cells from each other creating ultrathin blisters and large wounds. Both of these disorders are diagnosed by demonstrating IgG and C3 deposition in skin (Black et al., 1989), in pemphigoid along the basal membrane (Fig. 2B) and in pemphigus vulgaris intercellularly in the epidermis (Fig. 2C).

6. Conclusion

Complement analysis in the clinic and in research is a rapidly expanding field with substantial challenges for the complement society. Highly specialized analyses developed in different laboratories enable unique possibilities for inter-group collaboration to improve diagnostics and move research forward. On the other hand, simple and reliable screening tests for complement activity makes primary diagnosis of complement deficiency available close to the patient, avoiding storage and transportation of the samples, which is critical for reliable results. However, there is no consensus on criteria for analysing complement in disease and standardization and quality control of assays is to a large extent missing. We suggest that reference laboratories for different complement tests are established around Europe, located at places with high competence and experience in the different methodological fields of complement analysis. Future perspectives include proteomics and genetics to further explore the role of complement in disease, as well as establishing methods to evaluate the effect of complement therapeutics in patients with complement related diseases (Mollnes and Kirschfink, 2006).

References

- Arumugam, T.V., Shiels, I.A., Woodruff, T.M., Granger, D.N., Taylor, S.M., 2004. The role of the complement system in ischemia-reperfusion injury. *Shock* 21, 401–409.
- Atkinson, J.P., Frank, M.M., 2006. Bypassing complement: evolutionary lessons and future implications. *J. Clin. Invest.* 116, 1215–1218.
- Bergh, K., Iversen, O.J., 1989. Measurement of complement activation in rabbit plasma or serum using monoclonal antibodies against C5a. *Scand. J. Immunol.* 29, 333–341.
- Beyers, E.M., Zwaal, R.F., Willems, G.M., 2004. The effect of phospholipids on the formation of immune complexes between autoantibodies and beta-2-glycoprotein I or prothrombin. *Clin. Immunol.* 112, 150–160.
- Black, M.M., Bhogal, B.S., Willsted, E., 1989. Immunopathological techniques in the diagnosis of bullous disorders. *Acta Derm. Venereol. Suppl. (Stockh)* 151, 96–105.
- Borrebaeck, C.A., 2006. Antibody microarray-based oncoproteomics. *Expert Opin. Biol. Ther.* 6, 833–838.
- Bouwman, L.H., Roep, B.O., Roos, A., 2006. Mannose-binding lectin: clinical implications for infection, transplantation, and autoimmunity. *Human Immunol.* 67, 247–256.
- Brodsky, R.A., Hu, R., 2006. PIG-A mutations in paroxysmal nocturnal hemoglobinuria and in normal hematopoiesis. *Leuk. Lymphoma* 47, 1215–1221.
- Caprioli, J., Bettinaglio, P., Zipfel, P.F., Amadei, B., Daina, E., Gamba, S., Skerka, C., Marziliano, N., Remuzzi, G., Noris, M., 2001. The molecular basis of familial hemolytic uremic syndrome: mutation analysis of factor H gene reveals a hot spot in short consensus repeat 20. *J. Am. Soc. Nephrol.* 12, 297–307.
- Caprioli, J., Castelletti, F., Bucchioni, S., Bettinaglio, P., Bresin, E., Pianetti, G., Gamba, S., Brioschi, S., Daina, E., Remuzzi, G., Noris, M., 2003. Complement factor H mutations and gene polymorphisms in haemolytic uraemic syndrome: the C-257T, the A2089G and the G2881T polymorphisms are strongly associated with the disease. *Hum. Mol. Genet.* 12, 3385–3395.
- Couser, W.G., 1993. Pathogenesis of glomerulonephritis. *Kidney Int.* 44, S19–S26.
- Crowson, A.N., Mihm Jr., M.C., Magro, C.M., 2003. Cutaneous vasculitis: a review. *J. Cutan. Pathol.* 30, 161–173.
- Davis, M.D., Brewer, J.D., 2004. Urticarial vasculitis and hypocomplementemic urticarial vasculitis syndrome. *Immunol. Allergy Clin. North Am.* 24, 183–213, vi.
- Deppisch, R., Schmitt, V., Bommer, J., Hansch, G.M., Ritz, E., Rauterberg, E.W., 1990. Fluid phase generation of terminal complement complex as a novel index of bioincompatibility. *Kidney Int.* 37, 696–706.
- Dragon-Durey, M.A., Loirat, C., Cloarec, S., Macher, M.A., Blouin, J., Nivet, H., Weiss, L., Fridman, W.H., Fremeaux-Bacchi, V., 2005. Anti-factor H autoantibodies associated with atypical hemolytic uremic syndrome. *J. Am. Soc. Nephrol.* 16, 555–563.
- Edwards, A.O., Ritter III, R., Abel, K.J., Manning, A., Panhuysen, C., Farrer, L.A., 2005. Complement factor H polymorphism and age-related macular degeneration. *Science* 308, 421–424.
- Ekdahl, K.N., Norberg, D., Bengtsson, A.A., Sturfelt, G., Nilsson, U.R., Nilsson, B., 2007. A rapid, inexpensive, and easy-to-perform hemolytic complement assay using serum or buffer-changed EDTA-Plasma: its use for differential diagnosis and follow-up of patients with systemic lupus erythematosus. *Clin. Vaccine Immunol.* 14, 549–555.
- Esparza-Gordillo, J., Goicoechea de Jorge, E., Buil, A., Carreras, B.L., Lopez-Trascasa, M., Sanchez-Corral, P., Rodriguez de Cordoba, S., 2005. Predisposition to atypical hemolytic uremic syndrome involves the concurrence of different susceptibility alleles in the regulators of complement activation gene cluster in 1q32. *Hum. Mol. Genet.* 14, 703–712.
- Esparza-Gordillo, J., Goicoechea de Jorge, E., Garrido, C.A., Carreras, L., Lopez-Trascasa, M., Sanchez-Corral, P., Rodriguez de Cordoba, S., 2006. Insights into hemolytic uremic syndrome: segregation of three independent predisposition factors in a large, multiple affected pedigree. *Mol. Immunol.* 43, 1769–1775.
- Figuerola, J.E., Densen, P., 1991. Infectious diseases associated with complement deficiencies. *Clin. Microbiol. Rev.* 4, 359–395.
- Fredrikson, G.N., Truedsson, L., Sjoholm, A.G., 1993. New procedure for the detection of complement deficiency by ELISA—analysis of activation pathways and circumvention of rheumatoid factor influence. *J. Immunol. Methods* 166, 263–270.
- Fremeaux-Bacchi, V., Dragon-Durey, M.A., Blouin, J., Vigneau, C., Kuypers, D., Boudailliez, B., Loirat, C., Rondeau, E., Fridman, W.H., 2004. Complement factor I: a susceptibility gene for atypical haemolytic uraemic syndrome. *J. Med. Genet.* 41, E84.
- Fremeaux-Bacchi, V., Kemp, E.J., Goodship, J.A., Dragon-Durey, M.A., Strain, L., Loirat, C., Deng, H.W., Goodship, T.H., 2005. The development of atypical haemolytic-uraemic syndrome is influenced by susceptibility factors in factor H and membrane cofactor protein: evidence from two independent cohorts. *J. Med. Genet.* 42, 852–856.
- Fung, M., Loubser, P.G., Undar, A., Mueller, M., Sun, C., Sun, W.N., Vaughn, W.K., Fraser Jr., C.D., 2001. Inhibition of complement, neutrophil, and platelet activation by an anti-factor D monoclonal antibody in simulated cardiopulmonary bypass circuits. *J. Thorac. Cardiovasc. Surg.* 122, 113–122.
- Fure, H., Nielsen, E.W., Hack, C.E., Mollnes, T.E., 1997. A neoepitope-based enzyme immunoassay for quantification of C1-inhibitor in complex with C1r and C1s. *Scand. J. Immunol.* 46, 553–557.
- Garratty, G., 1984. The significance of complement in immunohematology. *Crit. Rev. Clin. Lab. Sci.* 20, 25–56.
- Garred, P., Larsen, F., Seyfarth, J., Fujita, R., Madsen, H.O., 2006. Mannose-binding lectin and its genetic variants. *Genes Immun.* 7, 85–94.
- Gertz, M.A., 2005. Cold agglutinin disease and cryoglobulinemia. *Clin. Lymphoma* 5, 290–293.
- Goicoechea de Jorge, E., Harris, C.L., Esparza-Gordillo, J., Carreras, L., Arranz, E.A., Garrido, C.A., Lopez-Trascasa, M., Sanchez-Corral, P., Morgan, B.P., Rodriguez de Cordoba, S., 2007. Gain-of-function mutations in complement factor B are associated with atypical hemolytic uremic syndrome. *Proc. Natl. Acad. Sci. U.S.A.* 104, 240–245.
- Gold, B., Merriam, J.E., Zernant, J., Hancox, L.S., Taiber, A.J., Gehrs, K., Cramer, K., Neel, J., Bergeron, J., Barile, G.R., Smith, R.T., Hageman, G.S., Dean, M., Allikmets, R., Chang, S., Yannuzzi, L.A., Merriam, J.C., Barbazetto, I., Lerner, L.E., Russell, S., Hoballah, J., Hageman, J., Stockman, H., 2006. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. *Nat. Genet.* 38, 458–462.
- Hageman, G.S., Anderson, D.H., Johnson, L.V., Hancox, L.S., Taiber, A.J., Hardisty, L.L., Hageman, J.L., Stockman, H.A., Borchardt, J.D., Gehrs, K.M., Smith, R.J., Silvestri, G., Russell, S.R., Klaver, C.C., Barbazetto, I., Chang, S., Yannuzzi, L.A., Barile, G.R., Merriam, J.C., Smith, R.T., Olsh, A.K., Bergeron, J., Zernant, J., Merriam, J.E., Gold, B., Dean, M., Allikmets, R., 2005. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. *Proc. Natl. Acad. Sci. U.S.A.* 102, 7227–7232.
- Haines, J.L., Hauser, M.A., Schmidt, S., Scott, W.K., Olson, L.M., Gallins, P., Spencer, K.L., Kwan, S.Y., Noureddine, M., Gilbert, J.R., Schetz-Boutaud, N., Agarwal, A., Postel, E.A., Pericak-Vance, M.A., 2005. Complement factor H variant increases the risk of age-related macular degeneration. *Science* 308, 419–421.
- Hartmann, H., Lubbers, B., Casaretto, M., Bautsch, W., Klos, A., Kohl, J., 1993. Rapid quantification of C3A and C5A using a combination of chromatographic and immunoassay procedures. *J. Immunol. Methods* 166, 35–44.
- Hawlich, H., Vilsendorf, M.A.Z., Bautsch, W., Klos, A., Kohl, J., 2000. Guinea pig C3 specific rabbit single chain Fv antibodies from bone marrow, spleen and blood derived phage libraries. *J. Immunol. Methods* 236, 117–131.
- Hay, E.M., 1995. Systemic lupus erythematosus. *Baillieres Clin. Rheumatol.* 9, 437–470.
- Hillmen, P., Hall, C., Marsh, J.C., Elebute, M., Bombara, M.P., Petro, B.E., Cullen, M.J., Richards, S.J., Rollins, S.A., Mojcik, C.F., Rother, R.P., 2004. Effect of eculizumab on hemolysis and transfusion requirements in patients with paroxysmal nocturnal hemoglobinuria. *N. Engl. J. Med.* 350, 552–559.
- Hiramatsu, Y., Gikakis, N., Gorman III, J.H., Khan, M.M., Hack, C.E., Velthuis, H.T., Sun, L., Marcinkiewicz, C., Rao, A.K., Niewiarowski, S., Colman, R.W., Edmunds Jr., L.H., Anderson III, H.L., 1997. A baboon model for hematologic studies of cardiopulmonary bypass. *J. Lab. Clin. Med.* 130, 412–420.

- Hughes, A.E., Orr, N., Esfandiary, H., az-Torres, M., Goodship, T., Chakravarthy, U., 2006. A common CFH haplotype, with deletion of CFHR1 and CFHR3, is associated with lower risk of age-related macular degeneration. *Nat. Genet.* 38, 1173–1177.
- Jansen, J.H., Hogasen, K., Mollnes, T.E., 1993. Extensive complement activation in hereditary porcine membranoproliferative glomerulonephritis type-II (porcine dense deposit disease). *Am. J. Pathol.* 143, 1356–1365.
- Janzi, M., Odling, J., Pan-Hammarstrom, Q., Sundberg, M., Lundeberg, J., Uhlen, M., Hammarstrom, L., Nilsson, P., 2005. Serum microarrays for large scale screening of protein levels. *Mol. Cell Proteomics* 4, 1942–1947.
- Johnson, U., Truedsson, L., Gustavii, B., 1983. Complement components in 100 newborns and their mothers determined by electroimmunoassay. *APMIS* 91, 147–150.
- Kavanagh, D., Kemp, E.J., Mayland, E., Winney, R.J., Duffield, J.S., Warwick, G., Richards, A., Ward, R., Goodship, J.A., Goodship, T.H., 2005. Mutations in complement factor I predispose to development of atypical hemolytic uremic syndrome. *J. Am. Soc. Nephrol.* 16, 2150–2155.
- Klein, R.J., Zeiss, C., Chew, E.Y., Tsai, J.Y., Sackler, R.S., Haynes, C., Henning, A.K., Sangiovanni, J.P., Mane, S.M., Mayne, S.T., Bracken, M.B., Ferris, F.L., Ott, J., Barnstable, C., Hoh, J., 2005. Complement factor H polymorphism in age-related macular degeneration. *Science* 308, 385–389.
- Kuipers, S., Aerts, P.C., Sjöholm, A.G., Harmsen, T., Vandijk, H., 2002. A hemolytic assay for the estimation of functional mannose-binding lectin levels in human serum. *J. Immunol. Methods* 268, 149–157.
- Kurzen, H., Brenner, S., 2006. Significance of autoimmunity to non-desmoglein targets in pemphigus. *Autoimmunity* 39, 549–556.
- Lederer, S.R., Kluth-Pepper, B., Schneeberger, H., Albert, E., Land, W., Feucht, H.E., 2001. Impact of humoral alloreactivity early after transplantation on the long-term survival of renal allografts. *Kidney Int.* 59, 334–341.
- Link, C., Hawlisch, H., Vilsendorf, A.M.Z., Gyleruz, S., Nagel, E., Kohl, J., 1999. Selection of phage-displayed anti-guinea pig C5 or C5a antibodies and their application in xenotransplantation. *Mol. Immunol.* 36, 1235–1247.
- Liu, Z., Diaz, L.A., 2001. Bullous pemphigoid: end of the century overview. *J. Dermatol.* 28, 647–650.
- Luzzatto, L., Gianfaldoni, G., 2006. Recent advances in biological and clinical aspects of paroxysmal nocturnal hemoglobinuria. *Int. J. Hematol.* 84, 104–112.
- Majewski, J., Schultz, D.W., Weleber, R.G., Schain, M.B., Edwards, A.O., Matisse, T.C., Acott, T.S., Ott, J., Klein, M.L., 2003. Age-related macular degeneration—a genome scan in extended families. *Am. J. Hum. Genet.* 73, 540–550.
- Maller, J., George, S., Purcell, S., Fagerness, J., Altshuler, D., Daly, M.J., Seddon, J.M., 2006. Common variation in three genes, including a noncoding variant in CFH, strongly influences risk of age-related macular degeneration. *Nat. Genet.* 38, 1055–1059.
- Manuelian, T., Hellwege, J., Meri, S., Caprioli, J., Noris, M., Heinen, S., Jozsi, M., Neumann, H.P.H., Remuzzi, G., Zipfel, P.F., 2003. Mutations in factor H reduce binding affinity to C3b and heparin and surface attachment to endothelial cells in hemolytic uremic syndrome. *J. Clin. Invest.* 111, 1181–1190.
- Martensson, U., Sjöholm, A.G., Sturfelt, G., Truedsson, L., Laurell, A.B., 1992. Western blot analysis of human IgG reactive with the collagenous portion of C1q: evidence of distinct binding specificities. *Scand. J. Immunol.* 35, 735–744.
- Mastellos, D., Prechl, J., Laszlo, G., Papp, K., Olah, E., Argyropoulos, E., Franchini, S., Tudoran, R., Markiewski, M., Lambris, J.D., Erdei, A., 2004. Novel monoclonal antibodies against mouse C3 interfering with complement activation: description of fine specificity and applications to various immunoassays. *Mol. Immunol.* 40, 1213–1221.
- Moller-Kristensen, M., Jensenius, J.C., Jensen, L., Thielens, N., Rossi, V., Arlaud, G., Thiel, S., 2003. Levels of mannan-binding lectin-associated serine protease-2 in healthy individuals. *J. Immunol. Methods* 282, 159–167.
- Mollnes, T.E., 1985. Early- and late-phase activation of complement evaluated by plasma levels of C3d, g and the terminal complement complex. *Complement* 2, 156–164.
- Mollnes, T.E., 1997. Analysis of in vivo complement activation. In: Herzenberg, L.A., Weir, D.M., Herzenberg, L., Blackwell, C. (Eds.), *Weir's Handbook of Experimental Immunology*. Blackwell Science, Boston, pp. 78.1–78.8.
- Mollnes, T.E., Harboe, M., 1993. Neoepitope expression during complement activation—a model for detecting antigenic changes in proteins and activation of cascades. *Immunologist* 1, 43–49.
- Mollnes, T.E., Kirschfink, M., 2006. Strategies of therapeutic complement inhibition. *Mol. Immunol.* 43, 107–121.
- Mollnes, T.E., Lea, T., Frøland, S.S., Harboe, M., 1985. Quantification of the terminal complement complex in human plasma by an enzyme-linked immunosorbent assay based on monoclonal antibodies against a neoantigen of the complex. *Scand. J. Immunol.* 22, 197–202.
- Mollnes, T.E., Garred, P., Bergseth, G., 1988. Effect of time, temperature and anticoagulants on in vitro complement activation: consequences for collection and preservation of samples to be examined for complement activation. *Clin. Exp. Immunol.* 73, 484–488.
- Mollnes, T.E., Redl, H., Hogasen, K., Bengtsson, A., Garred, P., Speilberg, L., Lea, T., Oppermann, M., Gotze, O., Schlag, G., 1993. Complement activation in septic baboons detected by neoepitope-specific assays for C3b/iC3b/C3c, C5a and the terminal C5b-9 complement complex (TCC). *Clin. Exp. Immunol.* 91, 295–300.
- Mollnes, T.E., Brekke, O.L., Fung, M., Fure, H., Christiansen, D., Bergseth, G., Videm, V., Lappégard, K.T., Kohl, J., Lambris, J.D., 2002. Essential role of the C5a receptor in *E. coli*-induced oxidative burst and phagocytosis revealed by a novel lepirudin-based human whole blood model of inflammation. *Blood* 100, 1869–1877.
- Munkvad, S., Jespersen, J., Gram, J., Overgaard, K., Ranby, M., 1990. Effects of methylamine and heparin on a rapid chromogenic assay of C1-esterase inhibitor in plasma. *Clin. Chem.* 36, 737–741.
- Navratil, J.S., Manzi, S., Kao, A.H., Krishnaswami, S., Liu, C.C., Ruffing, M.J., Shaw, P.S., Nilson, A.C., Dryden, E.R., Johnson, J.J., Ahearn, J.M., 2006. Platelet C4d is highly specific for systemic lupus erythematosus. *Arthritis Rheum.* 54, 670–674.
- Nilsson, U.R., Nilsson, B., 1984. Simplified assays of hemolytic activity of the classical and alternative complement pathways. *J. Immunol. Methods* 72, 49–59.
- Noris, M., Brioschi, S., Caprioli, J., Todeschini, M., Bresin, E., Porrati, F., Gamba, S., Remuzzi, G., 2003. Familial haemolytic uraemic syndrome and an MCP mutation. *Lancet* 362, 1542–1547.
- Notarangelo, L., Casanova, J.L., Fischer, A., Puck, J., Rosen, F., Seger, R., Geha, R., 2004. Primary immunodeficiency diseases: an update. *J. Allergy Clin. Immunol.* 114, 677–687.
- Nurnberger, W., Bhakdi, S., 1984. Plasma C3d/C3 quotient as a parameter for in vivo complement activation. *J. Immunol. Methods* 74, 87–91.
- Odorcuk, M., Keil, L.B., Debari, V.A., 1999. Binding of complement component C1q to anti-beta2 glycoprotein I antibodies from patients with antiphospholipid syndrome. *Inflamm. Res.* 48, 524–526.
- Oppermann, M., Gotze, O., 1994. Plasma clearance of the human C5a anaphylatoxin by binding to leucocyte C5a receptors. *Immunology* 82, 516–521.
- Papp, K., Szekeres, Z., Terenyi, N., Isaak, A., Erdei, A., Prechl, J., 2007. On-chip complement activation adds an extra dimension to antigen microarrays. *Mol. Cell Proteomics* 6, 133–140.
- Pascual, M., Mach-Pascual, S., Schifferli, J.A., 1997. Paraproteins and complement depletion: pathogenesis and clinical syndromes. *Semin. Hematol.* 34, 40–48.
- Pengo, V., Ruffatti, A., Iliceto, S., 2006. The diagnosis of the antiphospholipid syndrome. *Pathophysiol. Haemost. Thromb.* 35, 175–180.
- Perez-Caballero, D., Gonzalez-Rubio, C., Gallardo, M.E., Vera, M., Lopez-Trascasa, M., Rodriguez de Cordoba, S., Sanchez-Corral, P., 2001. Clustering of missense mutations in the C-terminal region of factor H in atypical hemolytic uremic syndrome. *Am. J. Hum. Genet.* 68, 478–484.
- Petersen, S.V., Thiel, S., Jensen, L., Steffensen, R., Jensenius, J.C., 2001. An assay for the mannan-binding lectin pathway of complement activation. *J. Immunol. Methods* 257, 107–116.
- Pfeifer, P.H., Kawahara, M.S., Hugli, T.E., 1999. Possible mechanism for in vitro complement activation in blood and plasma samples: Futhan/EDTA controls in vitro complement activation. *Clin. Chem.* 45, 1190–1199.

- Provost, T.T., Reichlin, M., 1988. Immunopathologic studies of cutaneous lupus erythematosus. *J. Clin. Immunol.* 8, 223–233.
- Ramos-Casals, M., Campoamor, M.T., Chamorro, A., Salvador, G., Segura, S., Botero, J.C., Yague, J., Cervera, R., Ingelmo, M., Font, J., 2004. Hypocomplementemia in systemic lupus erythematosus and primary antiphospholipid syndrome: prevalence and clinical significance in 667 patients. *Lupus* 13, 777–783.
- Richards, A., Buddles, M.R., Donne, R.L., Kaplan, B.S., Kirk, E., Venning, M.C., Tielemans, C.L., Goodship, J.A., Goodship, T.H., 2001. Factor H mutations in hemolytic uremic syndrome cluster in exons 18–20, a domain important for host cell recognition. *Am. J. Hum. Genet.* 68, 485–490.
- Richards, A., Kemp, E.J., Liszewski, M.K., Goodship, J.A., Lampe, A.K., Decorte, R., Muslumanoglu, M.H., Kavukcu, S., Filler, G., Pirson, Y., Wen, L.S., Atkinson, J.P., Goodship, T.H.J., 2003. Mutations in human complement regulator, membrane cofactor protein (CD46), predispose to development of familial hemolytic uremic syndrome. *Proc. Natl. Acad. Sci. U.S.A.* 100, 12966–12971.
- Richards, A., Kathryn, L.M., Kavanagh, D., Fang, C.J., Moulton, E., Fremeaux-Bacchi, V., Remuzzi, G., Noris, M., Goodship, T.H., Atkinson, J.P., 2007. Implications of the initial mutations in membrane cofactor protein (MCP; CD46) leading to atypical hemolytic uremic syndrome. *Mol. Immunol.* 44, 111–122.
- Roach, B., Kim, Y., Jerome, E., Michael, A.F., 1981. Influence of age and sex on serum complement components in children. *Am. J. Dis. Child.* 135, 918–920.
- Roos, A., Bouwman, L.H., Munoz, J., Zuiverloon, T., FaberKrol, M.C., Fallaux-vandenHouten, F.C., Klarmohamad, N., Hack, C.E., Tilanus, M.G., Daha, M.R., 2003. Functional characterization of the lectin pathway of complement in human serum. *Mol. Immunol.* 39, 655–668.
- Salmon, J.E., Girardi, G., 2004. The role of complement in the antiphospholipid syndrome. *Curr. Dir. Autoimmun.* 7, 133–148.
- Samano, E.S., Ribeiro, L.M., Gorescu, R.G., Rocha, K.C., Grumach, A.S., 2004. Involvement of C4 allotypes in the pathogenesis of human diseases. *Rev. Hosp. Clin. Fac. Med. Sao Paulo* 59, 138–144.
- Sanchez-Corral, P., Gonzalez-Rubio, C., Rodriguez de Cordoba, S., Lopez-Trascasa, M., 2004. Functional analysis in serum from atypical hemolytic uremic syndrome patients reveals impaired protection of host cells associated with mutations in factor H. *Mol. Immunol.* 41, 81–84.
- Schulze, M., Baker, P.J., Perkinson, D.T., Johnson, R.J., Ochi, R.F., Stahl, R.A., Couser, W.G., 1989. Increased urinary excretion of C5b-9 distinguishes passive Heymann nephritis in the rat. *Kidney Int.* 35, 60–68.
- Seelen, M.A., Roos, A., Wieslander, J., Mollnes, T.E., Sjolholm, A.G., Wurznier, R., Loos, M., Tedesco, F., Sim, R.B., Garred, P., Alexopoulos, E., Turner, M.W., Daha, M.R., 2005. Functional analysis of the classical, alternative, and MBL pathways of the complement system: standardization and validation of a simple ELISA. *J. Immunol. Methods* 296, 187–198.
- Seino, J., Kinoshita, Y., Sudo, K., Horigome, I., Sato, H., Narita, M., Noshiro, H., Kudo, K., Fukuda, K., Saito, T., et al., 1990. Quantitation of C4 nephritic factor by an enzyme-linked immunosorbent assay. *J. Immunol. Methods* 128, 101–108.
- Sjolholm, A.G., Selander, B., Ostenson, S., Holmstrom, E., Soderstrom, C., 1991. Normal human serum depleted of C1q, factor D and properdin: its use in studies of complement activation. *APMIS* 99, 1120–1128.
- Sjolholm, A.G., Jonsson, G., Braconier, J.H., Sturfelt, G., Truedsson, L., 2006. Complement deficiency and disease: an update. *Mol. Immunol.* 43, 78–85.
- Soderlind, E., Strandberg, L., Jirholt, P., Kobayashi, N., Alexeiva, V., Aberg, A.M., Nilsson, A., Jansson, B., Ohlin, M., Wingren, C., Danielsson, L., Carlsson, R., Borrebaeck, C.A., 2000. Recombining germline-derived CDR sequences for creating diverse single-framework antibody libraries. *Nat. Biotechnol.* 18, 852–856.
- Sonntag, J., Brandenburg, U., Polzehl, D., Strauss, E., Vogel, M., Dudenhausen, J.W., Obladen, M., 1998. Complement system in healthy term newborns: reference values in umbilical cord blood. *Pediatr. Dev. Pathol.* 1, 131–135.
- Stove, S., Klos, A., Bautsch, W., Kohl, J., 1995. Re-evaluation of the storage conditions for blood samples which are used for determination of complement activation. *J. Immunol. Methods* 182, 1–5.
- Sturfelt, G., Sjolholm, A.G., 1984. Complement components, complement activation, and acute phase response in systemic lupus erythematosus. *Int. Arch. Allergy Appl. Immunol.* 75, 75–83.
- Teisner, B., Brandslund, I., Grunnet, N., Hansen, L.K., Thellesen, J., Svehag, S.E., 1983. Acute complement activation during an anaphylactoid reaction to blood transfusion and the disappearance rate of C3c and C3d from the circulation. *J. Clin. Lab. Immunol.* 12, 63–67.
- Thiel, S., Frederiksen, P.D., Jensenius, J.C., 2006. Clinical manifestations of mannan-binding lectin deficiency. *Mol. Immunol.* 43, 86–96.
- Trendelenburg, M., 2005. Antibodies against C1q in patients with systemic lupus erythematosus. *Springer Semin. Immunopathol.* 27, 276–285.
- Truedsson, L., Sjolholm, A.G., Laurell, A.B., 1981. Screening for deficiencies in the classical and alternative pathways of complement by hemolysis in gel. *Acta Pathol. Microbiol. Scand. [C.]* 89, 161–166.
- Turner, M.W., 2003. The role of mannose-binding lectin in health and disease. *Mol. Immunol.* 40, 423–429.
- Vargha, R., Mueller, T., Arbeiter, K., Regele, H., Exner, M., Csaicsich, D., Aufricht, C., 2006. C4d in pediatric renal allograft biopsies: a marker for negative outcome in steroid-resistant rejection. *Pediatr. Transplant.* 10, 449–453.
- Venables, J.P., Strain, L., Routledge, D., Bourn, D., Powell, H.M., Warwicker, P., az-Torres, M.L., Sampson, A., Mead, P., Webb, M., Pirson, Y., Jackson, M.S., Hughes, A., Wood, K.M., Goodship, J.A., Goodship, T.H., 2006. Atypical haemolytic uraemic syndrome associated with a hybrid complement gene. *PLoS Med.* 3, e431.
- Wagner, J.L., Hugli, T.E., 1984. Radioimmunoassay for anaphylatoxins: a sensitive method for determining complement activation products in biological fluids. *Anal. Biochem.* 136, 75–88.
- Warwicker, P., Goodship, T.H.J., Donne, R.L., Pirson, Y., Nicholls, A., Ward, R.M., Turnpenny, P., Goodship, J.A., 1998. Genetic studies into inherited and sporadic hemolytic uremic syndrome. *Kidney Int.* 53, 836–844.
- Weiler, C.R., van Dellen, R.G., 2006. Genetic test indications and interpretations in patients with hereditary angioedema. *Mayo Clin. Proc.* 81, 958–972.
- West, C.D., 1989. The complement profile in clinical medicine. Inherited and acquired conditions lowering the serum concentrations of complement component and control proteins. *Complement. Inflamm.* 6, 49–64.
- West, C.D., 1994. Nephritic factors predispose to chronic glomerulonephritis. *Am. J. Kidney Dis.* 24, 956–963 [see comments].
- Wingren, C., Borrebaeck, C.A., 2006. Antibody microarrays: current status and key technological advances. *OMICS* 10, 411–427.
- Wouters, D., Wiessenberg, H.D., Hart, M., Bruins, P., Voskuyl, A., Daha, M.R., Hack, C.E., 2005. Complexes between C1q and C3 or C4: novel and specific markers for classical complement pathway activation. *J. Immunol. Methods* 298, 35–45.
- Yang, Y., Chung, E.K., Zhou, B., Lhotta, K., Hebert, L.A., Birmingham, D.J., Rovin, B.H., Yu, C.Y., 2004. The intricate role of complement component C4 in human systemic lupus erythematosus. *Curr. Dir. Autoimmun.* 7, 98–132.
- Zecevic, R.D., Pavlovic, M.D., Stefanovic, D., 2006. Lupus band test and disease activity in systemic lupus erythematosus: does it still matter? *Clin. Exp. Dermatol.* 31, 358–360.
- Zipfel, P.F., Skerka, C., 2006. Complement dysfunction in hemolytic uremic syndrome. *Curr. Opin. Rheumatol.* 18, 548–555.
- Zwirner, J., Felber, E., Reiter, C., Riethmuller, G., Feucht, H.E., 1989. Deposition of complement activation products on plastic-adsorbed immunoglobulins. A simple ELISA technique for the detection of defined complement deficiencies. *J. Immunol. Methods* 124, 121–129.