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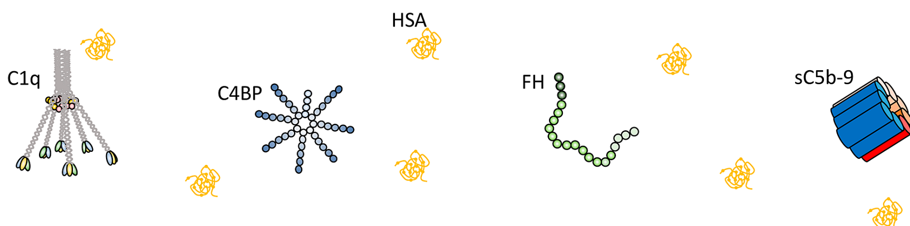
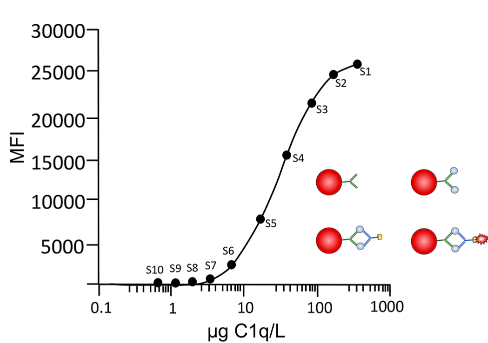
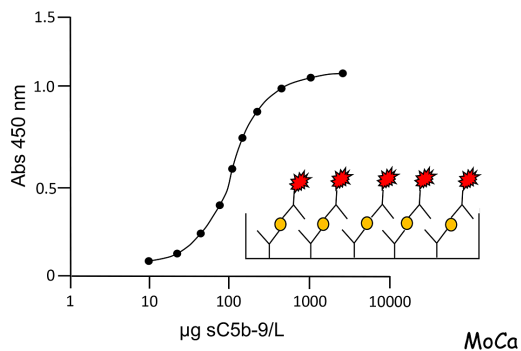
KERSTIN SANDHOLM

DEVELOPMENT AND EVALUATION OF IMMUNOASSAYS FOR COMPLEMENT DIAGNOSTICS







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Development and evaluation of immunoassays for complement diagnostics

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Kerstin Sandholm

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Development and evaluation of immunoassays for complement diagnostics
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Abstract

Sandholm, Kerstin (2019). *Development and evaluation of immunoassays for complement diagnostics:,* Linnaeus University Dissertations No 369/2019, ISBN: 978-91-89081-14-7 (print), 978-91-89081-15-4 (pdf).

Laboratory analyses of human body fluids play an important role in clinical diagnosis. This thesis comprises projects in which various immune assays have been developed and evaluated as complement diagnostics in both plasma and cerebrospinal fluid (CSF). Various methods have been used, such as ELISA, Western blot, flow cytometry, and xMAP technology.

In **paper 1**, we monitored complement parameters in EDTA-plasma and CSF from patients with suspected neuroborreliosis (NB) by using in-house sandwich ELISAs. We found significantly elevated levels of C1q, C4, C3, and C3a in CSF, but not in plasma, suggesting that complement plays a role in the intrathecal immune response in NB.

Complement is a main player in early inflammation, and **in paper 2**, we investigated the role of complement activation in phagocytosis and the release of cytokines and chemokines in response to two clinical isolates: *Borrelia afzelii* K78 and *Borrelia garinii* LU59. Our results show that complement activation plays an important role in the initial process of phagocytosis, but not in the subsequent cytokine release that occurs in response to live *Borrelia* spirochetes. C1q, a valuable biomarker of disease activity in systemic lupus erythematosus (SLE), can be quantitated using a number of different immunochemical techniques. In **paper 3**, we developed and validated a magnetic bead-based immunoassay for quantifying C1q in EDTA-plasma and CSF. In contrast to soluble immunoprecipitation assays such as nephelometry and turbidimetry, this new assay was not hampered by the interaction between C1q and detecting antibodies. The novel assay was shown to give a clear correlation between nephritis and SLEDAI score in SLE.

Warfarin is a commonly used but complicated treatment in patients with thrombosis. It reduces the function of vitamin K-dependent coagulation proteins, including protein S, which is a ligand for C4b-binding protein (C4BP). In **paper 4**, we demonstrated a decrease in various isoforms of C4BP that resulted in a strong complement activation in patients treated with warfarin, but not in patients treated with other anticoagulants.

Taken together, the results from the papers included in this thesis stress the importance of validated assays with high sensitivity and specificity in enabling accurate diagnosis in patients with various inflammatory diseases.

Keywords: Immune assays, complement diagnostics, ELISA, xMAP-technology, C1q, preanalytical factors, method validation

Bättre sent än aldrig!

POPULÄRVETENSKAPLIG SAMMANFATTNING

Laboratorieanalyser av olika kroppsvätskor är viktiga för att kunna ställa rätt diagnos vid olika sjukdomstillstånd och även för att kunna följa effekten av en behandling. För att kunna göra detta krävs tillförlitliga analysmetoder. Immunkemiska analysmetoder, där antikroppar är viktiga redskap och reagens, är mycket vanliga på kliniska laboratorier och inom forskning.
I denna avhandling har olika immunkemiska analyser utvecklats och utvärderats för komplementanalyser i både plasma och cerebrospinalvätska (CSV).

Komplementsystemet är en viktig del av vårt medfödda immunförsvar och består av ett 50-tal cellbundna och lösliga proteiner. Dessa proteiner aktiveras när de träffar på bakterier, virus eller skadade celler eftersom deras uppgift är att oskadliggöra dessa genom olika åtgärder bl.a genom att starta en inflammation. Brist på någon komplementfaktor eller en okontrollerad aktivering av komplementsystemet, kan leda till allvarliga skador på våra egna celler och vävnader, som i sin tur kan leda till olika infektioner, autoimmuna sjukdomar eller transplantations-komplikationer.

För att kunna mäta koncentrationen och funktionen av dessa komplementproteiner behövs det noggranna, pålitliga och väl utvärderade analysmetoder. När nya metoder utvecklas är det viktigt att de optimeras och valideras dvs. att testa noggrannhet, linjäritet och detektionsgräns. Dessutom måste man vara medveten om alla faktorer som kan påverka analysresultatet, från provtagning till slutlig analys och bedömning av resultatet.

I **artikel 1** har vi analyserat ett stort antal plasmaprover och CSV från patienter som sökt sjukvård för misstänkt neuroborrelios. Ett antal komplementproteiner och en aktiveringsmarkör analyserades med egenutvecklade mätmetoder. Vi kunde påvisa förhöjda koncentrationer i CSV av vissa komplementproteiner och att en aktivering hade skett i centrala nervsystemet. I plasman var det normala värden.

I **artikel 2** undersöktes komplementsystemets roll när *Borrelia*-bakterier ska oskadliggöras och hur det påverkar utsöndringen av cytokiner (små proteiner som medverkar vid inflammation). Detta gjordes genom att blanda blod *in vitro* med två olika *Borrelia*- stammar. En *Borrelia*-stam var känslig och den andra var motståndskraftig mot komplementaktivering. Med hjälp av olika immun-

kemiska metoder kunde vi konstatera att komplementsystemet är viktigt men inte nödvändigt för att oskadliggöra *Borrelia*-bakterier och att det inte bildades mer cytokiner när komplementsystemet är aktivt.

Komplementfaktor C1q, som är en viktig sjukdomsmarkör vid sjukdomen systemisk lupus erythematosis (SLE), kan mätas i plasma med olika immunkemiska metoder. I **artikel 3** utvecklade, optimerade och utvärderade vi en ny metod för att mäta C1q i både plasma och CSV. C1q resultat från plasmaprover och CSV från patienter jämfördes med resultat från andra metoder bl.a nefelometri som ofta används på kliniska laboratorier. Vår nya metod gav resultat som överensstämde mycket bra med andra metoder dock inte med nefelometri som därför vi inte rekommenderar för C1q analys.

Warfarin (waran) är en vanlig men komplicerad behandling av patienter med tromboser. Detta läkemedel påverkar de koagulationsfaktorer som är vitamin K-beroende dvs de som behöver vitamin K för att bildas på ett korrekt sätt. Protein S är ett sådant protein och det är bundet till C4b bindande protein (C4BP) ett viktigt reglerande protein i komplementsystemet. I **artikel 4** har vi analyserat C4BP i plasma från patienter under och efter warfarinbehandling och kan visa att dessa patienter under denna behandling har sänkt koncentration av C4BP och en kraftig komplementaktivering. Som jämförelse analyserades även plasma från patienter med annan behandling av tromboser och friska givare, men i dessa plasmaprover ses inga sänkta C4BP nivåer eller någon komplementaktivering. I denna studie kunde vi analysera koncentrationen C4BP och dess olika former tack vare att vi utvecklade egna mätmetoder för dessa.

Målet med denna avhandling var utveckling, optimering och utvärdering av olika mättekniker för att identifiera och följa störningar i komplementsystemet, som är en viktig komponent av människans naturliga immunsystem.

LIST OF PUBLICATIONS

This thesis is based on the following articles:

1. Complement activation in Lyme neuroborreliosis – increased levels of C1q and C3a in cerebrospinal fluid indicate complement activation in the CNS. Henningsson AJ, Ernerudh J, **Sandholm K**, Carlsson SA, Granlund H, Jansson C, Nyman D, Forsberg P, Nilsson Ekdahl K. *J Neuroimmunol. 2007 Feb;183(1-2):200-7*

2. Early cytokine release in resonse to live Borrelia burgdorferi Sensu Lato Spirochetes is largely complement independent. **Sandholm K**, Henningsson AJ, Säve S, Bergström S, Forsberg P, Jonsson N, Ernerudh J, Ekdahl KN. *PLoS One. 2014 Sep 29;9(9)*

3. Evaluation of a Novel Immunoassay for Quantification of C1q for Clinical Diagnostic Use. **Sandholm K**, Persson B, Skattum L, Eggertsen G, Nyman D, Gunnarsson I, Svenungson E, Nilsson B, Ekdahl KN. *Front Immunol. 2019 Jan 25;10:7.*

4. Warfarin treatment downregulates C4BP and correlates with increased complement activation in contrast to Factor Xa inhibitors. **Sandholm K**, Ibrahim M, Hårdstedt M, Antovic M, Grosso G, Svenungsson E, Nilsson B, Morange P, Bruzelius M, Nilsson Ekdahl K. *Manuscript*

Additional work outside the scope of this thesis

1. **Sandholm, K**, Persson, B, Abdalla, S, Mohlin, C, Nilsson, B, Ekdahl, KN. Quantification of complement proteins with special reference to C1q: Multiplex vs ELISA vs rocket immune electrophoresis vs nephelometry. 2019. *In The complement system - innovative diagnostic and research protocols. Methods in molecular biology (Springer Nature) in press*

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ABBREVIATIONS

AP	Alternative pathway
C3Nef	C3 Nephritic factor
C4BP	C4b-binding protein
C4BPt	Total C4BP
CCD	Charge-coupled device
CDR	Complementarity determining region
CNS	Central nervous system
CP	Classical pathway
CR1	Complement receptor 1
CRASPs	Complement regulator acquiring surface proteins
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CV	Coefficient of variation
DAF	Decay acceleration factor
DOAC	Direct oral anticoagulant
EDAC	1-ethyl-3(3-dimethylaminopropyl) carboimide hydrochloride
EDTA	Ethylendiaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FH	Factor H
FHL-1	Factor H-like protein
FHR	Factor H-related protein
FITC	Fluorescein isothiocyanate
H	Heavy
IC	Immune complex
ICS	International complement standard
Ig	Immunoglobulin
L	Light
LB	Lyme borreliosis
LED	Light-emitting diode
LP	Lectin pathway
MAC	Membrane attack complex
MASP	MBL-associated serine protease
MBL	Mannose binding lectin
MBSI	Magnetic bead-based immunoassay
MCP	Membrane cofactor protein
MFI	Mean fluorescence intensity
PS	Protein S
PTX3	Pentraxin 3
RID	Radial immunodiffusion

RIE	Rocket immunoelectrophoresis
S-NHS	N-hydroxy-sulfosuccinimide
SA-PE	Streptavidin-phycoerytin
SAP	Serum amyloid P
SCR	Short consensus repeats
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SLE	Systemic lupus erythematosus
VKA	Vitamin-K antagonist
xMAP	x=analyte MAP=multi-analyte profiling

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INTRODUCTION

Laboratory diagnostics of human fluids (plasma, serum, urine, and cerebrospinal fluid [CSF]) play an important role in the clinical diagnosis of various diseases, as well as in monitoring the effect of treatments. Immunochemical methods are valuable tools in diagnostic work, and the focus of this thesis has been to develop and validate various immunoassays for complement diagnostics.

Before a new method can be used in clinical diagnostics, many parameters need to be investigated, optimized, and validated. In addition, the impact of preanalytical factors and method interference, which may affect the test results, must be considered. In order to achieve a reliable and robust immune assay, all steps from sample collection to test results must be checked, and they have to be traceable (Figure 1).

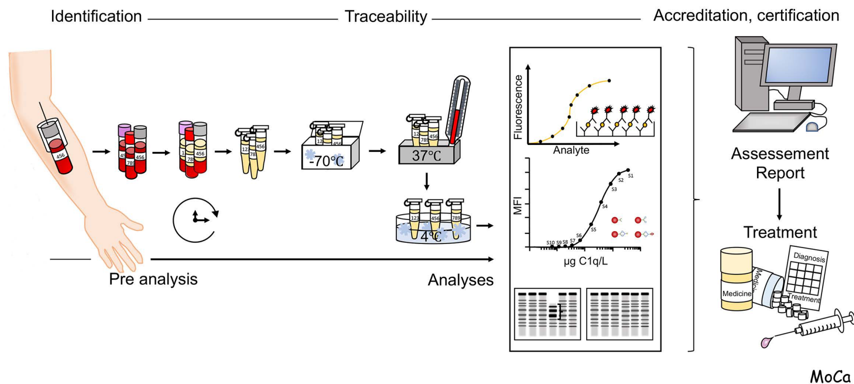

Figure 1. *Overview of the flow from sampling, sample handling and preparation, storage, analysis and assessment, which when properly controlled lead to a correct diagnosis and treatment. Critical factors include proper sampling and appropriate sampling tubes, sampling preparation such as temperature, speed and time for centrifugation, storage and thawing temperature and analysis with a validated method. Detailed information is given in the section "Pre-analytical factors". Adapted from Mohebnasab M, Eriksson O, Persson B, Sandholm K, Mohlin C, Huber-Lang M, Keating BJ, Ekdahl KN, Nilsson B. (2019) Current and future approaches for monitoring responses to anti-complement therapeutics. Front.Immunol. 10:2539*

The complement system is a cornerstone of our innate immune system and consists of at least 50 soluble and membrane-bound proteins. The main role of complement in host defense is to attack and harm pathogens and to remove damaged cells, e.g., by mediating inflammation. Complement deficiencies as well as excessive complement activation can cause pathological conditions. Therefore, it is of the highest importance to make accurate diagnoses in patients with various inflammatory diseases by using sensitive, specific, and reliable methods. (Activation and regulation of the complement system are comprehensively described below).

Antibodies

Antibody structure

Antibodies, also called immunoglobulins (Igs), are a large family of glycoproteins produced by terminally differentiated B lymphocytes known as plasma cells. All antibodies have the same basic structure: two identical heavy (H) and two identical light (L) chains linked by disulfide and noncovalent bonds (Figure 2A). The antibodies in mammals are divided into five classes (IgA, IgD, IgE, IgG, IgM) according to their heavy chain structure. The IgG and IgA isotypes can be divided into additional subclasses, which are based on differences in their heavy chain. In humans, IgG has 4 subclasses (IgG1, IgG2, IgG3, IgG4) and IgA 2 subclasses (IgA1, IgA2). Antibodies have two important functional domains: the antigen binding site on the variable portion of the molecule, known as the Fab (fragment, antigen binding) domain, and the Fc (fragment, crystallizable) domain on the constant portion. The Fc domain binds to Fc receptors on various cell types, and the effect of this binding can be an activation of natural killer cells, activation of the classical pathway of complement, and phagocytosis. There are two identical antigen binding sites where each site binds to an epitope on the antigen being recognized by the Ig. In the variable region of each chain (H and L) there are three hypervariable sequences called complementarity determining regions (CDRs). These CDRs form a loop structure, and the six CDRs on each arm form the antigen-binding site. Antibodies can be monomers (IgG), dimers (IgA) and pentamers (IgM), which means that their total binding strength (avidity) increases with the number of antigen-binding site on the antibody molecule (1).

Polyclonal and monoclonal antibodies

Most antigens are complex molecules, presenting many epitopes (i.e., small regions of the antigen) for recognition by specific antibodies. These epitopes are recognized by a large number of antibody molecules that originate from different clones of B lymphocytes; hence, such antibodies are known as

polyclonal antibodies (Figure 2B). Monoclonal antibodies, on the other hand, are antibodies produced by the same clone of B lymphocytes; therefore, these antibodies all bind to the same epitope. New epitopes (called neoantigenic epitopes) can be exposed when an antigen is altered by a chemical modification such as proteolysis, and these epitopes may be recognized by specific antibodies (1, 2). Antibodies of this kind are used, for example, in the analysis of C3a, a complement activation marker, in which a neoepitope on the C3a moiety is exposed after cleavage of the C3 molecule (3).

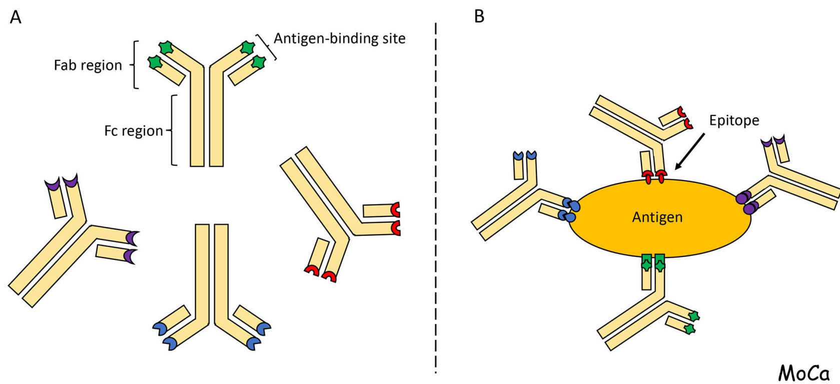

***Figure 2**. Schematic overview of antibodies of IgG isotype in a polyclonal antiserum **(A)**. Polyclonal antibodies bound to epitopes on an antigen **(B)**.*

Minor variations in the antigenic structure can fine-tune the specificity of an antibody and its strength, or affinity, in binding to an antigen. Since an antibody only recognizes a small part of an antigen (epitopes are often 6-8 amino acids), it can sometimes bind to similar epitopes as well; i.e., it can exhibit cross-reactivity with one or more other antigen(s). Compared to antibodies against proteins, antibodies against carbohydrates have low affinity (because the producing B-cells clones do not undergo affinity maturation) but their avidity is high because they are of IgM isotype (because the B-cells have not undergone isotype switch) (4, 5).

Production of antibodies

Polyclonal antibodies can be produced in various animals, such as rat, rabbit, goat, or sheep, by immunization with the relevant antigen, together with an adjuvant to initiate and boost antibody production. Weeks after immunization, blood is collected from the animal and allowed to clot, and polyclonal antibody

is purified from the resulting serum. The immunized animal will produce antibodies to epitopes in the administered antigen that differ from those in its own antigen. Therefore, the choice of animal depends on how dissimilar the corresponding molecule is between the two species, and also on the volume of antibody that is desired (6). Instead of a native protein being used for the antigen, a peptide composed of a known amino acid sequence from the antigen can be synthesized in high purity and used for immunization. For this purpose, the peptide must be covalently linked to a carrier protein, a large molecule capable of stimulating an immune response (7, 8).

In 1975, Köhler and Milstein first described how they fused splenic B cells from an immunized mouse with myeloma cells to generate immortal hybridoma cells, each producing a unique monoclonal antibody (9). This technique was initially used to produce mouse monoclonal antibodies, but more recently rat monoclonal antibodies have been produced to enable studies of mouse antigens. Polyclonal, monoclonal, and peptide antibodies are all used in diagnostics, and they have advantages as well as disadvantages in terms of specificity, production time, and cost. The production of polyclonal antibodies is less expensive and faster than the production of monoclonal antibodies. An advantage of producing monoclonal antibodies is that they can be produced in large quantities with high batch-to-batch homogeneity, whereas the quality and specificity of polyclonal antibodies can vary greatly, depending on their production in different animals at different times. Polyclonal antibodies have higher potential for cross-reactivity because of their ability to recognize multiple epitopes, but this cross-reactivity can also generate a high background noise in an assay. To avoid this potential problem, the polyclonal antibodies can be affinity-purified against the antigen of choice. Another option to decrease cross-reactivity is to use a synthetic peptide as immunogen, since polyclonal antisera to synthetic peptides have a specificity comparable to that of monoclonal antibodies. However, polyclonal antibodies are often more robust; for example, they can better withstand pH and buffer changes (2, 10). Consequently, there are many factors to consider when choosing antibodies for different applications and methods.

Immunochemical assays

An immunochemical method can be defined as a process that utilizes high-specificity antibodies to detect an antigen in tissue, cells, or fluid. Immunochemical assays have been used in clinical laboratories and in research for a long time. These methods are often simple, rapid, and robust. Depending on the format, immunoassays can be qualitative or quantitative. Many of these

techniques are currently used for the detection of intact complement proteins or activation fragments in serum, plasma, urine, and/or CSF (11, 12).

The binding between antigen and antibody consists of reversible non-covalent bonds, which means that immunoassays involve weak interactions such as van der Waals forces, hydrogen bonds, electrostatic forces, and hydrophobic interactions. If the number or strength of the bonds is inadequate, stable antigen-antibody complexes cannot be formed, and the affinity of the antibody is low (1). Antigen-antibody binding is influenced by several factors, such as temperature, pH, and ionic strength, and these conditions are therefore important to consider in an immunoassay (13, 14).

Radial immunodiffusion (RID)

This method was developed by Mancini and coworkers in 1964 (15) and depends on the fact that a ring of precipitate forms over time when an unknown amount of antigen is allowed to diffuse radially from a well in an agar or gel that contains specific antibody recognizing that antigen. The resulting area enclosed by the precipitate is proportional to the amount of antigen present. This method was quickly established within diagnostic laboratories and is still used (Figure 3).

Rocket immunoelectrophoresis (RIE)

Rocket immunoelectrophoresis also uses agarose gels containing specific antibody against the antigen to be analyzed. Diluted serum samples are applied to holes punched in the gel, which contains the monospecific antibody. Instead of diffusion, an electric current induces the migration of the antigen, and a precipitate forms that assumes a rocket- like shape at the sites in the gel where the antigen and antibody meet in optimal proportions. The height of the precipitation front is proportional to concentration of the antigen (16, 17) (Figure 3).

Turbidimetry and nephelometry

Turbidimetry and nephelometry are based on the principle that small particles in suspension, in this case antigen-antibody complexes, will disperse the light passing through the suspension (Figure 3). The complexes are formed when the detecting antibody is mixed with the sample; a high-intensity light source is used to pass through the reaction vessel where the reagent is present. In turbidimetry, the light beam transmitted through the sample is measured, and in nephelometry, light that is scattered at an angle away from the beam is

measured. Photodetectors collect the light, and the amount of light is compared with the amount of light from known concentrations of the analyte (18).

Enzyme-linked immunosorbent assay (ELISA)

In 1971, Engvall and Perlmann (19-21) developed the ELISA, one of the most powerful immunoassay approaches that can be used to quantify an antibody or antigen. This method is routinely used in diagnostics and research because of its high sensitivity and ease of handling. Different types of ELISA are used, depending on what needs to be analyzed, with the most frequently used variants including the indirect, sandwich, and competitive ELISAs. For analyzing an antigen, sandwich ELISA is commonly used; this technique is initiated by adsorbing a specific capture antibody to a solid phase, usually the wells of a microtiter plate. The antigen to be analyzed is added to the wells, where it binds to the antibody. An enzyme-labeled detecting antibody is then added, and a colorless substrate is allowed to react with the enzyme to generate a color whose absorbance is proportional to the amount of bound antigen. Many companies provide commercial kits that include everything needed for ELISA analyses, such as microtiter plates, calibrators, controls, antibodies, enzyme substrates, and buffers. The package insert for these methods often describes the validation of the method and gives the reference interval for normal values.

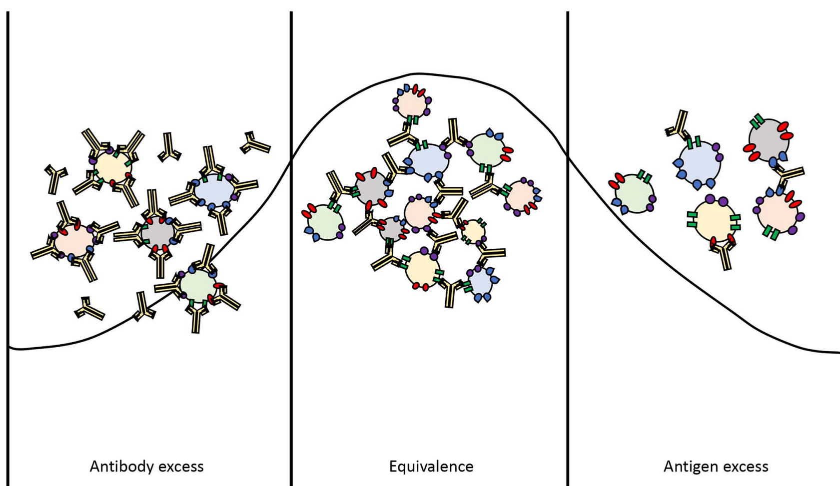

***Figure 3**. Immunoprecipitation in solution. Bivalent or multivalent antibodies can form complexes with antigen molecules which form complexes. Precipitates are formed at the point of equivalence where neither the antibody nor the antigen are in excess. This general reaction is the basis for all immunochemical assays.*

Western blotting

In Western blotting, a specific antibody is used to detect the protein to be identified, after the protein has been made to migrate according to its molecular size. Since the protein is present as part of a mixture of different molecules, it must first undergo this analytical separation according to size via electrophoresis on a polyacrylamide gel (SDS-PAGE). Thereafter, all the separated proteins are transferred to a membrane, and then the position of the protein antigen on the membrane can be detected by the binding of specific antibody. The detecting antibody may be labeled with an enzyme that can be used to visualize the antigen via a colored precipitate or chemiluminescent signal (22, 23). This technique gives reliable information as to whether a protein has been proteolytically digested to smaller fragments, but it is at best semi-quantitative regarding concentration.

Bead-based immunoassay - xMAP technology

About 20 years ago, the Luminex Corporation® (Austin, TX, US) developed the multiplex assay, a technique that made it possible to analyze several analytes simultaneously. This methodology, known as xMAP technology (x=analyte, MAP=multi-analyte profiling), uses a combination of color-coding microspheres (beads), allowing up to 500 different assays to be performed simultaneously on the same sample. This multi-assay approach is possible because these beads are dyed with different ratios of two or three different fluorescent colors, and each region of the beads can be detected by a xMAP instrument.

All of these assays are performed in microtiter plates, and between each of the additions, an incubation for an appropriate time occurs, and then a wash is used to remove the excess reagent that has not been bound.

There are two types of beads used, non-magnetic and magnetic, but all the microspheres consist of a polystyrene core and a polymer layer. The beads are physically identical and have the same size but differ in the amounts of the internal classification dyes present. The non-magnetic beads have a diameter of 5.6 µm, and the magnetic beads have a magnetic coating and a diameter of 6.5 µm (24, 25).

Suppliers are constantly developing new commercial immunoassay kits to measure various biomarkers in plasma, urine, and CSF with this technology. Currently, there are immunoassay panels available for cytokines, chemokines, cancer markers, hormones, growth factors, diabetes, inflammation, and sepsis, among others.

Because it is an open and flexible platform, xMAP technology also makes it possible to develop custom-designed and analyte-specific methods (26-28). There are a number of different microspheres, and they all have different molecules coupled to the surface that give them their special properties.
Avidin-coupled beads (MagPlex®-Avidin) can bind biotinylated capture reagents and are useful when working with small molecules such as peptides. MagPlex-TAG™ beads have a 24-base DNA sequence called anti-TAG covalently coupled to their surfaces that can bind a complementary TAG sequence on a primer or probe (29).

There are three different microspheres that are carboxylated polystyrene microparticles: MicroPlex® micropheres (nonmagnetic), Sero-MAP™ microspheres (non-magnetic), and MagPlex® microspheres (paramagnetic). Sero-MAP™ microspheres have been optimized to reduce non-specific binding in serology assays (30).

MagPlex® microspheres with carboxyl groups allow the formation of covalent coupling with proteins and are useful for sandwich immunoassays, which involve pairs of antibodies (31, 32). These carboxyl groups must be activated with carbodiimide derivatives before the coupling of specific protein. For this purpose, 1-ethyl-3 (3-dimethylaminopropyl) carboimide hydrochloride (EDAC) is used, and an active form of an O-acylisourea intermediate is formed. To form a stable ester, N-hydroxy-sulfosuccinimide (S-NHS) is then used to react with NH_2 groups to form a covalent amide bond between the beads and, for example, a capture antibody (33). Specific antigen to be detected binds to the capture antibody, and thereafter to a detecting biotinylated antibody. In order to bind to and detect the biotin-bearing antigen-antibody complexes, a streptavidin-phycoerythrin (SA-PE) conjugate is then added (Figure 4).

The Luminex company offers three different xMAP®-based instruments with different capacities and technology. In the MAGPIX® instrument, there are light-emitting diodes (LEDs) with two different path lengths that can detect the color of the bead region (at 635 nm) and the amount of bound SA-PE (at 525 nm), corresponding to the concentration of bound analyte. A charge-coupled device (CCD) camera is used, and MAGPIX uses a magnet to illuminate and image a monolayer of immobilized magnetic beads, with all the beads being read at once (24).

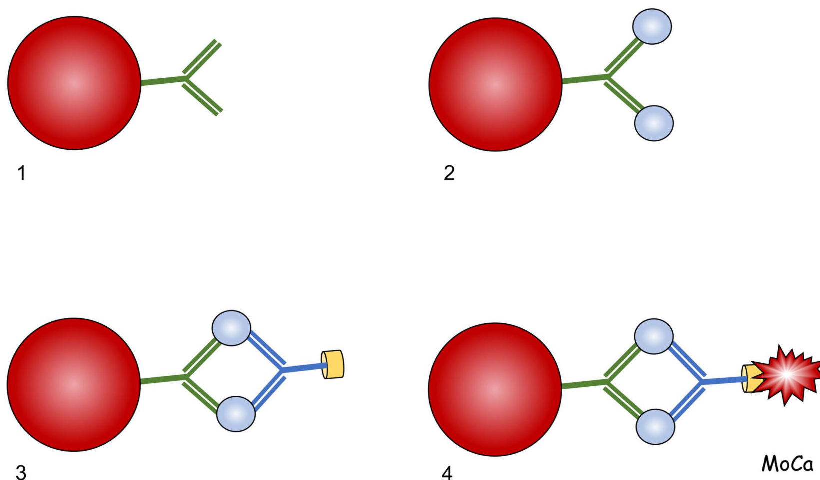

Figure 4. *Principle for magnetic bead sandwich immunoassay.* ***1:*** *bead with conjugated capture antibody (Ab);* ***2:*** *bound analyte;* ***3:*** *detecting, biotinylated Ab;* ***4:*** *fluorophore (phycoerythrin) labelled streptavidin provides the signal where the mean fluorescence intensity is proportional to the concentration analyte.*

Types of interference in immunoassays

Interference is defined as *"the effect of a substance present in the sample that alters the correct value of the result"* (34).
A plethora of original and review articles have described interference in clinical laboratory analyses, and not least immunoassays (13, 35-40). The frequent occurrence of this sort of problem underscores how important it is to be aware of these factors in order to be able to trust the results, as well as to minimize the risk of misinterpreting the results. What makes interference so complicated is that the effect of the interference can differ from method to method, from test to test, and from patient to patient. Factors that can cause problems in an immunoassay include: a) cross-reacting substances, b) heterophilic antibodies and autoantibodies, and c) antigen excess (i.e, the high-dose Hook effect).

Cross-reactivity

Because antibodies only bind to a small portion of their antigens, small structural differences can cause cross-reactions with other similar analytes in the sample. The specificity of each immunoassay is primarily determined by the specificity of the antibodies used. Therefore, it is possible for compounds other than the target analyte to elicit a response in an immunoassay if they are similar

enough in structure to the analyte being assayed. A cross-reaction can either be positive or negative; that is, the result can be incorrect in being either too high or too low (37).

Heterophilic antibodies

The existence of human antibodies with affinities for assay antibodies was reported as early as 1973 (41). In laboratory diagnostics, these antibodies are known as heterophilic antibodies, and they can be divided into two groups (42): One group consists of human anti-animal antibodies, which are formed in response to animal antibodies injected for diagnostic or therapeutic purposes. They are high-affinity antibodies and can create analytical problems (43).
Another group consists of antibodies that have an affinity for animal antibodies (most common IgG and IgM/RF related) and are found in patients without previous exposure to these animal antibodies. These antibodies are often presumed to have a low affinity and a broad specificity (38, 44).

Autoantibodies are antibodies directed against a self-antigen and often generated against neoepitopes. Many examples of such autoantibodies to analytes have been described, such as those to insulin (45) or thyroid (46). Many patients also have autoantibodies to various complement proteins; these autoantibodies include anti-C1q (47, 48), anti-factor H (49), and the autoantibody C3Nef, which binds to the alternative pathway convertase (50). Rheumatoid factor (RF) are autoantibodies, usually of the IgM class, that are directed against the Fc region of a patient´s own IgG. These antibodies are found in many autoimmune diseases, including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and scleroderma (13, 38).

Antigen excess

Antigen excess, or the high-dose Hook effect, is a phenomenon caused by antigen concentrations that are high in relation to the amount of antibody present (51) (Figure 3). As early as 1935, Heidelberger and Kendall showed that in antigen excess, insufficiently diluted sera can produce falsely low results (52). This effect can be seen not only in methods in which equivalent antigen-antibody complexes must be formed, but also in sandwich assays in which both the capture and detection antibodies are saturated by the high analyte concentration. In such cases, no “sandwich” can be formed by the capturing antibody, antigen, and detection antibody (39).

Pre-analytical factors

Many factors can influence the result of an analysis before a blood sample has arrived in the laboratory. In order to achieve high patient safety, high quality, and reliable laboratory results, it is important that all sampling and sample handling be done accurately and with the correct technology (53). The ISO 15189:2012 defines the pre-analytical phase as *"steps starting in chronological order, from the clinician´s request and including the examination requisition, preparation of the patient, collection of primary samples, and transportation to and within the laboratory, and ending when the analytical examination procedure begins"* (54). Pre-analytical errors account for about 60-70% of all problems with laboratory diagnostics (55). Major of these errors can be attributed to insufficient or incorrect patient preparation and/or collection or preparation, transportation, and/or storage of the sample. It is obvious that the patient's identity must be confirmed and that the patient be informed about required preparation, such as the last allowable time for intake of food, liquid, and medicine. Appropriate sampling materials for the intended analysis must always be used, and the sampling, handling, and storage must be carried out correctly (56, 57) (Figure 1).

Pre-analytical factors in complement analysis

Prior to complement analyses, it is extremely important to properly collect and process all body fluids. This caution applies to both functional analyses and quantification of complement proteins as complement activation markers.
EDTA-plasma should be used for the quantification of complement proteins and their activation products. The only anticoagulant that completely inhibits complement activation *ex vivo* is EDTA. Heparin and citrate are not sufficient inhibitors and should therefore not be used for complement analysis. To avoid *ex vivo* activation in other body fluids such as urine and CSF, EDTA should be added during sampling (58, 59). For functional complement studies, serum must be used (60), or if plasma is used, it must be anticoagulated with a specific thrombin inhibitor such as lepirudin (61)(Figure 5).

Serum and plasma should be separated by centrifugation at 4°C within 4 hours of collection, and then quickly frozen at -70°C. If the sample needs to be transported, it should be sent in packages containing dry ice. Before analysis, proper thawing is important: slowly on ice, or quickly at 37°C, and the samples then immediately placed into an ice-water bath (12, 58, 59, 62).

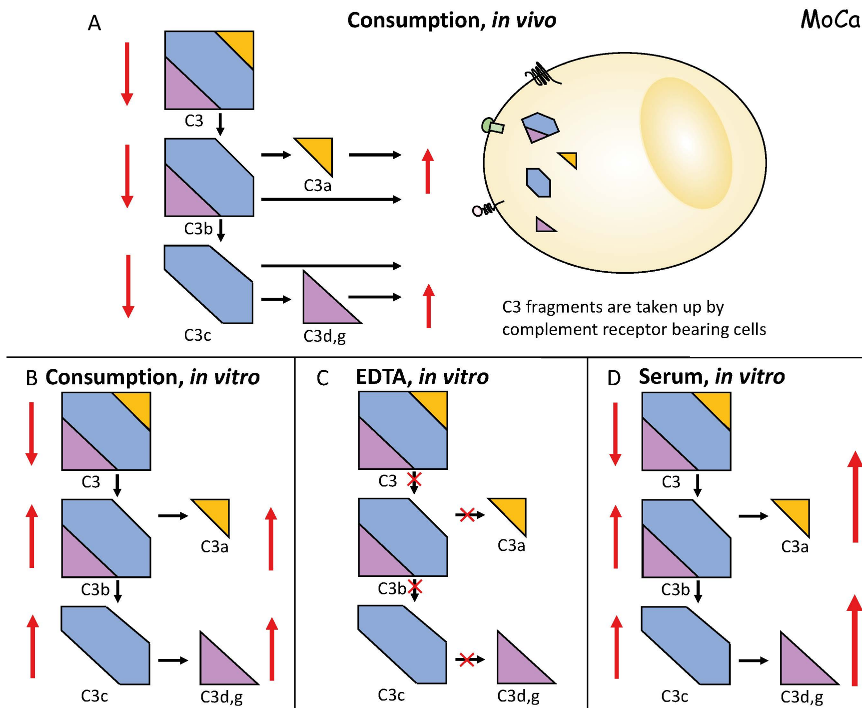

Figure 5. *Activation and consumption of complement in vivo and in vitro. The activation products when C3 is activated are C3a, C3b/iC3b, C3d,g and C3c. In vivo these fragments may get eliminated by complement receptor bearing cells (A). When the complement system is activated in vitro in during coagulation to obtain serum (D) or after sampling with inadequate anticoagulants e.g. citrate or heparin (B) the activation products remain in the fluid phase. To quantitatively inhibit complement activation in vitro blood can be drawn in the presence of EDTA which inhibits all activation pathways (C). The length of the arrows symbolizes the degree of C3 cleavage. From Ekdahl KN, Persson B, Mohlin C, Sandholm K, Skattum L, Nilsson B. Interpretation of Serological Complement Biomarkers in Disease. Front Immunol. 2018;9:2237.*

Development of in-house sandwich immunoassays for complement diagnostics

Many companies offer commercial ELISA kits for analysis of various complement factors, but there are few suppliers who can offer bead-based immunoassays for these analytes. One alternative approach is to develop the needed assays in-house. Many parameters should be investigated and

optimized, and there are basic requirements that need to be fulfilled when developing an immunoassay to be used in clinical diagnostics (63-65). Some examples of parameters that need to be defined are:

- *Assay format*

Which analysis format is appropriate? Microtiter plate or beads? Which plate type, which beads?

- *Capture and detecting antibodies*

The antibodies should show specific binding to the target antigen and no detectable cross-reactivity to other related molecules in the assay. If two monoclonal antibodies are used in a sandwich assay, it is important that they do not bind to adjacent epitopes or to each other. Monoclonal antibodies are preferred for achieving maximum sensitivity and specificity because a monoclonal antibody is produced by one clone. If polyclonal antibodies are used, they should be affinity-purified to reduce the background. To identify the optimal assay conditions, the concentration of both the capture and the detecting antibody must be titrated.

- *Reagent*

In both ELISAs and bead-based immunoassays, a dilution buffer is used to dilute standards, controls, samples, and detecting antibodies. Other buffers are used for blocking and washing, and it is therefore important to examine the content of these buffers to make sure they function optimally. A blocking buffer is a buffer containing inert proteins that bind or adsorb to free surfaces, i.e., non-coated surfaces such as those in a microtiter plate. Blocking of the free surfaces makes the method more sensitive because it reduces the risk of nonspecific interactions and thus produces a lower background.

- *Calibration, standard curves, and sample dilution*

A standard serum containing a known concentration of the relevant analyte must be used to prepare dilutions to yield a standard curve. For each analyte, an appropriate measurement interval must be ascertained, and a suitable sample dilution must be determined.

- *Optimizing temperature and incubation times*

The analysis must be robust, with only slight variations in its operating conditions, such as temperature, pH, and incubation time.

Validation and quality control

When a method has been developed and optimized, it must also be validated to demonstrate that it is accurate and appropriate for the intended purpose (65). Among other things, this validation involves checking the assay's accuracy, limits of quantification, linearity, selectivity, and sample stability (66, 67). The term accuracy includes both precision and trueness. Many different factors can contribute to the variation in a measurement result. These factors include the skill of the technician, the equipment, the measuring instrument, the calibration, and the reagent lot, as well as the environmental conditions such as temperature and humidity.

Precision is measured as both repeatability and reproducibility and is a qualitative concept. In order to convey the precision of an assay, its imprecision is expressed in terms of both the standard deviation (SD) and the coefficient of variation (CV), often expressed as CV % = SD/mean*100. When repeatability is examined, as many factors as possible must remain constant, with the same sample being repeatedly analyzed in the same evaluation. This evaluation will show the smallest variation in the analytic method and is referred to as intra-assay variation. To measure the greatest variation in the method, the reproducibility is determined by conducting repeated measurements on the same samples on different days, when it is possible for different factors to vary; this variation is referred to as inter-assay variation. Since these intra- and inter-assay variations are unpredictable, they are considered random errors (68, 69).

Trueness is defined as *"the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value"* (69). This closeness is expressed as the mean difference between the measured and the true values. It is called systematic error because it is predictable and can best be checked by comparing the result with other accepted methods of analysis (70-72).

When the method has been validated and implemented in a clinical laboratory, both internal and external controls should be included to achieve reliable and accuracy test results. Internal control monitors day-to-day reproducibility of the method; and by using an external control from a national (e.g., Equalis) or international (e.g., United Kingdom External Quality Assessment Services (UKNEQAS)) quality assurance program, constant differences can be detected between the laboratory results in different laboratories. By using controls, analytic errors can be monitored and noted to indicate whether a test result can be accepted.

Furthermore, discrepancies are found between results obtained from different laboratories using different analytical techniques which underscores the need for an international calibrator which ideally should be made available to laboratories involved in complement determination and diagnostics.

The complement system

Complement activation

The complement system is a complex system consisting of about 50 proteins that is activated in a cascade-like manner. These proteins are located in the fluid phase of the body, where they promote inflammation, opsonize microbes, stimulate the recruitment of phagocytes, and also kill microbes directly. The complement proteins may also be bound to cells, where they function as receptors or regulators of complement activation. To activate the complement system, molecules on the surface of a microbe must be recognized through one of three pathways (Figure 6A):

1) The classical pathway (CP) is activated via complement protein C1q, which binds to antibodies bound to the microbe's surface. The CP can also be initiated by pentraxins (e.g., C-reactive protein [CRP]) or negatively charged molecules (e.g., DNA and heparin).

2) The lectin pathway (LP) is activated when the plasma protein mannose-binding lectin (MBL) or ficolins recognizes carbohydrate patterns on the surface of microorganisms.

3) The alternative pathway (AP) is activated by foreign surfaces such as biomaterials and certain microbial structures, e.g. lipopolysaccharide (LPS). Complement protein C3 undergoes a conformational change to C3b and then covalently binds to these surfaces.

When C1q is activated, the C1 complex (or mannose binding lectin [MBL] and ficolins, which activate MASP1-2) cleaves C4 and C2. One of the cleavage fragments, C4b, binds covalently to nearby surfaces and together with C2a forms the CP convertase (C4bC2a). The AP convertase is formed when covalently bound C3b in the fluid phase binds factor B. Factor B is then cleaved by factor D, and together with properdin forms the AP convertase (C3bBbP).
The convertases cleave C3 in to the functional fragments C3a and C3b.; C3a is an anaphylatoxin that mediates inflammation; C3b acts as an opsonin, i.e., it enables phagocytosis. Both C3 convertases can induce an amplification loop of the AP via the activation of C3, which causes more C3 to be cleaved and leads to amplification of the activation. When additional C3b molecules are added to

the C3 convertases, the enzymatic specificity changes from C3 to C5. Upon cleavage of C5, C5b and the anaphylatoxin C5a (more potent than C3a) are formed. This step is the start of the terminal pathway, whose end product is C5b-9, the membrane attack complex (MAC), which can be inserted into the cell membrane and induce cell lysis. The MAC complex can also remain in plasma as soluble C5b-9 (sC5b-9), which together with C3a and C5a can be used as a marker for complement activation in research and diagnostics (73-75).

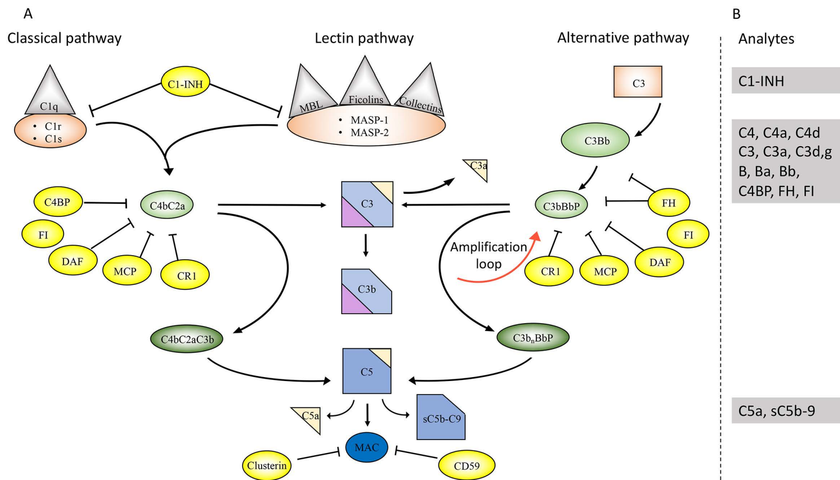

Figure 6. *Overview over the complement system. (A) The complement cascade can be activated by three different pathways: the classical, the lectin and the alternative pathway. A number of membrane-bound and fluid phase inhibitors regulate the complement activation. Color coding: grey triangles, recognition molecules; orange symbols: initiators i.e., serine proteases and C3; green ovals: convertases; bright yellow ovals: inhibitors; dim yellow triangles: anaphylatoxins. (B) Selected analytes that are suitable for use to assess of complement function and activation. From Ekdahl KN, Persson B, Mohlin C, Sandholm K, Skattum L, Nilsson B. Interpretation of Serological Complement Biomarkers in Disease. Front Immunol. 2018;9:2237.*

Complement regulation

An uncontrolled activation of the complement system can lead to serious damage to autologous cells and tissues that can develop into inflammation and ultimately autoimmune disease. To prevent this uncontrolled progression and to discriminate between the surface of pathogenic cells and healthy human cells, complement activation is balanced by both membrane-bound and soluble regulators. This regulation focuses on the most important steps at various levels

in the complement cascade: initiation, amplification (leading to cleavage of C3 and C5), and formation of the MAC (76).
C1 inhibitor (C1INH) is a proteinase inhibitor that inactivates a variety of proteases in both the complement and contact/coagulation cascades. In the complement cascade, C1INH regulates the proteases C1r/C1s and MASP-1/MASP-2 in the CP and LP, respectively, as well as proteases in the contact system such as Factor (F)XIIa, FXIa, and kallikrein (77).

Anaphylatoxins can be cleaved and thereby inhibited by serum carboxypeptidase N (78). The cell-linked regulators that inactivate the C3 convertase from the various activation pathways are complement receptor 1 (CR1), decay acceleration factor (DAF, also known as CD55) and membrane cofactor protein (MCP; CD46). Examples of soluble regulators are C4b binding protein (C4BP), which regulates the CP/LP convertase, and factor H (FH) and factor H-like protein 1 (FHL1), which are recruited to the host cell surface to downregulate the AP convertase. The terminal pathway is regulated by CD59 and clusterin, both of which are cell-bound, and vitronectin, which is soluble. All of these molecules inhibit the formation and assembly of the MAC (73, 74, 76).

Factor H (FH)

Factor H, identified as β1H globulin in 1965 (79), is a 150-kDa single-chain glycoprotein whose concentration in plasma is about 500 mg/L. It is mainly a regulator of the AP and belongs to a protein family that includes complement FH-like protein 1 (FHL1) and five FH-related proteins (FHR1-5). FH consists of 20 individually folded protein domains, termed short consensus repeats (SCRs), or complement control protein (CCP) domains. (80-82). The function of FH is to regulate C3b by acting as a cofactor for factor I (a serine protease), which cleaves C3b and C3(H_2O) to inactivated C3b (iC3b); by competing with factor B for binding to C3b, it also prevents the formation of both the C3 and C5 convertases. FH also has decay-accelerating activity, enhancing the dissociation of the C3bBb complex. Its various domains have different functions: The N-terminal domains 1-4 are responsible for the regulatory activity, and the C-terminal domains 19-20 are responsible for the recognition of C3b on self surfaces. FH also has domains that binds heparin and pentraxins (83-85).

C4b binding protein (C4BP)

C4b-binding protein is a potent soluble inhibitor of the CP and LP, circulating in plasma at a concentration of ~200 mg/L. It is a large molecule (500 kDa), and

its major isoform in plasma (75-80%) consists of seven identical α-chains and one β-chain, with the chains being linked by disulfide bridges (86, 87). Other isoforms that occur contain either six α-chains and one β-chain, or six or seven α-chains exclusively. All the chains are composed of multiple CCP domains, with the α-chains having eight and the β-chain three CCPs. The function of C4BP is to regulate C4b-mediated responses in various ways, in part by being a cofactor of factor I, which cleaves both soluble and cell-bound C4b. In addition, it prevents the formation of the CP/LP C3 convertase (C4bC2a) by binding C4b and also accelerates the natural decay of this convertase. Each α-chain has a binding site for one C4b, but because of steric hindrance, one C4BP can only bind four C4b molecules simultaneously. In addition, all the circulating C4BP molecules containing a β-chain are in complex with the vitamin K-dependent anticoagulant protein Protein S (PS), whereas PS exists in both a free (~30 %) and a bound form. The ability of C4BP to act as an inhibitor of the complement system is not affected by the bound PS, but the bound PS has lost some anticoagulant activity (88, 89).

Cytokines and chemokines

The cells of the immune system interact with each other, and many of these interactions are mediated by cytokines. Cytokines are a heterogeneous group of soluble proteins and peptides with different structures and function that are secreted by many different cell types and bind to receptors on various target cells, thereby regulating and coordinating immunological and inflammatory processes in both the innate and adaptive immune systems. Chemokines are a group of low molecular weight cytokines that stimulate and regulate the migration of leukocytes from the blood to the tissues. This phenomenon, called chemotaxis, is possible because chemokines create a concentration gradient that guides leukocytes to areas of inflammation. The release of certain cytokines and chemokines may serve as an important marker of inflammation in experiments both *in vitro* and *in vivo* (78, 90, 91). A limited selection of cytokines and chemokines of interest for this thesis is given in table I.

Table I. Innate cytokines/chemokines relevant for this thesis and determined in Paper 2.

Cytokine	**Source**	**Selected effects**	**Increase from basal levels (results from paper 2)**
IL-1β	Macrophages	Pro-inflammatory, acute phase protein induction, coagulation	10,000-fold
IL-6	Macrophages	Pro-inflammatory, acute phase protein induction, Th17 differentiation	400-fold
TNF, tumor necrosis factor	Macrophages, T-cells	Pro-inflammatory, acute phase protein induction, coagulation	140-fold
MIP3-α macrophage inflammatory protein-3α (CCL20)	T-cells, monocytes	Chemokine, mixed leukocyte recruitment	40-fold
IL-10	Macrophages, lymphocytes	Anti-inflammatory, macrophage inhibition	35-fold
IL-8 (CXCL8)	Macrophages, monocytes	Chemokine, neutrophil recruitment	15-fold
GM-CSF, granulocyte-macrophage colony stimulating factor	Macrophages, T-cells	Growth factor granulocyte differentiation	10-fold
IP-10, interferon gamma induced protein 10 (CXCL10)	Macrophages, endothelial cells	Chemokine, effector T-cell recruitment	8-fold
IL-23	Macrophages, dendritic cells	Th17 differentiation	7-fold
IL-12(p70)	Macrophages, dendritic cells	Th1 differentiation	4-fold
GRO-α (CXCL1)	Macrophages, neutrophils	Chemokine, neutrophil recruitment	4-fold
IL-17 A	Th17 cells	Cytokine production, inflammation	1.5-fold
MDC (CCL22)	Macrophages, dendritic cells	Chemokine, T-cell recruitment	No change
MCP-1 monocyte chemotactic protein (CCL2)	Macrophages, endothelial cells	Chemokine, mixed leukocyte recruitment	No change

Pathology of complement

The complement system is primarily perceived as a host defense system, but there is also a potentially more damaging side to the innate immune pathway. The ability of the complement system to control threats from pathogens and to eliminate cellular debris can quickly make it a detrimental factor for its host. Deficiencies in the complement system and insufficient regulation can cause many pathophysiological processes in a number of diseases and biomedical interventions, including borreliosis infection (mentioned below), inflammation, autoimmune disease, and allogeneic and xenogenic transplantation (92, 93).

In many inflammatory diseases, a defect in a complement factor or excessive complement activation caused by insufficient regulation can occur. There are a number of diseases in which the complement system has a role, some of which are illustrated in figure 7. There are a limited number of biomarkers that are used for the diagnosis of diseases in which the complement system is involved. These markers can be used to monitor individual patients in their disease course and evaluate their treatment if the baseline levels of the markers are known (11, 94).

Complement diagnostics

To obtain a complete picture of the status of a patient's complement system, several different analyses must be performed (Figure 6B). Functional analyses are done to screen for deficiencies in the CP and AP, either with hemolytic assays or ELISAs. To determine which component is deficient, analyses of the concentrations of individual factors (e.g., C1q, C3, C4, FB) are performed frequently by means of various types of immunoassays. In clinical practice, nephelometry and turbidimetry are most frequently used.
Upon complement activation, activation products are formed that can be measured with both commercial immunoassays and in-house assays. Assays for these activation products (C3a, C3b/iC3b/C3c, C4a, C4b, C4d, Ba, Bb, C5a, sC5b-9) most often use monoclonal antibodies that recognize a neoepitope, i.e., an epitope that becomes available upon cleavage of the native protein. If polyclonal antibodies are used, a fractionation by size must be done. For example, if C3d,g is to be analyzed, precipitation must be used to remove the large forms of C3 before analysis. This step is required because a polyclonal anti-C3d,g antibody also recognizes C3, C3b, and iC3b. When complement activation *in vivo* is analyzed, it is important to determine the ratio of the C3a or C3d,g level to the total level of C3 (C3a or C3d,g/C3), in part because there is a continuous generation of C3 activation fragments via the AP, but primarily

because the basal levels of C3 can vary considerably, either because of consumption or because C3 is an acute-phase protein. If samples intended for the detection of complement activation are collected and handled in the wrong way (see Pre-analytical factors), complement activation markers can be produced *in vitro* (11).

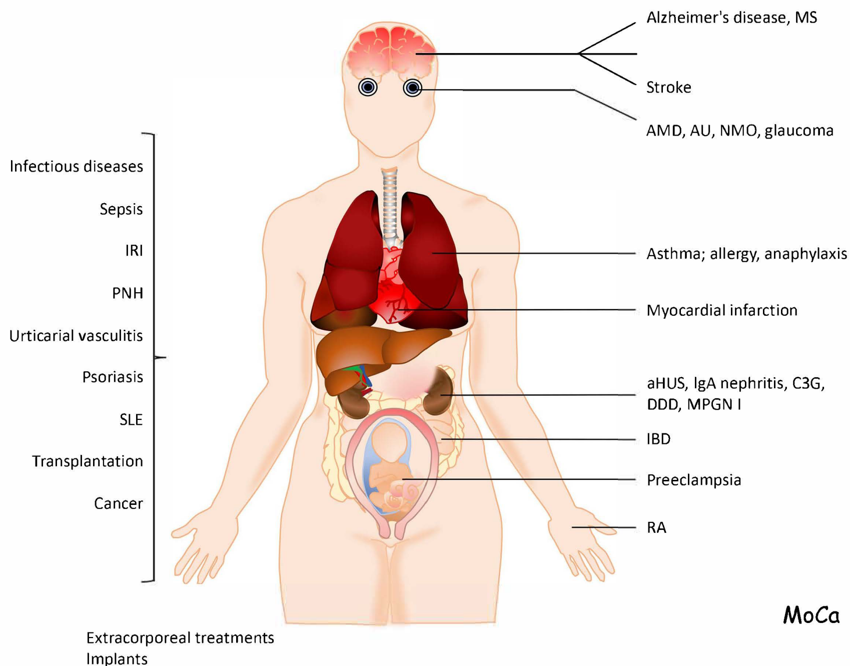

Figure 7. *Examples of diseases and treatments in which the complement system is involved. Some of these pathological conditions are systemic while others are organ-specific. IRI; ischemia reperfusion injury; PNH, paroxysmal nocturnal hemoglobinuria; SLE, systemic lupus erythematosus; MS, multiple sclerosis, AMD, age-related macular degeneration; AU, autoimmune uveitis; NMO, neuromyelitis optica; aHUS, atypical haemolytic uremic syndrome; C3GN, C3 glomerulonephritis; DDD, dense deposit disease; MPGN, membranoproliferative glomerulonephritis; IBD, irritable bowel disease; RA, rheumatoid arthritis. From Ekdahl KN, Persson B, Mohlin C, Sandholm K, Skattum L, Nilsson B. Interpretation of Serological Complement Biomarkers in Disease. Front Immunol. 2018;9:2237 (11)*

Borrelia infection

Lyme borreliosis (LB) is a tick-borne disease caused by spirochetes belonging to the *Borrelia burgdorferi* sensu lato complex. These infections occur in temperate regions of the Northern Hemisphere. LB is caused by at least three genospecies, particularly *Borrelia burgdorfei sensu stricto*, *B. afzelli,* and *B. garinii* (95). The first symptoms of LB may appear as an annular migratory rash (erythema migrans) in the skin, starting as a small spot at the site of the tick bite. If the infection is not treated, the spirochetes can spread to other organs in the body and cause serious conditions. *B. afzelii* is most associated with skin manifestations, *B. garinii* causes neuroborreliosis, and *B. burgdorferi* appears to be the most arthritogenic (96).

Borrelia genospecies are classified as either serum-sensitive or serum-resistant according to their ability to survive in the presence of human complement. To avoid complement activation, many pathogens have developed mechanisms to use the host's complement regulators on the cell surface. Their hijacking of host regulators is possible because these pathogens express complement regulator-acquiring surface proteins (CRASPs). These proteins are lipoproteins expressed on the outer surface of *Borrelia* that bind one or more host proteins of the factor H family (97-99); binding of C4BP had also been observed (100).

C1q

Complement protein C1q is a large complex glycoprotein with a molecular size of 460 kDa and a plasma concentration of ~180 mg/L. It is a hexamer with a tulip-like structure and consists of 18 polypeptide chains, each containing 3 different chains (A, B, and C). The N-termini of the chains consist of a collagen-like region, and the C-terminal portions of the chains combine to form a globular domain that has affinity for the Fc portion of IgG and IgM. C1q is part of the large Ca^{2+} -dependent C1 complex that also includes the serine proteases C1r and C1s (101, 102). The complement proteins are mostly synthesized in the liver, but C1q can be synthesized by a variety of cells, including monocytes/macrophages (103), epithelial cells (104), dendritic cells (105), microglial cells (106), and endothelial cells (107).

C1q has been assigned functions related to both antibody-dependent and -independent immunity. Immune complexes (ICs) and other molecules (e.g., C-reactive protein [CRP], serum amyloid P component [SAP], and pentraxin-3 [PTX3]) are recognized by C1q and activate the CP, which effectively eliminates pathogens and apoptotic cells (108).

C1q is also involved in a rapidly expanding list of pathological conditions, including autoimmunity, preeclampsia, and cancer, as well as in trophoblast invasion (109, 110).

C1q is often deficient in systemic lupus erythematosus (SLE), a systemic autoimmune disease whose symptoms can include skin rash, arthritis, chronic fatigue, glomerulonephritis, and CNS involvement. The formation of autoantibodies against DNA and histones is a major mechanism of this disease. ICs can be formed, which activate and consume the proteins in the CP during exacerbation of the disease. These ICs can be deposited in the kidney glomeruli, leading to complement activation that damages the tissue, in turn leading to SLE with nephritis (111). Homozygous hereditary deficiency of C1q is associated with a very high risk of SLE development. Studies have shown that 88% of those with C1q deficiency develop SLE, and 30% have glomerulonephritis. It is therefore important to obtain information about C1q levels, since they may be of great value for monitoring SLE disease activity in these patients (112, 113).

AIMS

This thesis comprises projects in which various immune assays have been developed and evaluated as tools for complement diagnostics both in plasma and in cerebrospinal fluid (CSF). Various methods have been used, including ELISA, Western blot, flow cytometry, and xMAP technology.
The overall emphasis of the work is the importance of validating assays with high sensitivity and specificity to enable accurate diagnosis in patients with various inflammatory conditions and suspected complement perturbations.

MATERIALS AND METHODS

In the papers included in this thesis, various immunochemical methods have been used to qualitatively investigate and quantitatively measure the concentration of complement proteins and cytokines/chemokines in human plasma and cerebrospinal fluid (CSF). In a few cases we have used commercial kits, but for most analytes we have developed, optimized, and used our own in-house methods utilizing antibodies, both monoclonal and polyclonal, from various companies.

Material

Patients

Paper 1

Plasma + CSF from 298 patients with suspected neuroborreliosis (Åland Central Hospital, Finland)

Paper 2

Lepirudin-treated blood and plasma from 8 healthy blood donors (Linnaeus University, Kalmar, Sweden)

Paper 3

1: Serum from 85 patients with different diagnoses (Region Skåne, Lund, Sweden)
2: Plasma from 379 SLE patients (with/without nephritis) and 322 controls (Karolinska University Hospital, Solna, Sweden)
3: CSF from 31 patients with suspected neuroborreliosis (Åland Central Hospital, Finland)

Paper 4

1: Paired plasma samples from 22 patients with and without warfarin (VKA) treatment; cross-over study (Université de la Méditerraneé, Marseille, France).
2: Paired plasma samples from 34 patients with and without direct oral anticoagulant (DOAC) treatment; cross-over study (Université de la Méditerraneé, Marseille, France).
3: Samples from 28 healthy controls from Clinical Research Dalarna - Uppsala University, Falun, Sweden.

In this thesis, blood supplemented with three different anticoagulant agents has been used.

In papers 1, 3, and 4, blood was collected in ethylendiaminetetraacetic acid **(EDTA)** vacutainer tubes, and the resulting plasma was used for determination of concentrations of intact complement proteins and activation products. CSF was collected, and EDTA was added to a final concentration of 10 mM when the samples were thawed prior to analysis. The mechanism of action of EDTA is to chelate metal ions, including Ca^{2+} and Mg^{2+}, which are necessary for activation of the coagulation system (Ca^{2+}) and the complement system (both Ca^{2+} and Mg^{2+}).

In paper 2, blood was collected in plastic vacutainer tubes with the addition of **hirudin** (Refludan®, BD Bioscience, Plymouth, UK) at a final concentration of 50 µg/mL blood. Hirudin is a small protein (65 amino acids) that was originally isolated from the medicinal leech *Hirudo medicinalis* but is now produced recombinantly (lepirudin, Refludan®). Hirudin inactivates thrombin so that fibrinogen cannot be cleaved to fibrin, thereby inhibiting clot formation (114). Hirudin/lepirudin has very limited effects on complement activation and has been recommended as an anticoagulant in models to study complement *in vitro* (61, 115).

In Paper 4, **citrate plasma**, which is widely used in the analysis of coagulation function, was used. Citrate is a chelating agent that binds Ca^{2+}, but its effect can be reversed by the addition of Ca^{2+} to the sample.

Methods

Enzyme linked immunosorbent assays (papers 1,2, 3, and 4)

C1q, C3, and C4

To measure fluid-phase C1q, C3/C3 fragments and C4/C4 fragments in EDTA-plasma and CSF, three sandwich ELISAs were developed (paper 1). Microtiter plates were coated with polyclonal antibodies against anti-Hu-C1q (rabbit) (Dako A/S, Glostrup, Denmark) anti-Hu-C3c (rabbit) (Dako) and anti-Hu-C4c (rabbit) (Dako) antibodies. The same antibodies, but biotinylated, were used as detecting antibodies, followed by streptavidin-HRP (GE Healthcare, Little Chalfort, U.K.).
Plasma with known concentrations of the complement proteins was used as a calibrator, and a control of pooled plasma was included in each determination.
In paper 3, a C1q ELISA kit from Hycult (Uden, Netherlands) was used when the results were compared to those obtained with other C1q methods.

Complement activation markers C3a, C4a, and sC5b-9

The complement activation products C3a (papers 1, 2) and sC5b-9 (paper 2) were measured with in-house sandwich ELISAs as previously described (3, 116) The monoclonal antibody 4SD17.3, recognizing a neoepitope on C3a, was used as the capture antibody, and detection was performed using a biotinylated polyclonal anti-Hu-C3a (rabbit) antibody. To measure the sC5b-9 complex, a monoclonal anti-Hu-C9 aEII antibody (Bioporto Diagnostics A/S, Hellerup, Denmark) was used for capture, and polyclonal biotinylated anti-Hu-C5 (sheep) antibody BP373 (Acris, Herford, Germany) for detection. Zymosan-activated serum calibrated against known C3a and sC5b-9 concentrations served as the standards, and a pool of activated serum was used as a control. To measure the activation of the classical and lectin pathways (paper 4), C4a was analyzed with a commercial EIA kit (A036, Quidel, San Diego, CA, USA).

Quantification of complement proteins bound to spirochetes

An ELISA-like assay was developed to measure C3 and C4 fragments, proteins of the FH family, and C4BP that bound to spirochetes during incubation with lepirudin-plasma (paper 2). The bound proteins were detected with polyclonal antibodies anti-C3c anti-C4c from Dako and anti-FH and anti-C4BP from The Binding Site (Birmingham, UK) which were biotinylated prior to use.

Rocket immunoelectrophoresis (paper 3)

To compare the quantification of C1q with a newly developed method in paper 3, two in-house rocket immunoelectrophoresis methods were used. These assays were performed in Lund, Region Skåne, Sweden and in Karolinska University Hospital, Solna, Sweden.

Western blot analysis (papers 1, 2)

Western blotting was used in paper 1 to investigate which forms of C3 could be found in CSF in the patient samples. For this purpose, an anti-Hu-C3c (rabbit) antibody (Dako) that detects C3, C3b, and iC3b, in addition to C3c, was used. In paper 2, Western blotting was used to study the structure of C3 bound to the spirochetes and to determine whether Factor H-like protein 1 (FHL-1) and/or FH-related proteins (FHR) bound, in addition to FH. For this work, anti-Hu-C3c (rabbit) antibody (Dako) and anti-Hu-FH (sheep) antibody (The Binding Site) were used.

Cytokine and chemokine analysis (paper 2)

To measure the cytokine and chemokine concentrations in plasma after incubation with *Borrelia* spirochetes in lepirudin-treated blood, Luminex xMAP technology (Milliplex Human Cytokine/Chemokine kit, Merck Millipore, Burlington, MA, USA) was used. The analyzed cytokines and chemokines were Il-1β, IL-6, IL-10, IL-12 (p70), IL-17A, IL-23, TNF, GM-CSF, CXCL10 (IP-10), CXCL1 (GRO-α), CCL22 (MDC), CXCL8 (IL-8), CCL20 (MIP3-α) and CCL2 (MCP-1).

Flow cytometry (paper 2)

Borrelia spirochetes were grown in Barbour-Stoenner-Kelly II medium (117) until the density reached 10^8-10^9 cells/mL. To enable the study of phagocytosis by leukocytes, the spirochetes were washed and labeled with fluorescein (118). Measurement of the fluorescence was performed with a flow cytometer (Cell Lab Quanta SC, Beckman Coulter, Miami, FL, USA), with granulocytes and monocytes being analyzed according to size (forward scatter) and granularity (side scatter). About 5000 granulocytes and ~500 monocytes were collected, and the data are presented as mean fluorescence intensity (MFI).

Magnetic bead-based immunoassay (papers 3, 4)

Paramagnetic carboxylated microspheres (xMAP microspheres) (Bio-Plex Pro Magnetic COOH Beads, BIO-RAD, Hercules, CA, USA) and a commercially available Amine Coupling Kit (BIO-RAD) were used when the antibodies were coupled to the beads. The manufacturer´s instructions were followed, and titration of the antibody concentration/batch of beads (1.25 x 10^6 beads) was done to obtain optimal assay performance. The detecting antibodies had to be biotinylated, and titrations were made to determine the optimum concentration of the biotinylated antibodies to be used. International Complement Standard #1 (ICS#1), pooled serum from 45 blood donors (12, 119), was used as a calibrator in all xMAP methods. Table II shows which antibodies and what concentrations were used as capturing and detecting antibodies in each in-house xMAP. Measurement intervals and sample dilutions are also presented.

Procedure

The assay was performed in 96-well flat-bottom black microtiter plates (Bio-Plex Pro Flat Bottom Plates, BIO-RAD), and antibody-coupled beads were used at a concentration of 2500 beads per analyte/well. All standards, controls, samples, and antibodies were diluted in assay buffer PBS (10 mM sodium phosphate buffer, pH 7.4, with 0.145 M NaCl) supplemented with 0.1 % bovine

serum albumin (BSA; Sigma, St. Louis, MO, USA) and 0.02% Tween 20 (Sigma) and tested in duplicate (50 µL/well). Between each addition of sample, detecting antibody, or streptavidin PE (1:100) (BIO-RAD), the plate was incubated for 30 min at room temperature, in the dark and under rotation at 850 rpm. The plates were washed with PBS + 0.05% Tween 20 after each incubation. The beads were resuspended in 125 µL assay buffer/well, and the mean fluorescence intensity (MFI) was measured in a BioPlex MAGPIX Multiplex Reader (BIO-RAD) using Bio-Plex manager MP Software and Bio-Plex Manager 6.1 (BIO-RAD) to calculate the results.

Table II. Concentrations of antibodies, measurement intervals and sample dilutions in the xMAP analyses in this thesis.

	C1q	**C4BPtotal**	**C4BPβ chain**	**C4BP-protein S**	**sC5b-9**
Capture antibody	anti-Hu C1q mAb clone WL02 Hycult	anti-Hu C4BPα mAb clone 10-07 Bio-Rad	anti-Hu C4BPα mAb clone 10-07 Bio-Rad	anti-Hu C4BPα mAb clone 10-07 Bio-Rad	anti-Hu C5b-9 mAb Clone aE11 BioPorto
Conc.capture ab/1.25 x 10^6 beads	3µg	3µg	3µg	3µg	3µg
Detecting biotinylated Ab.	anti-Hu C1q mAb clone DJ01, Hycult	anti-Hu C4BPα mAb clone 10-07 Bio-Rad	anti-Hu C4BPβ (rabbit) pAb MyBioSource	anti-Hu protein S (sheep) pAb Bio-Rad	anti-Hu C5 (sheep) pAb Origene
Conc. detecting ab.	1 µg/mL	1 µg/mL	2 µg/mL	13 µg/mL	4 µg/mL
Measurement interval	0.37-375 µg/L	0.78-400 µg/L	0.07- 37.5 AU/mL	0.07- 37.5 AU/mL	1.92-985 µg/L
Sample dilution	1:5000	1:5000	1:10 000	1:10 000	1:5

Nephelometry (papers 3 and 4)

In paper 3, complement C1q was analyzed by nephelometry in two ways: either with a BN Pro Spec system (Siemens Healthcare, Erlangen, Germany) using reagents from Siemens or with an in-house method with polyclonal sheep anti-Hu C1q antibodies (The Binding Site) with an IMMAGE nephelometer (Beckman Coulter, Bromma, Sweden). Complement factors C3 and C4, in

paper 4, were measured on an IMMAGE nephelometer (Beckman Coulter) using Complement C3 reagent (446450) and Complement C4 reagent (446490) (Beckman Coulter).

RESULTS AND DISCUSSION

The aims of **study 1** were to characterize the pattern of activation in patients with well-defined neuroborreliosis (NB); i.e., whether complement is activated and whether activation occurs systemically and/or intrathecally. We therefore analyzed the concentrations of complement factors C1q, C3, and C4 and the activation marker C3a in EDTA-plasma and CSF from a total of 298 patients who were admitted to hospital with symptoms of suspected NB. Of these, 23 were subsequently diagnosed with NB, 47 with other Borrelia infection (B), 20 with CNS involvement of non-Borrelia origin (CNS), 16 with other CNS-infections (I), and 192 were considered controls (C), i.e., no diagnosis was reached.

To avoid complement activation *in vitro*, the samples were centrifuged and frozen at -70°C within 4 hours and transported on dry ice to the laboratory. At the time of analysis, the samples were rapidly thawed in a 37°C water bath and thereafter stored in an ice-water bath. EDTA (10 mM final concentration) was added to the CSF samples immediately after thawing. In this study we had five different groups (NB, B, CNS, I, C) of patients, the analyses were performed blinded, and every sample was analyzed in duplicate. Complement activation was monitored in terms of the generation of C3a, as previously described (3). To determine the concentrations of C1q, C3, and C4 in plasma and CSF, we developed sandwich ELISAs for each analyte. In each assay, polyclonal antibodies directed against C3c, C4c, or C1q were used for both capture and detection.

Before being used to analyze patient samples, the assays were optimized regarding the concentration of capturing and detecting antibody. In addition, the linearity, precision, measure intervals, and sample dilutions were determined for each assay. Daily internal controls were determined, and the inter-assay CV% values for the three assays were 7.1% for C1q, 19.0% for C3, and 12.9% for C4. In the literature, the recommended levels regarding precision and accuracy in immunoassay are generally in the 10 to 25% range (63, 120). When commercially available ELISA kits are used, the company provides recommendations on the levels of precision that should be approved.

We did not find any significant differences in the plasma levels of C1q, C3, C4, or C3a between the control group and the other four groups. In contrast, in CSF, the levels of C1q, C3, C4, and C3a were significantly elevated in patients with

NB when compared to the control group, as well as to the other groups (paper 1, Table 3, Fig. 1A). We also concluded that complement activation had occurred in the CNS, since we found significantly elevated values for C3a in CSF from NB patients when compared to the control group (paper 1, Fig. 1B). Complement activation in the CSF has been studied in the host defense against both bacterial and viral infections and also recently against tick-borne encephalitis (121-123). All these studies have used ELISA kits from different companies to analyze complement proteins and activation products. However, as yet there is no international complement calibrator available, so the absolute levels of complement parameters in CSF/plasma cannot be compared in studies from different laboratories. Our results concerning C1q generation in the CNS are in accordance with previous studies, e.g., (124, 125), suggesting that our in-house assays are reliable and accurate.

We can conclude that in human Lyme NB, complement activation occurs in CNS, since our results have shown increased intrathecal levels of C1q, C3/C3 fragments, C4/C4 fragments, C3a, and we also found a positive correlation between C1q and C3a in the CSF. No systemic complement activation could be detected, since normal levels of C1q, C3, C4, and C3a were found in the plasma.

In **paper 2,** we aimed to investigate the role of complement activation in phagocytosis and the release of cytokines and chemokines in response to two clinical isolates of *Borrelia. Borrelia afzelii* K78 (complement-resistant) and *B. garinii* LU59 (complement-sensitive) were incubated with lepirudin-treated plasma, and the complement activation was monitored in terms of the generation of C3a and sC5b-9.

Higher levels of both C3a and sC5b-9 were found in plasma incubated with *B. garinii* LU59 than in plasma incubated with *B. afzelii* K78 (paper 2, Fig. 1A). This difference may be explained by the fact that *B. afzelii* K78 has a better ability to protect itself from complement activation because of its expression of several CRASPs, which can bind the complement inhibitors FH and FHL-1. This situation in contrast to that in *B. garinii* LU59, which only expresses one form of CRASP that has been reported to have a weak affinity for FH and FHL-1 (97).

In order to study the binding of various complement proteins, we developed an ELISA-like assay in which the bacteria served as the solid phase, then confirmed the results by Western blotting. In the ELISA-like assay, spirochetes from the two *Borrelia* strains were incubated in lepirudin-plasma, followed by precipitation and repeated washing of the spirochete pellet by centrifugation. Thereafter, the amounts of C3/C3 fragments, C4/C4 fragments, FH family

proteins, and C4BP were (semi-)quantified using polyclonal antibodies. Since the detecting C3c antibody recognizes native C3, C3b, iC3b, and C3c, we used Western blotting to verify that the bound C3 was quantitatively degraded to iC3b. iC3b is inactive because it cannot participate in the alternative convertase, but it still functions as an opsonizing fragment, thereby promoting phagocytosis. With both methods, we found that *B. afzelii* K78 bound more proteins belonging to the FH family (including FHL-1 and FHR proteins) than did *B. garinii* LU49; therefore, we saw higher binding of C3 fragments to *B. garinii* LU49 (paper 2, Fig. 1B-F). Both C4 fragments and C4BP were detected at low levels, but with no significant difference between the two *Borrelia* strains.

To investigate to what extent complement activation is necessary for phagocytosis, FITC-labeled spirochetes (*B. afzelii* K78, *B. garinii* LU49) were incubated in lepirudin-treated blood, with or without various inhibitors: EDTA (which binds Ca^{2+} and Mg^{2+} to completely inhibit complement activation); compstatin (a synthetic peptide C3 inhibitor) (126); C5aRa (a C5a receptor antagonist); and a scrambled peptide of C5aRa, which was used as control. To study phagocytosis, we developed a flow cytometry-based method with which we monitored the increase in fluorescence in granulocytes and monocytes as a measure of the phagocytosis of FITC-labeled spirochetes. Under our experimental conditions, *B. garinii* LU59 was phagocytosed to a lower degree (~70%) than was *B. afzelii* K78, and both compstatin and C5aRa resulted in a 50-80% reduction in phagocytosis of both *Borrelia* strains, suggesting that complement activation is important for phagocytosis, but not absolutely necessary (paper 2, Fig. 2B).

We then investigated the significance of complement activation for cytokine and chemokine generation and release in response to the two *Borrelia* strains and were able to demonstrate that 6 of 14 selected cytokines/chemokines (IL-1β, IL-6, TNF, CCL20, IL-10, and CXCL8) showed a 10- to 10,000-fold increase when compared to baseline values (Introduction, Tab.I), with no difference between the two *Borrelia* strains. By including inhibitors for activation at the C3 and C5 levels, we were able to show that complement activation had no effect on cytokine/chemokine release, except for CXCL8, which showed a significant decrease for *B. garinii* LU59 in the presence of compstatin (paper 2, Fig. 3).

Based on these results, we can conclude that 1) the complement-resistant K78 strain of *Borellia* recruits more FH and activates complement to a lesser extent than does the complement-sensitive Lu59 strain; 2) complement activation is important for the phagocytosis of *Borrelia* spirochetes, but not crucial; and

3) inhibition of complement activation has no effect on the secretion of the cytokines/chemokines studied in this *in vitro* system.

In **paper 3,** we developed and evaluated an immunoassay for the quantification of C1q for clinical diagnostic use. C1q is a useful marker of disease activity and efficacy of treatments in various diseases, not only in SLE but also in neurological and psychiatric conditions (109).

C1q is a complex molecule that binds to immune complexes (IC) and antigen-bound antibodies. Many methods that are often used in clinical laboratories, including nephelometry and turbidimetry, are based on IC formation, but they may give misleading results as a result of the antibody-binding properties of C1q. In order to address this issue, we developed and evaluated an in-house magnetic bead-based sandwich immunoassay (MBSI) to measure C1q in serum/plasma and CSF.

In our new method based on xMAP technology (24), we used a monoclonal antibody pair that bound different epitopes on C1q but did not interfere with C1q's binding to the antigen. The capture antibody, in different concentrations, was coupled to paramagnetic carboxylated microspheres. To obtain an optimal assay, we titrated the concentrations of both the capturing and detecting antibody. To develop and optimize our method, we determined the best assay buffer, measurement interval, limit of quantification, linearity, intra- and inter CV% (paper 3, Table 2) and calculated a reference interval based on 100 healthy blood donors (paper 3, Fig. 5). Our optimizations indicated that PBS supplemented with 0.1% BSA and 0.02% Tween 20 performed well as the assay buffer; coupled beads with 3 µg anti-C1q/1.25 ^ 10^6 beads, together with 1 µg /ml biotinylated anti-C1q as detecting antibody provided the conditions for a good method for measuring C1q. Complement standard # 1 (150 mg/L) was serially diluted in two-fold dilutions from 1: 400 in nine steps. To measure the background, assay buffer alone was used. Plasma/serum samples were diluted 1:5000, and CSF was diluted 1:50, both in assay buffer. The measurement interval was 375 µg/L to 0.73 µg/L (paper 3, Fig. 2A). The raw data acquired from a MAGPIX Multiplex reader were expressed as mean fluorescence intensity (MFI), and the standard curve had a wide range of MFI 28000 – 50, making the values reliable and reproducible even at low concentrations (paper 3, Fig. 2B). This high reliability and reproducibility can be seen in our comparison of previous C1q analyses in CSF to our in-house ELISA, in which the previous analyses showed a good correlation with our new method (paper 3, Fig. 4). The concentration in CSF was only ~1/100 of the concentration in plasma, but the lower sample dilution and wide measurement range of our new

method gave reliable values at low MFI as well as at higher concentrations of C1q.

We then went on to compare this novel assay with various techniques currently used to evaluate C1q in the clinic. In the first study, 40 serum samples with different C1q levels that had been analyzed by rocket immunoelectrophoresis (RIE) were compared to the values obtained for the same samples from nephelometry, a commercially available ELISA from Hycult, and a previously developed in-house ELISA using polyclonal antibodies (paper 3, Fig. 1). We then decided to use the antibody pair from Hycult in our new MBSI, since we found a high correlation between RIE and the Hycult ELISA (r = 0.880), but only a negligible correlation between RIE and nephelometry (r = 0.011), (paper 3, Fig.1A-C).

We then did a second study with another 45 serum samples to compare our new MBSI with RIE, nephelometry, and our in-house ELISA. As before, the MBSI correlated strongly with the results from both RIE (r=0.896; ρ=< 0.0001) and ELISA (r= 0.960; ρ=< 0.0001), but we found that nephelometry was not suitable for C1q analysis (paper 3, Fig. 3A-C).

We then used our MBSI to evaluate the C1q concentration in a large group of SLE patients (n = 379) with (n=69) or without (n=310) ongoing nephritis, who had previously been assessed by RIE. The renal disease was assessed by using the BILAG index (127). We also used MBSI to quantify C1q in age-and sex-matched controls (patients without SLE, n=322). The C1q levels of the SLE patients were significantly lower than those in the control group. Both methods showed a significant difference in C1q concentration between the SLE patients with and without nephritis (paper 3, Fig. 6A-B). We also found a relationship between the C1q concentration and the SLE disease activity index (SLEDAI) value (128), with patients who had a high SLEDAI having consistently lower C1q concentrations (paper 3, Fig. 7A-B).

We also did a minor study to determine whether the concentration of C1q differs between EDTA plasma and serum taken from same donors at the same time and analyzed in parallel. We noted that the C1q concentration was about 5% lower in serum, which can be explained by the fact that C1q binds to the surface of activated platelets (129, 130), which are removed as part of the centrifugation process.

Finally, we were able to establish that this novel assay is not affected by C1q autoantibodies (n = 5) (paper 3, Fig. 3A-C) and was not hampered by a nonspecific interaction between C1q and the detection antibodies, which often

occurs in the case of nephelometry or turbidimetry because their methodology is based on the formation of IC in the fluid phase.

A study has been performed by Rosenberg-Hasson to compare serum and plasma in the analysis of cytokines by multiplex immunoassay (131); this study has shown that plasma gives a lower non-specific background and that low-level changes can be detected in cytokine analysis. The authors also found that the nonspecific background differed between different individuals. Thus, we must be aware that different types or levels of interference may occur in different immunoassays.

In summary, we have developed an MBSI method for measuring C1q that correlates strongly with RIE and ELISA. Our MBSI has acceptable levels of intra- and inter-assay variation, indicating that the assay is accurate and reproducible. The new assay is highly sensitive, and consequently we can measure low concentrations of C1q in both plasma and CSF. C1q in plasma, analyzed with both RIE and our MDSI, shows a clear connection to nephritis and SLEDAI in SLE patients. On the basis of our findings, we suggest that analysis of C1q by nephelometry should be replaced with MBSI or ELISA in clinical diagnostic laboratories.

In **paper 4**, we developed xMAP methods to measure the total plasma concentrations of total C4BP (C4BPt), its β-chain content, and its complex with protein S (PS). C4BP consists of seven α-chains and one β-chain, but other isoforms can exist. In plasma, the β-chain is always bound to PS with high affinity. PS is a vitamin K-dependent protein and is the cofactor of activated protein C, a regulatory protein in the coagulation cascade.

The aim in paper 4 was to measure the concentration of C4BPt and complement activation in plasma from paired patients with and without treatment with a vitamin K antagonist (VKA) (n = 22) and with and without treatment with direct oral anticoagulants (DOAC) (n = 34). Complement activation was monitored by quantitating C3a and C4a (both by ELISA) as well as sC5b-9 after our previously developed in-house ELISA was converted to an MBSI using the same capturing and detecting antibodies. To measure C4BPt, a monoclonal anti-C4BPα was coupled to magnetic beads, and the same antibody was biotinylated and used as the detecting antibody. A specific antibody against the β-chain was used as a detecting antibody in the determination of C4BPβ, and an anti-PS antibody was used in the analysis of C4BP-PS complexes. Beads coupled with anti-C4BPα were used in all our analyses. All the methods were optimized and validated as previously discussed in paper 3. C3 and C4 were analyzed by nephelometry and C1q by our published MBSI method (paper 3).

Our results showed significantly lower concentrations of C4BPt in patients receiving VKA-treatment than in patients without VKA, which corresponds well with earlier reports (132, 133). We also saw a selective decrease in the levels of C4BPβ and of C4BP-PS complexes in VKA-treated patients (paper 4, Fig. 1). The lower levels of C4BP during VKA treatment were associated with complement activation, as indicated by significant increases in the C3a/C3 ratio and sC5-9 levels (paper 3, Fig. 3A,B). In the samples from DOAC-treated patients, we did not detect any decrease in the levels of C4BPt, C4BPβ, C4BP-PS, or any complement activation Our results did show a significantly reduced C1q concentration with VKA treatment, but not in the DOAC samples. These results support the activation and consumption of the classical pathway during VKA treatment, which we suspect is a result of decreased levels of the complement regulator C4BP.

In this study we were interested in the total C4BP concentration, the β-chain content, and the levels of C4BP-PS complexes. By developing our own xMAP methods, we were able to demonstrate that when C4BPt decreases, so do the levels of C4BPβ and the C4BP-PS complex in plasma. These decreases have been explained by Carlsson et al. (134) in terms of the formation of complexes of PS and C4BP in the cells, because the β-chain is very hydrophobic, and the isoform of C4BP with a β-chain requires PS for secretion.

Our new methods use coupled beads with the same capture antibody (anti-C4BPα), but we chose different detection antibodies for each analyte. For each assay, the linearity was good, and the intra-assay variation (<5%) and inter-assay variation (<15%) were acceptable. We found a strong correlation between C4BPβ and C4BP-PS complexes in our plasma samples (paper 4, Fig. 2), corroborating the conclusion that the protein S we measured is bound to the β-chain in C4BP

In our summary, we point out that our results question whether VKA is an appropriate treatment for patients with enhanced complement activation, such as IC-mediated diseases or during treatment with various biomaterials, as in hemodialysis.

CONCLUSIONS

In this thesis we have used and developed traditional sandwich ELISAs and novel xMAP methods to analyze various complement proteins and complement activation products. The xMAP technology has many advantages and great development opportunities.

Important points that should be considered in the development and use of immunoassays, in general, include:

- The specificity of the antibodies; polyclonal antibodies are batch-dependent, and the specificity can vary with production in different animals
- The antibody concentration must be optimized by titration
- Care must be taken regarding pre-analytical factors and interference that can produce unreliable results
- Thorough washing must be performed between each addition of sample dilution and antibody to avoid cross-reactions and high background
- Good quality control, reagent traceability, and internal controls are necessary for each assay
- A normal material must be analyzed for each analyte, to obtain a reference interval, since no international complement calibration is yet available

Some advantages of xMAP technology compared to other immunoassays include:

- Short assay time and low sample volume
- Low detection limits, enabling complement proteins and activation products to be assayed at low concentrations in both plasma and CSF
- High sample dilution, which reduces the problems of heterophilic antibodies and matrix interference
- Enabling the analysis of several relevant biomarkers for a specific disease in the same sample simultaneously.

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