The role of C1q in (auto) immunity

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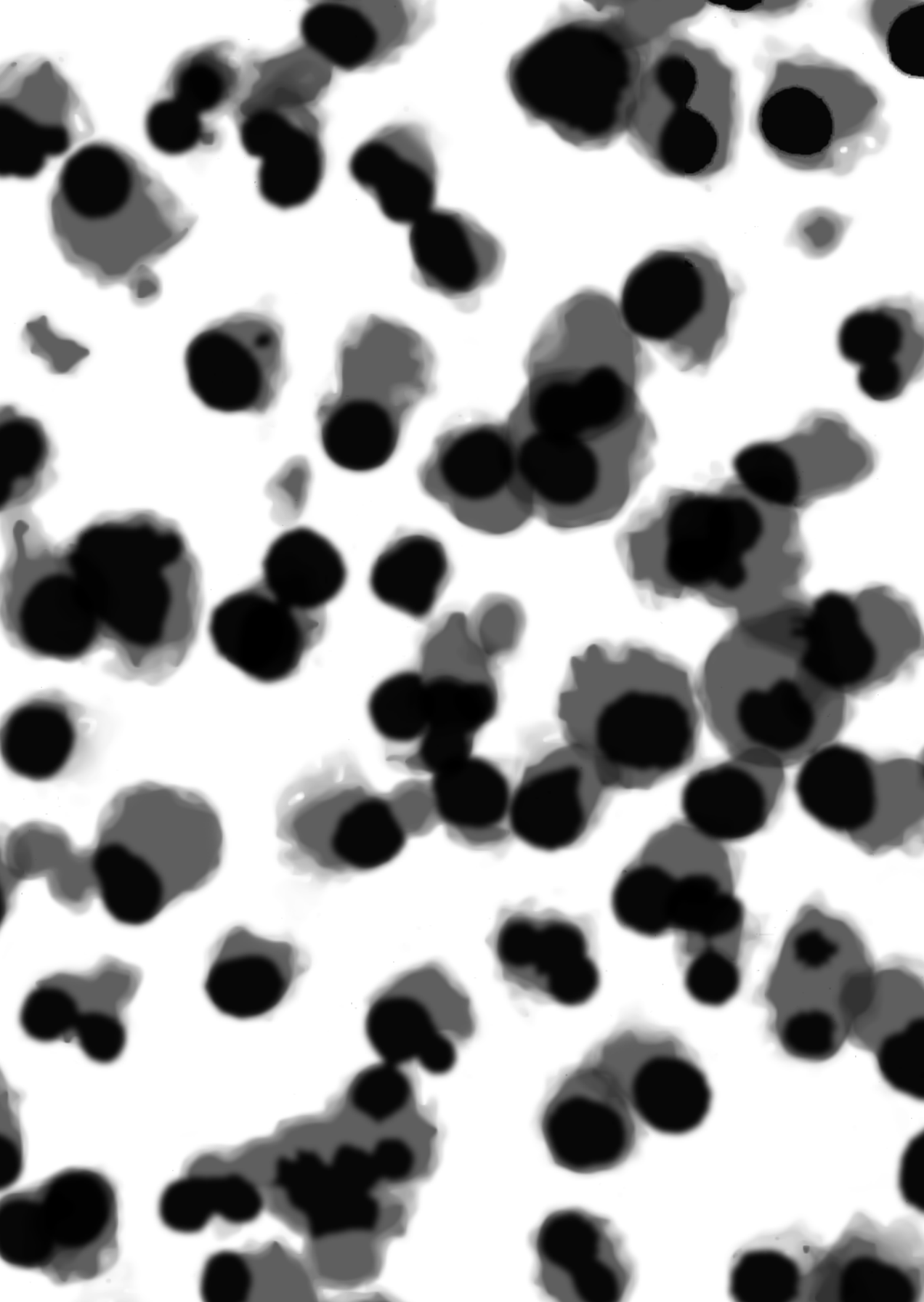

Chapter 1

Introduction

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Front Immunol. 2013 May 14;4:117

Introduction

The immune system

The immune system plays an important role in protecting the body against infectious agents. The immune response is delicately regulated and should only be initiated when necessary. This also requires timely termination, since inappropriate immune activation can result in persistent inflammatory disease or autoimmunity [1]. The immune system can be divided into the innate system and the adaptive system. The innate immune system is the first line of defence that provides a quick non-specific response compared to the adaptive immune system. The adaptive immune response is a specific response that is mainly established by two cell types, the B and T cells. The B cells are able to recognize their antigen in their native form. The antigen recognition together with activation signals will activate the B cells and differentiate them in antibody secreting plasma cells [2]. T cells are responsible for producing these activation signals, but are also able to produce inflammatory cytokines and kill infected cells. In contrast with B cells, T cells are only able to recognize processed antigens presented by antigen presenting molecules also known as the human leukocyte antigens (HLA) [3]

The innate immune system

The innate part of the immune system is known to respond quickly and non-specifically. Behind the mechanical and biological barriers, including the skin and mucus in the lungs and intestine, innate immune cells can be activated in case of infection with a pathogen. The innate immune cells can be triggered via pattern recognition molecules (PAMPS) or via signals of damaged and stressed cells called danger-associated molecular pattern molecules (DAMPS). Characteristics of the innate immune system are activation of the complement system, the recruitment and activation of immune cells by cytokines, recognition and clearance of pathogens in the tissue, and induction of the adaptive immune response via antigen presentation. Innate immune cells are, amongst others, macrophages, dendritic cells, neutrophils, mast cells, basophils and natural killer cells. The complement system is seen as a part of the innate immune system because the activation of the complement system can be quick and non-specific. Macrophages, dendritic cells and mast cells are important cells in the production of complement components and thereby also important in facilitating proper functioning of the complement system [4-7].

Macrophages

Macrophages are large mononuclear phagocytic cells that are able to present antigens, but are also very important to engulf and degrade cellular debris and pathogens. Myeloid progenitor cells are derived from the bone marrow that differentiate into monocytes. The monocytes will migrate into tissues were they can maturate towards macrophages. One of the important functions of the macrophage is to engulf and to kill microorganisms. Macrophages were identified in the 70's as major producers of C1q [8]. Next to macrophages, it is now known that immature dendritic cells are also a major source of C1q production [9, 10]. C1q produced by macrophages and dendritic cells play an important role in the clearance of dead cells and cellular debris [11, 12].

Dendritic cells

Dendritic cells (DCs) are derived from the same myeloid progenitor cells as macrophages and are the most potent antigen-presenting cells (APCs). Derived from the bone marrow, monocytic cells travel through the circulation into the tissue where they become immature DCs. In the tissue, the immature DCs are able to interact with microbial-associated molecules that can activate the DCs. Subsequently, DCs can migrate towards secondary lymph nodes under influence of cytokines and chemokines. During the migration the DCs will undergo a process of maturation. After entering the lymph nodes, the DCs are capable to present antigens in combination with co-stimulatory molecules resulting in T-cell activation. Immature DCs are an important source of C1q production and after reaching a mature stage, the C1q production is diminished [9, 10].

Mast cells

Mast cells (MCs) are best known for their role in anaphylactic allergy responses and to protect the body against parasitic worms. They are predominantly present in mucosal and skin surfaces and thereby close to the host/environment interface [13-15]. As effector cells they can also play an important role in other diseases like atherosclerosis, contact dermatitis, cancer and arthritis [16-19]. One of the markers of MCs is the high affinity IgE receptor (FcεRI). Via this receptor MCs can become activated followed by degranulation. As IgE is already bound to the high-affinity FcεRI, crosslinking is of FcεRI is a quick process leading to a swift activation of MCs. Degranulation can also occur after triggering by the complement components C3a and C5a. Interaction of C3a or C5a with mast cells will activate the mast cells resulting in degranulation where cytokines, chemokines and bioactive lipids will be released[20-23].

The complement system

The complement system is an essential part of the innate immune system of the human body. It consists of a set of soluble proteins, mainly present in blood and other body fluids, as well as an intricate system of membrane bound complement receptors and regulators. Complement activation can take place via three distinct pathways; the classical pathway, the lectin pathway and the alternative pathway. Each pathway is activated in a different manner. Together, the complement system consists around 30 proteins and via a cascade of enzymatic reactions it results in the production of biologically active fragments.

The three pathways have their own activation and recognition mechanisms that lead to the formation of C3 convertases where the central complement component C3 will be cleaved into anaphylatoxin C3a and opsonin C3b. After formation of the C3 convertases C4b2a or C3bBb, the terminal pathway will be initiated, which starts with the formation of a C5 convertase. This convertase cleaves C5 into C5b and the anaphylatoxin C5a. The fragment C5b will interact with C6 and C7 and form a complex, which is able to bind to cell membrane where it can interact with C8 that will insert into the cell membrane. To form a lytic pore, several C9 fragments are needed and will finally form the terminal complement complex (TCC, C5b9, membrane attack complex (MAC)) (Figure 1).

Classical pathway

The initiator molecule C1q, which forms, together with the serine proteases C1r and C1s, the C1 complex, activates the classical pathway. C1q can bind to different ligands like antigen-bound IgG and IgM, apoptotic cells, cellular debris and acute phase proteins. When C1q binds to a ligand it undergoes a conformational change, which allows the serine proteases C1r and C1s to become activated. The serine protease C1s will subsequently cleave C4 into C4a and C4b. The covalent binding of C4b follows this on the target together with C2a, which is a cleavage product of C2 that is cleaved by C1s into C2a and C2b, forming the C4b2a complex. This classical pathway C3-convertase cleaves C3 in to C3a and C3b. C3b can covalent bind to its target and C3b act as a opsonin, C3a functions as an anaphylotoxin. After the formation of the C3 convertase the C5 convertase will be formed, leading to the formation of the terminal pathway [24].

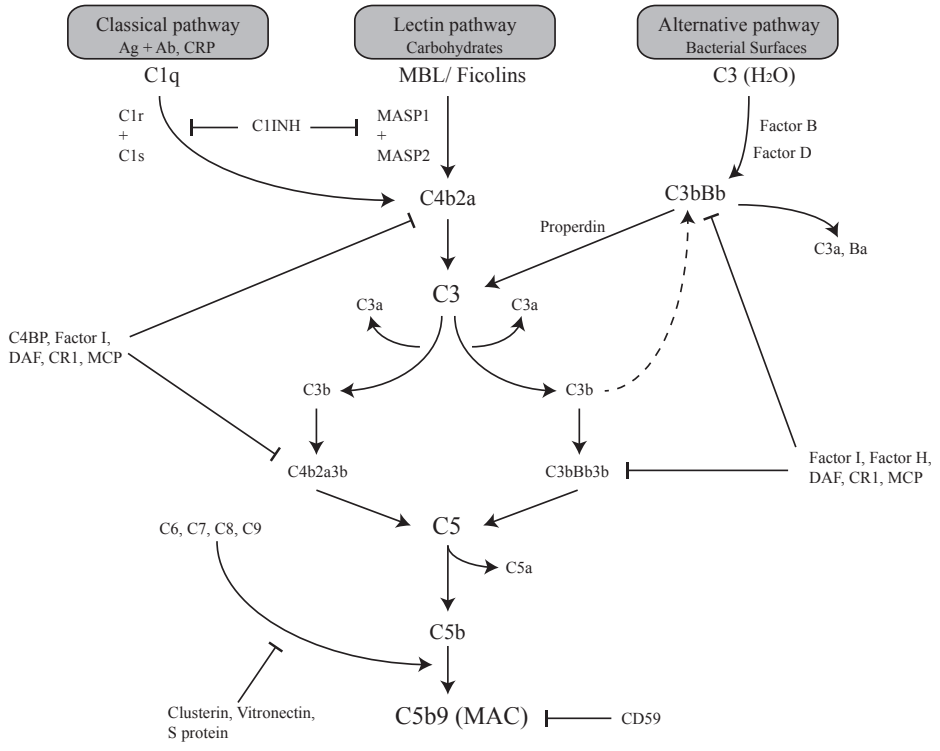


Figure 1. Activation pathways of the complement system. The central complement component C3 is activated via three pathways. The classical pathway is activated by binding of immune surveillance molecules on the activating surface different from the lectin pathway that is initiated by carbohydrate residues on the activating surface. Direct binding of C3b to the activating surface triggers the alternative pathway. All the three pathways lead to an enzymatic convertases that cleaves C3 (into C3a and C3b) either C5 (into C5a and C5b). Furthermore, C5b triggers the terminal pathway, which lead to the formation of a multimeric membrane attack complex (C5b-9), resulting in a pore in the target cell membrane.

Lectin pathway

The lectin pathway acts in a similar way in activation as the classical pathway because the recognition molecule of the lectin pathway, mannose-binding lectin (MBL) is structurally highly related to C1q and interacts also with serine proteases. However, it recognizes other targets. The lectin pathway will be activated by the recognition of certain carbohydrates ligands by mannose binding lectin (MBL) as well as H-ficolin or L-ficolin. When MBL is bound to mannose residues on pathogens, serine proteases (MASPs) will become activated in the same manner as C1r and C1s. The MASPs associated with MBL are MASP-1, MASP-2 and MASP-3. MASP-2 is responsible for activating C4 and C2 will generate the C3 convertase C4b2a, which is similar to the C3 convertase in the classical pathway.

Alternative pathway

The alternative pathway is spontaneously activated by hydrolysis of C3 in the circulation. This activation process is also known as “tick-over”. Here, C3 is spontaneously hydrolysed into C3(H20) or C3i. This hydrolysis can be amplified when C3 is interacting with biological and artificial interfaces like gas bubbles, biomaterial surfaces, and lipid surfaces and complexes [25-28]. Upon hydrolysis the C3 conformation changes allowing factor B (FB) to bind to C3(H20). This will be cleaved by the serine proteases Factor D (FD) resulting in the cleavage product C3(H20)Bb. This C3-convertase is able to continuously cleave C3 into C3a and C3b fragments. In the presence of an activating surface, C3b will covalently bind to the surface and initiate a positive feedback loop to the alternative pathway [28].

Complement regulation

During activation of the complement system several regulators are involved. In the classical pathway and the lectin pathway the soluble regulator C1 inhibitor (C1-INH) plays a role. The major role of C1INH in the complement system is the inhibition of proteases including as C1s, C1r and MASP2 [29, 30]. Furthermore, C1-INH is also able to interfere with the interaction of C3b with FB via a reversible interaction and inhibit the alternative pathway [31]. Further down the classical pathway, complement regulators like C4b-binding protein (C4BP), Complement Receptor type 1 (CR1) and Decay-Accelerating Factor (DAF, CD55), which all can bind to C4b and to C3b and can inhibit further activation of the classical and the lectin pathway [32-34].

In the alternative pathway the major fluid phase complement regulators are Factor H (FH), and properdin [35]. Factor H acts as a co-factor of Factor I mediating the cleavage of C3 and decay accelerating activities for the alternative pathway C3 convertases [36]. Properdin regulates the alternative pathway by stabilizing the short lived C3 convertase C3bBb [37]. Furthermore, the alternative pathway can be regulated on the cell surface including, membrane co-factor protein (MCP, CD46), Complement receptor 1 (CR1, CD35) and DAF. MCP acts as a co-factor for factor I and thereby mediates the cleavage of C3b and C4b. CR1 is a receptor for C3b and C4b and enhances the phagocytic activity of neutrophil-mediated phagocytosis [38]. DAF can accelerate the decay of C3 convertases as described above, but is also able to accelerating the decay of C5 convertases [33, 39].

The terminal pathway of the complement system will result in the formation of the MAC. During this formation several regulatory proteins can regulate this process. CD59, vitronectin and clusterin are regulatory proteins that inhibit the formation of the MAC [40-42].

Systemic Lupus Erythematosus (SLE)

Systemic Lupus Erythematosus (SLE) is an autoimmune disease, which can affect various organs and thereby can be characterized by clinical features including glomerulonephritis, rashes, serositis, haemolytic anaemia, thrombocytopenia and the central nervous system can also be involved. SLE is characterized by B-cell hyper-activation, autoantibody production and immune complex formation [43, 44]. Patients with SLE have a long list of clinical presentations, which can make it difficult for the clinician to diagnose the patient with SLE. The American College of Rheumatology (ACR) established a set of criteria for the diagnosis of SLE as well as for reasons of scientific research [45]. Overall 20-150 in 100.000 people are affected by SLE and it predominantly affects women (80%) [46].

The exact aetiology of SLE is unknown but it involves different factors like the genetic background and environmental factors [47, 48]. Deficiencies in the classical pathway of the complement system is highly associated with the development of SLE [49]. In C1q deficiency around 80% will develop lupus, in C1r/C1s deficiency the lupus incidence is around 68%, in C4 deficiency 75% and in C2 deficiency 10%[50-52].

Antibodies against a wide variety of self-antigens are present in SLE patients. Some of these autoantibodies can be already present in serum years before onset of disease [53]. Many of the antibodies are targeting nuclear antigens and are known as anti-nuclear antibodies (ANA's) [53]. ANA's can be divided into several subtypes including anti-dsDNA antibodies, anti-Ro antibodies, anti-histone antibodies, anti-Sm antibodies and anti-hnRNP antibodies. Other autoantibodies, which can be present in SLE patients, are directed against complement components like C1q (anti-C1q) or anti-C1s antibodies [54, 55].

In the circulation autoantibodies can form immune complexes and deposits in tissues like the skin or the kidney or immune complexes can form locally in tissues. Studies show that SLE patients have higher immunoglobulins in serum compared to healthy controls which correlates with disease activity [56].

Due to the unknown cause and complexity of SLE, patients are mainly treated on an individual basis. Most of the patients receive immunosuppressive drugs like prednisone. In case of deficiency in a complement component like C1q, patients benefit from treatment with Intra-venous Immuno-Globulins (IVIG) [57] and fresh frozen plasma [58]. A more radical treatment is Haematopoietic Stem cell transplantation in which has been successfully in two SLE patients with C1q deficiency [59, 60].

Neuropsychiatric Systemic Lupus Erythematosus (NPSLE)

One of the manifestations in SLE is the involvement of the nervous system, which includes a diversity of neurological and psychiatric events. Around 39-50% of SLE patients are presenting NPSLE symptoms [61]. There is a difference in NPSLE manifestation attributed to SLE compared to neurological manifestations as a complication of the disease or due the treatment of the SLE. The American College of Rheumatology (ACR) established in 1999 criteria which the clinicians use for the diagnosis of NPSLE [62]. In these criteria clinicians can segregate the NPSLE in diffuse and focal NPSLE.

Patients with NPSLE are treated in many different ways, due to the different types of disease and inflammation, also the type of symptoms in NPSLE play an important role in the type of treatment [63].

Complement factors are known to contribute to the pathology of inflammatory central nervous system (CNS) and neurodegenerative diseases [64]. In cerebrospinal fluid (CSF) an increase of complement components C3, C4 and C5b9 were found [65, 66]. This may impact on disease progression as in mice that were deficient for alternative complement pathway activity, less lupus cerebritis was seen as compared to controls [67].

Recently, research groups demonstrated that activated microglia are important producers of complement components, which can be of importance in inflammatory conditions in the brain like depression, but also in NPSLE [68].

Furthermore, the breakdown of the blood brain barrier (BBB) is prominent in NPSLE as well as the presence of autoantibodies against brain antigens like glutamate receptors. These antibodies against glutamate receptors are known to play an important role in neurodegenerative diseases [69-71]. Also autoantibodies against complement components could play a role in NPSLE, but the exact mechanism is still unknown.

The role of complement in arthritis

There is strong evidence that complement plays an important role in the pathogenesis of rheumatoid arthritis (RA) and osteoarthritis (OA) [72]. One study in 2013 showed that single nucleotide polymorphisms (SNPs) in and around the C1q genes were associated with the susceptibility of RA. These results could be explained because the genetic variants would increase the production of C1q leading to an increased capacity to activate the classical pathway [73], and suggest that more complement activation associates with more joint damage. As seen in several studies, a decreased haemolytic activity and complement components was found in the synovial fluid, indicating local complement activation and consumption [74-76]. In

mouse models of inflammatory arthritis it was found that complement activation is involved in joint damage especially complement component C5a [77].
One of the factors activating the classical pathway is binding of C1q to immune complexes and to microparticles from apoptotic granulocytes, where deposition of C1, C3 and C4 was seen in synovial fluid [78]. The alternative pathway has been suggested to be more involved in the complement activation in the joint for there was an increase Bb fragments found in RA synovial fluid [79] and in rodent arthritis models it is demonstrated that factor B or C5 deficient mice were protected from arthritis whereas C4 deficient mice not [80, 81]. Cartilage fragments are recently implicated as complement regulators. Several studies indicate that several components of the extracellular matrix (ECM) are enhancing and others are inhibiting complement activation [82-84].

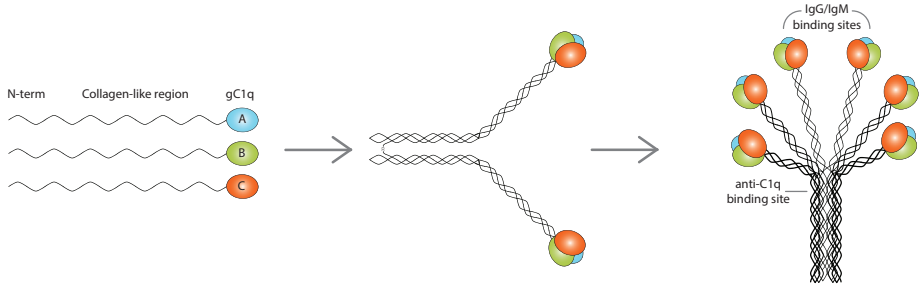


Figure 2. Schematic overview of the complex build-up of the C1q molecule.

Complement component C1q

C1q gene and protein

The genes C1QA, C1QB and C1QC are located on the p-arm of chromosome 1 (chromosomal location: 1p34-1p36.3). These genes encode for the constituents of the C1q molecule, the C1q A, B and C peptide chains that each form one helical strand and all contain a short N-terminal region, a collagen-like region and a C-terminal globular region (gC1q domain) (Figure 1). The collagen-like regions assemble into helical conformations. Six of these structural triple helix units form, because of non-covalent interactions via two C-chains, the hexameric C1q molecule that has an overall structure that resembles a bouquet of tulips [85] or an 'Eiffel-tower'-like structure [86]. Structurally the C1q molecule (460 kDa) is composed of 18 polypeptide chains (6xA, 6xB and 6xC).

C1q production

In contrast to most other complement factors, C1q is not produced by hepatocytes but mainly by macrophages and immature dendritic cells [9, 10]. Following their maturation, dendritic cells completely shut down C1q production [9, 10] suggesting a role for C1q in adaptive immune responses [4, 7]. Indeed, a role for C1q in adaptive immunity can also be concluded from in-vivo studies regarding antigen presentation [87], cellular activation of immune cells [88, 89] and setting a tolerance threshold [90-92].

C1q binding to immunoglobulins; monomeric versus hexameric

C1 driven complement activation can be triggered via the binding of C1q to the Fc-region of IgM or IgG and activation of C1r-C1s tetramer [93]. For IgM, the most efficient activation is associated with the hexameric form, representing a minor fraction of total IgM (5% of total IgM) [94]. Hexameric IgM (IgMh) differs from pentameric IgM (IgMp) in the absence of J-chain, which is an evolutionary conserved peptide that covalently links pentameric IgM and functions to its transepithelial transport by binding the poly-Ig receptor. The lack of J-chain in IgMh possibly represents a mechanism to prevent polymeric Ig, with high complement activation potential (approximately one-log compared to IgMp, [95], to be transported to the mucosal surfaces where the antigenic load is generally high [96]. The physiological function of human IgMh is still largely unknown. Melis et al. propose that IgMp and IgMh should be seen as two distinct subclasses [97] and not as a side product of evolution.

The C1q binding sites are hidden in IgMp, but are forced outwards by a star-to-staple conformational change upon antigen binding [98-100] and at the same time the complex glycans are turned inside. These conformational changes may decrease the clearance rate of IgMp and initiate complement activation after binding of C1q [101, 102]. The hexameric form, on the other hand, appears to be a planar molecule in which all of the variable regions are available for antigen and Fc-binding [101, 103].

The binding of one of the C1q head domains to an IgG-Fc part is of low affinity [104, 105]. Activation of the classical pathway by IgG requires that at least two IgG molecules bind to C1q (at least for non-nucleated cells). Only in rare cases certain paraproteins have been reported to induce C1 activation while in monomeric form [106]. Attachment of IgG to antigens in close proximity to each other, allows C1q to bind more than one Fc-region, thereby activating complement [98, 107]. Simple proximity binding however appears to be insufficient for maximal complement activation by IgG. Diebolder et al. recently demonstrated that IgG molecules form

ordered hexameric structures after they bind cell surface-expressed antigen. These hexamers form a docking station for C1 binding and activation. Mutations that interrupted hexamer formation led to strongly decreased C1q binding and CDC. All features to potentiate levels of complement activation described above, together with the structural data for C1q, IgG and IgM hexamers therefore fit with the notion that Ig hexamerization is required for potent activation of the classical pathway of complement [86, 108].

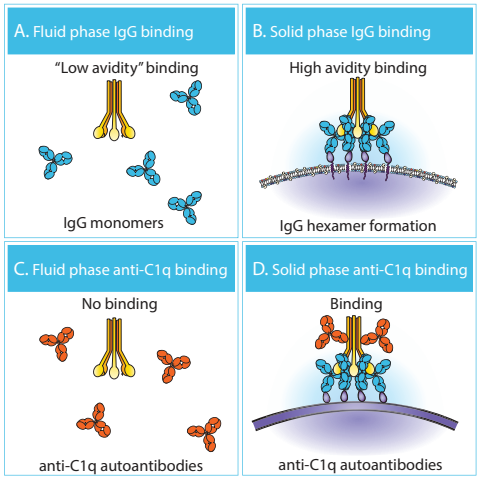


Figure 3. Schematic overview of the interactions between C1q and immunoglobulins
A. In fluid-phase; C1q avidity too low for binding to monomeric IgG molecules under physiological conditions and therefore no activation. **B.** Antibody binding to antigens allows hexamerisation via Fc-Fc interactions, this generates an optimal platform for C1q binding and activation. **C.** Anti-C1q autoantibodies do not bind to fluid-phase C1q and are therefore non-depleting. **D.** Anti-C1q autoantibodies bind to solid-phase C1q and amplify complement activation [109].

C1q traditional and non-traditional roles

The traditional view on the role of C1q is restricted to the activation of the classical pathway following its binding to ligand bound IgM or multimeric IgG [110]. The list of ligands for C1q has grown considerably, now also including DNA [111], CRP [112], and matrix molecules such as decorin [83, 113, 114].

In the observation regarding the accumulation of apoptotic cells received a lot of attention. Normally apoptotic cells are hardly detectable in tissues as these are rapidly eliminated by e.g. macrophages. Hence the "waste disposal" theory was introduced suggesting that C1q, by binding to dying and dead cells would stimulate phagocytosis [115]. Indeed dedicated in-vitro experiments revealed that early, and especially late apoptotic cells could bind C1q [116, 117], which resulted in more efficient uptake by phagocytes [118, 119]. The uptake of C1q-opsonized

apoptotic cells by phagocytes not only clears a potential source of autoantigens but also impacts on the immune status of the phagocyte [120]. The complement activation that occurs as a result of C1q binding to dead cells needs to be well controlled to allow for efficient opsonisation, including C3b fragments, without the risk of lysis and release of autoantigens [121, 122]. Next to C1q, apoptotic and necrotic cells were also shown to bind MBL [123] and properdin [124] to enhance complement mediated opsonisation. The process of apoptotic cell clearance is essential in physiology and is characterized by redundancy. Therefore it was postulated that C1q may instead, or additionally, impact on the threshold for auto-reactivity in the adaptive immune response. Indeed both in-vitro studies and studies in C1q-deficient mice indicate that C1q impacts on the adaptive immune response [87, 90-92, 125].

Apart from the effects of C1q as part of the complement activation cascade described above, several reports have implicated C1q in processes that may not per se involve additional complement activation. For example C1q has been reported to play an important role in nerve pruning [126] Wnt/Beta-catenin signalling [127, 128]. Also a role for C1q was reported on neoangiogenesis [129]. C1q could potentially exert such effects in the absence of complement activation by the activation of cellular C1q receptors. Over the years several receptors have been considered to be C1q receptors and several have since then been abandoned. The gC1q-Receptor and the cC1q-Receptor are still considered bonafide C1q receptors [130] and additionally a role for C1q binding to the inhibitory collagen receptor LAIR-1 was reported [131].

Genetic C1q deficiency

Genetic deficiency of C1q is strongly associated with development of Systemic Lupus Erythematosus (SLE) [132]. This observation defined the lupus-paradox, where in most SLE patients C1q contributes to tissue damage by inducing complement activation via tissue-deposited immune complexes (ICs), whereas genetic deficiency of C1q is also associated with development of SLE [133]. Clearly C1q is not essential for the clinical presentation of SLE, but how C1q contributes to the protection against the development of SLE remains to be solved. A major breakthrough was achieved when the first C1q knock-out mice were generated [134]. These mice developed a lupus-like disease and importantly the investigators observed increased numbers of apoptotic cells [134]. Later it was shown that the lupus-like disease observed in these first C1q-deficient mice was largely due to the breeding strategy in which a large genetic segment containing autoimmunity-prone genes was co-transferred [135]. Next to a variety of papers describing the effects

of complete genetic deficiency of C1q, several reports that describe associations between SNPs in C1q and clinical phenotypes have appeared. The presence of SNPs in the C1q genes has been associated with differences in response to rituximab treatment for lymphoma treatment [136, 137] as well as with development of SLE [138-143] or development of RA [73].

Antibodies against the C1 complex

Anti-C1q autoantibodies

The past four decades anti-C1q autoantibodies have been studied in a wide variety of autoimmune and renal conditions as well as in infectious diseases [144]. In the healthy population the prevalence of anti-C1q autoantibodies ranges between 2% and 8% [106, 145-148] and increases with age [149]. Hypocomplementemic Urticarial Vasculitis Syndrome (HUVS) represents the clinical condition with the highest percentage of anti-C1q positivity; 100% [150]. Other conditions characterized by high anti-C1q antibody prevalence are, mixed connective tissue disease (94%), Felty's syndrome (76%), and SLE (30–60%) [144, 146, 148, 151].
The occurrence of anti-C1q autoantibodies was shown to have familial clustering, indicating that there is a genetic risk factor that together with environmental cues may precipitate the production of these antibodies [152]. Anti-C1q autoantibodies have also been described to occur in infectious diseases although at a frequency of around, for example 13% of HIV infected individuals vs. 5% in healthy controls [153] or up to 26% in patients suffering from hepatitis C virus infection as compared to 10% of healthy controls [154].
Anti-C1q autoantibodies cannot be used as a diagnostic tool however, the presence of anti-C1q autoantibodies can provide prognostic information. This has been particularly instrumental in the renal complication of systemic lupus erythematosus (SLE), lupus nephritis (LN). Anti-C1q autoantibodies were reported to occur in 30–60% of the SLE patients and to be especially present in those patients that among the many clinical presentations also suffered from LN [144, 155].
Several studies provide evidence that anti-C1q autoantibodies are superior to other serological markers in identifying a flare of LN [156, 157]. However, other studies indicate that combinations of anti-C1q antibodies with other serological markers are superior to anti-C1q antibodies alone [158-160]. Especially striking is the strong negative predictive value of anti-C1q testing for LN. In the absence of anti-C1q autoantibodies it is very unlikely that a patient with LN will develop a flare [106, 156, 158, 161-163]. As many of these studies report on rather small patient populations from very diverse ethnic backgrounds ranging from Brazil [163], China [164], India

[165], and Egypt [166] it is likely that considerable variation exists in the strength at which anti-C1q antibodies are associated with and is predictive for LN flares. Several of the larger studies from Europe and Hong Kong point in the same direction [156, 159, 167] and also a recent meta-analysis confirmed the diagnostic value of serum anti-C1q antibodies for LN [168]. Whether or not anti-C1q antibodies are also associated with the disease activity of LN remains to be established, as currently there is no consensus on this issue [147, 159, 169]. Also in post-streptococcal nephritis anti-C1q autoantibodies were reported to associate with a worse outcome [170]. Collectively these data suggest that anti-C1q by itself may not be pathogenic to the kidney, as anti-C1q autoantibodies are also found in the healthy population. This would imply that only following a specific insult to the kidney these anti-C1q autoantibodies could enhance the local pathology, as seen in the enhanced LN in SLE and PSGN. Experimental evidence for a pathogenic contribution of anti-C1q autoantibodies came from murine studies [171-174]. Administration of anti-C1q antibodies to healthy mice results in a modest deposition of C1q and anti-C1q antibodies but this does not induce renal impairment [172]. In sharp contrast anti- C1q autoantibodies strongly aggravate immunecomplex-mediated nephritis [173]. The mode of action was depending on the presence of C1q in the immune complexes in the glomeruli, as anti-C1q autoantibodies were not able to aggravate glomerulonephritis induced by non-C1q fixing immune complexes [173]. Anti-C1q autoantibodies may also play pathogenic roles in other conditions such as autoimmune thyroid disease [148].

One mechanism to clear anti-C1q autoantibodies from the circulation is to use immunoabsorption on C1q-columns [175]. This method depleted next to circulating immune complexes also anti-C1q autoantibodies and was shown to be beneficial in SLE patients [176, 177].

Assays to detect anti-C1q

Over time several assays have been developed to detect anti-C1q autoantibodies both in humans and in experimental animal models. The first assays employed a direct coating of intact C1q, which necessitated the use of high salt conditions to discriminate between immune complex binding and anti-C1q autoantibody binding [178]. Already early in the history of anti-C1q autoantibodies it was discovered that the majority of these autoantibodies is directed against the collagen-like part of the C1q molecule [179]. From equilibrium studies and from the observation that anti-C1q antibodies can be found in the presence of freely circulating C1q it was argued that anti-C1q antibodies may interact with epitopes that are not exposed in C1q in fluid phase [180]. Later these arguments were supported by elegant studies

using phage display technology generated Fab fragments that only interacted with solid-phase C1q [181]. Next, assays have been developed that utilized only the C1q collagen-like region, generated by enzymatic digestions as antigen [145, 179]. This eliminated the need to use high ionic strength buffer. A recent paper reports on the use of peptides derived from C1q that have interesting properties to detect a major linear epitope in a high percentage of the patients in the absence of high-ionic strength buffer [182]. In contrast to the assays reported before that anti-C1q antibodies only target the collagen-like region of C1q in 2007 it was discovered that there are also antibodies that specifically target the globular head regions of C1q [183].

To study anti-C1q antibodies in experimental animal models, assays were developed that used coating of purified mouse C1q and high salt conditions similar to the human situation [171, 172]. Next, in order to circumvent the purification of mouse C1q, an assay was developed which employed a coating of C1q binding peptides, that captured C1q from Rag-/- serum, as the antigenic entity for the anti-C1q ELISA [173, 174]. (Table 1)

Year	Milestone	
1971/88	Identification of C1q as the target of autoantibodies	[179, 185]
1982	C1q in solid phase exposes neo-epitopes	[180]
1984	Identification of the collagen-like stalk as the main binding site of anti-C1q antibodies	[186, 187]
1987	Anti-C1q associates with the occurrence of LN	[188]reviewed a.o. [144]
1991	Identification of anti-C1q in mice	[122]
1996	Anti-C1q also present in healthy population and increase with age	[171]
1993	Anti-C1q also present in healthy population and increase with age	[149]
2004	Experimental evidence on how anti-C1q can be pathogenic to the kidney in LN but not in healthy individuals	[173]
2007	Identification of anti-C1q antibodies that target the globular heads	[183]

Table 1. History of anti-C1q antibodies

Anti-C1s autoantibodies

Autoantibodies targeting C1s have been described to occur in around 50% of the SLE patients [184]. The binding of these autoantibodies to C1s enhances its activity and has been suggested to be partially responsible for the reduced levels of C4 observed in SLE [184]. As these antibodies have only been described in one study, future studies will have to reveal the clinical implications of the presence of these antibodies.

Scope of the thesis

The scope of this thesis is to describe several aspects of the role of C1q in immunity and autoimmunity in a clinical- and cellular perspective.

The clinical association of C1q and anti-C1q in autoimmunity

C1q deficiencies are relatively rare, but have a strong association in the development of SLE. Around 80% of the C1q deficient patients will develop SLE, but the clinical outcome is variable. In **chapter 2**, we describe a Dutch child with C1q deficiency presenting only recurrent infections without any signs of autoimmunity. This made us curious about other C1q deficient patients and their clinical outcome. In **chapter 3**, we investigated the variability in clinical presentation and outcome of C1q deficient patients worldwide using questionnaires. Some C1q deficient patients with SLE are diagnosed with a genetic mutation resulting in the production of low levels of C1q that has a low molecular weight (LMW-C1q). In **chapter 4**, we describe a C1q deficient patient producing low levels of C1q with a low molecular weight who is diagnosed with SLE and NPSLE. The role of complement activation and NPSLE is not well known. The studies presented in **chapter 5**, describes if there is an association between complement activation and anti-C1q antibodies in patients with NPSLE.

Local Production of C1q by immune and non-immune cells

As described by several groups the main producers of C1q are immature dendritic cells and macrophages. Because both cells are from myeloid origin we focused on another cells originating from the same progenitor. In **chapter 6**, we describe the production of C1q in mast cells, indicating that mast cells can contribute to complement activation in tissue where mast cells are predominantly present like the skin or synovial tissue. In **chapter 7**, we discuss the role of C1q produced by chondrocytes, which are non-immune cells and are important in the formation of cartilage.

References

1. Nathan, C. and A. Ding, Nonresolving inflammation. Cell, 2010. 140(6): p. 871-82.
2. Chaplin, D.D., 1. Overview of the human immune response. J Allergy Clin Immunol, 2006. 117(2 Suppl Mini-Primer): p. S430-5.
3. Parham, P. and C. Janeway, The immune system. Fourth edition. ed. 2015, New York, NY: Garland Science, Taylor & Francis Group. 1 volume (various pagings).
4. Castellano, G., et al., Dendritic cells and complement: at the cross road of innate and adaptive immunity. Mol Immunol, 2004. 41(2-3): p. 133-40.
5. Morita, H., et al., Regulatory roles of mast cells in immune responses. Semin Immunopathol, 2016. 38(5): p. 623-9.
6. Xu, W., et al., Dendritic cell and macrophage subsets in the handling of dying cells. Immunobiology, 2006. 211(6-8): p. 567-75.
7. van Kooten, C., et al., Complement production and regulation by dendritic cells: molecular switches between tolerance and immunity. Mol Immunol, 2008. 45(16): p. 4064-72.
8. Muller, W., H. Hanauske-Abel, and M. Loos, Biosynthesis of the first component of complement by human and guinea pig peritoneal macrophages: evidence for an independent production of the C1 subunits. J Immunol, 1978. 121(4): p. 1578-84.
9. Castellano, G., et al., Infiltrating dendritic cells contribute to local synthesis of C1q in murine and human lupus nephritis. Mol Immunol, 2010. 47(11-12): p. 2129-37.
10. Castellano, G., et al., Maturation of dendritic cells abrogates C1q production in vivo and in vitro. Blood, 2004. 103(10): p. 3813-20.
11. Korb, L.C. and J.M. Ahearn, C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited. J.Immunol., 1997. 158(10): p. 4525-4528.
12. Nauta, A.J., et al., Direct binding of C1q to apoptotic cells and cell blebs induces complement activation. Eur.J.Immunol., 2002. 32(6): p. 1726-1736.
13. Galli, S.J., et al., Mast cells as “tunable” effector and immunoregulatory cells: recent advances. Annu Rev Immunol, 2005. 23: p. 749-86.
14. Galli, S.J., S. Nakae, and M. Tsai, Mast cells in the development of adaptive immune responses. Nat Immunol, 2005. 6(2): p. 135-42.
15. Voehringer, D., Protective and pathological roles of mast cells and basophils. Nat Rev Immunol, 2013. 13(5): p. 362-75.
16. Bot, I. and E.A. Biessen, Mast cells in atherosclerosis. Thromb Haemost, 2011. 106(5): p. 820-6.
17. Ribatti, D. and E. Crivellato, Mast cells, angiogenesis and cancer. Adv Exp Med Biol, 2011. 716: p. 270-88.
18. Vocanson, M., et al., Effector and regulatory mechanisms in allergic contact dermatitis. Allergy, 2009. 64(12): p. 1699-714.
19. Woolley, D.E., The mast cell in inflammatory arthritis. N Engl J Med, 2003. 348(17): p. 1709-11.
20. Johnson, A.R., T.E. Hugli, and H.J. Muller-Eberhard, Release of histamine from rat mast cells by the complement peptides C3a and C5a. Immunology, 1975. 28(6): p. 1067-80.
21. Klos, A., et al., The role of the anaphylatoxins in health and disease. Mol Immunol, 2009. 46(14): p. 2753-66.
22. Guo, Q., et al., Regulation of C3a receptor signaling in human mast cells by G protein coupled receptor kinases. PLoS One, 2011. 6(7): p. e22559.
23. Takafuji, S., et al., Degranulation from human eosinophils stimulated with C3a and C5a. Int Arch Allergy Immunol, 1994. 104 Suppl 1(1): p. 27-9.

24. Walport, M.J., Complement. First of two parts. N Engl J Med, 2001. 344(14): p. 1058-66.
25. Nilsson, B., et al., The role of complement in biomaterial-induced inflammation. Mol Immunol, 2007. 44(1-3): p. 82-94.
26. Nilsson, B. and K. Nilsson Ekdahl, The tick-over theory revisited: is C3 a contact-activated protein? Immunobiology, 2012. 217(11): p. 1106-10.
27. Nilsson Ekdahl, K., et al., Generation of iC3 at the interface between blood and gas. Scand J Immunol, 1992. 35(1): p. 85-91.
28. Pangburn, M.K. and H.J. Muller-Eberhard, Initiation of the alternative complement pathway due to spontaneous hydrolysis of the thioester of C3. Ann N Y Acad Sci, 1983. 421: p. 291-8.
29. Arlaud, G.J., et al., Interaction of C1-inhibitor with the C1r and C1s subcomponents in human C1. Biochim Biophys Acta, 1979. 576(1): p. 151-62.
30. Sim, R.B., G.J. Arlaud, and M.G. Colomb, C1 inhibitor-dependent dissociation of human complement component C1 bound to immune complexes. Biochem J, 1979. 179(3): p. 449-57.
31. Davis, A.E., 3rd, P. Mejia, and F. Lu, Biological activities of C1 inhibitor. Mol Immunol, 2008. 45(16): p. 4057-63.
32. Suankratay, C., et al., Mechanism of complement-dependent haemolysis via the lectin pathway: role of the complement regulatory proteins. Clin Exp Immunol, 1999. 117(3): p. 442-8.
33. Kim, D.D. and W.C. Song, Membrane complement regulatory proteins. Clin Immunol, 2006. 118(2-3): p. 127-36.
34. Hamer, I., et al., Soluble form of complement C3b/C4b receptor (CR1) results from a proteolytic cleavage in the C-terminal region of CR1 transmembrane domain. Biochem J, 1998. 329 (Pt 1): p. 183-90.
35. Kemper, C., J.P. Atkinson, and D.E. Hourcade, Properdin: emerging roles of a pattern-recognition molecule. Annu Rev Immunol, 2010. 28: p. 131-55.
36. Zipfel, P.F., et al., Factor H family proteins: on complement, microbes and human diseases. Biochem Soc Trans, 2002. 30(Pt 6): p. 971-8.
37. Fearon, D.T. and K.F. Austen, Properdin: binding to C3b and stabilization of the C3b-dependent C3 convertase. J Exp Med, 1975. 142(4): p. 856-63.
38. Krych-Goldberg, M. and J.P. Atkinson, Structure-function relationships of complement receptor type 1. Immunol Rev, 2001. 180: p. 112-22.
39. Holers, V.M., The spectrum of complement alternative pathway-mediated diseases. Immunol Rev, 2008. 223: p. 300-16.
40. Davies, A., et al., CD59, an LY-6-like protein expressed in human lymphoid cells, regulates the action of the complement membrane attack complex on homologous cells. J Exp Med, 1989. 170(3): p. 637-54.
41. Podack, E.R. and H.J. Muller-Eberhard, Isolation of human S-protein, an inhibitor of the membrane attack complex of complement. J Biol Chem, 1979. 254(19): p. 9808-14.
42. O'Bryan, M.K., et al., Human seminal clusterin (SP-40,40). Isolation and characterization. J Clin Invest, 1990. 85(5): p. 1477-86.
43. Lipsky, P.E., Systemic lupus erythematosus: an autoimmune disease of B cell hyperactivity. Nat Immunol, 2001. 2(9): p. 764-6.
44. Mok, C.C. and C.S. Lau, Pathogenesis of systemic lupus erythematosus. J Clin Pathol, 2003. 56(7): p. 481-90.
45. Hochberg, M.C., Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum, 1997. 40(9): p. 1725.
46. Mills, J.A., Systemic lupus erythematosus. N Engl J Med, 1994. 330(26): p. 1871-9.
47. Rullo, O.J. and B.P. Tsao, Recent insights into the genetic basis of systemic lupus erythematosus.

Ann Rheum Dis, 2013. 72 Suppl 2: p. ii56-61.
48. Cooper, G.S., et al., Occupational and environmental exposures and risk of systemic lupus erythematosus: silica, sunlight, solvents. Rheumatology (Oxford), 2010. 49(11): p. 2172-80.
49. Truedsson, L., A.A. Bengtsson, and G. Sturfelt, Complement deficiencies and systemic lupus erythematosus. Autoimmunity, 2007. 40(8): p. 560-6.
50. Walport, M.J., K.A. Davies, and M. Botto, C1q and systemic lupus erythematosus. Immunobiology, 1998. 199(2): p. 265-85.
51. Pickering, M.C., et al., Systemic lupus erythematosus, complement deficiency, and apoptosis. Adv Immunol, 2000. 76: p. 227-324.
52. Amano, M.T., et al., Genetic analysis of complement C1s deficiency associated with systemic lupus erythematosus highlights alternative splicing of normal C1s gene. Mol Immunol, 2008. 45(6): p. 1693-702.
53. Arbuckle, M.R., et al., Development of autoantibodies before the clinical onset of systemic lupus erythematosus. N Engl J Med, 2003. 349(16): p. 1526-33.
54. Orbai, A.M., et al., Anti-C1q antibodies in systemic lupus erythematosus. Lupus, 2015. 24(1): p. 42-9.
55. Suzuki, Y., et al., Selective deficiency of C1s associated with a systemic lupus erythematosus-like syndrome. Report of a case. Arthritis Rheum, 1992. 35(5): p. 576-9.
56. Ward, M.M., et al., The relationship between soluble interleukin 2 receptor levels and antidouble stranded DNA antibody levels in patients with systemic lupus erythematosus. J Rheumatol, 1991. 18(2): p. 235-40.
57. Jordan, S.C., Intravenous gamma-globulin therapy in systemic lupus erythematosus and immune complex disease. Clin Immunol Immunopathol, 1989. 53(2 Pt 2): p. S164-9.
58. Mehta, P., et al., SLE with C1q deficiency treated with fresh frozen plasma: a 10-year experience. Rheumatology (Oxford), 2010. 49(4): p. 823-4.
59. Arkwright, P.D., et al., Successful cure of C1q deficiency in human subjects treated with hematopoietic stem cell transplantation. J Allergy Clin Immunol, 2014. 133(1): p. 265-7.
60. Olsson, R.F., et al., Allogeneic Hematopoietic Stem Cell Transplantation in the Treatment of Human C1q Deficiency: The Karolinska Experience. Transplantation, 2016. 100(6): p. 1356-62.
61. Hanly, J.G., et al., Neuropsychiatric events at the time of diagnosis of systemic lupus erythematosus: an international inception cohort study. Arthritis Rheum, 2007. 56(1): p. 265-73.
62. The American College of Rheumatology nomenclature and case definitions for neuropsychiatric lupus syndromes. Arthritis Rheum, 1999. 42(4): p. 599-608.
63. Magro-Checa, C., et al., Management of Neuropsychiatric Systemic Lupus Erythematosus: Current Approaches and Future Perspectives. Drugs, 2016. 76(4): p. 459-83.
64. Hong, S., et al., Complement and microglia mediate early synapse loss in Alzheimer mouse models. Science, 2016. 352(6286): p. 712-6.
65. Sanders, M.E., et al., Detection of activated terminal complement (C5b-9) in cerebrospinal fluid from patients with central nervous system involvement of primary Sjogren's syndrome or systemic lupus erythematosus. J Immunol, 1987. 138(7): p. 2095-9.
66. Jongen, P.J., et al., Cerebrospinal fluid C3 and C4 indexes in immunological disorders of the central nervous system. Acta Neurol Scand, 2000. 101(2): p. 116-21.
67. Alexander, J.J., et al., Absence of functional alternative complement pathway alleviates lupus cerebritis. Eur J Immunol, 2007. 37(6): p. 1691-701.
68. Farber, K., et al., C1q, the recognition subcomponent of the classical pathway of complement, drives microglial activation. J Neurosci Res, 2009. 87(3): p. 644-52.
69. Hirohata, S., et al., Blood-brain barrier damages and intrathecal synthesis of anti-N-methyl-D-

aspartate receptor NR2 antibodies in diffuse psychiatric/neuropsychological syndromes in systemic lupus erythematosus. Arthritis Res Ther, 2014. 16(2): p. R77.
70. Diamond, B., et al., Losing your nerves? Maybe it's the antibodies. Nat Rev Immunol, 2009. 9(6): p. 449-56.
71. Trippe, J., et al., Autoantibodies to glutamate receptor antigens in multiple sclerosis and Rasmussen's encephalitis. Neuroimmunomodulation, 2014. 21(4): p. 189-94.
72. Wang, Q., et al., Identification of a central role for complement in osteoarthritis. Nat Med, 2011. 17(12): p. 1674-9.
73. Trouw, L.A., et al., Genetic variants in the region of the C1q genes are associated with rheumatoid arthritis. Clin Exp Immunol, 2013. 173(1): p. 76-83.
74. Sjoholm, A.G., et al., C1 activation, with C1q in excess of functional C1 in synovial fluid from patients with rheumatoid arthritis. Int Arch Allergy Appl Immunol, 1986. 79(2): p. 113-9.
75. Hedberg, H., B. Lundh, and A.B. Laurell, Studies of the third component of complement in synovial fluid from arthritic patients. II. Conversion and its relation to total complement. Clin Exp Immunol, 1970. 6(5): p. 707-12.
76. Pekin, T.J., Jr. and N.J. Zvaifler, Hemolytic Complement in Synovial Fluid. J Clin Invest, 1964. 43: p. 1372-82.
77. Okroj, M., et al., Rheumatoid arthritis and the complement system. Ann Med, 2007. 39(7): p. 517-30.
78. Berglund, K., et al., Complement activation, circulating C1q binding substances and inflammatory activity in rheumatoid arthritis: relations and changes on suppression of inflammation. J Clin Lab Immunol, 1980. 4(1): p. 7-14.
79. Brodeur, J.P., et al., Synovial fluid levels of complement SC5b-9 and fragment Bb are elevated in patients with rheumatoid arthritis. Arthritis Rheum, 1991. 34(12): p. 1531-7.
80. Wang, Y., et al., A role for complement in antibody-mediated inflammation: C5-deficient DBA/1 mice are resistant to collagen-induced arthritis. J Immunol, 2000. 164(8): p. 4340-7.
81. Banda, N.K., et al., Alternative complement pathway activation is essential for inflammation and joint destruction in the passive transfer model of collagen-induced arthritis. J Immunol, 2006. 177(3): p. 1904-12.
82. Happonen, K.E., et al., Regulation of complement by cartilage oligomeric matrix protein allows for a novel molecular diagnostic principle in rheumatoid arthritis. Arthritis Rheum, 2010. 62(12): p. 3574-83.
83. Groeneveld, T.W., et al., Interactions of the extracellular matrix proteoglycans decorin and biglycan with C1q and collectins. J Immunol, 2005. 175(7): p. 4715-23.
84. Melin Furst, C., et al., The C-type lectin of the aggrecan G3 domain activates complement. PLoS One, 2013. 8(4): p. e61407.
85. Kishore, U., et al., C1q and tumor necrosis factor superfamily: modularity and versatility. Trends Immunol, 2004. 25(10): p. 551-61.
86. Diebolder, C.A., et al., Complement is activated by IgG hexamers assembled at the cell surface. Science, 2014. 343(6176): p. 1260-3.
87. van Montfoort, N., et al., A novel role of complement factor C1q in augmenting the presentation of antigen captured in immune complexes to CD8+ T lymphocytes. J Immunol, 2007. 178(12): p. 7581-6.
88. Baruah, P., et al., C1q enhances IFN-gamma production by antigen-specific T cells via the CD40 costimulatory pathway on dendritic cells. Blood, 2009. 113(15): p. 3485-3493.
89. Trendelenburg, M., et al., Monocytosis and accelerated activation of lymphocytes in C1q-deficient autoimmune-prone mice. Immunology, 2004. 113(1): p. 80-88.
90. Baruah, P., et al., Mice lacking C1q or C3 show accelerated rejection of minor H disparate skin

grafts and resistance to induction of tolerance. Eur J Immunol, 2010. 40(6): p. 1758-67.
91. Ferry, H., et al., Increased positive selection of B1 cells and reduced B cell tolerance to intracellular antigens in c1q-deficient mice. J Immunol, 2007. 178(5): p. 2916-22.
92. Fossati-Jimack, L., et al., C1q deficiency promotes the production of transgenic-derived IgM and IgG3 autoantibodies in anti-DNA knock-in transgenic mice. Mol Immunol, 2008. 45(3): p. 787-95.
93. Gal, P., et al., Early complement proteases: C1r, C1s and MASPs. A structural insight into activation and functions. Mol Immunol, 2009. 46(14): p. 2745-52.
94. Randall, T.D., L.B. King, and R.B. Corley, The biological effects of IgM hexamer formation. Eur J Immunol, 1990. 20(9): p. 1971-9.
95. Collins, C., F.W. Tsui, and M.J. Shulman, Differential activation of human and guinea pig complement by pentameric and hexameric IgM. Eur J Immunol, 2002. 32(6): p. 1802-10.
96. Johansen, F.E., R. Braathen, and P. Brandtzaeg, Role of J chain in secretory immunoglobulin formation. Scand J Immunol, 2000. 52(3): p. 240-8.
97. Melis, J.P., et al., Complement in therapy and disease: Regulating the complement system with antibody-based therapeutics. Mol Immunol, 2015. 67(2 Pt A): p. 117-30.
98. Burton, D.R., Immunoglobulin G: functional sites. Mol Immunol, 1985. 22(3): p. 161-206.
99. Czajkowsky, D.M. and Z. Shao, The human IgM pentamer is a mushroom-shaped molecule with a flexural bias. Proc Natl Acad Sci U S A, 2009. 106(35): p. 14960-5.
100. Feinstein, A., N. Richardson, and M.I. Taussig, Immunoglobulin flexibility in complement activation. Immunol Today, 1986. 7(6): p. 169-74.
101. Arnold, J.N., et al., Human serum IgM glycosylation: identification of glycoforms that can bind to mannan-binding lectin. J Biol Chem, 2005. 280(32): p. 29080-7.
102. Perkins, S.J., et al., Solution structure of human and mouse immunoglobulin M by synchrotron X-ray scattering and molecular graphics modelling. A possible mechanism for complement activation. J Mol Biol, 1991. 221(4): p. 1345-66.
103. Muller, R., et al., High-resolution structures of the IgM Fc domains reveal principles of its hexamer formation. Proc Natl Acad Sci U S A, 2013. 110(25): p. 10183-8.
104. Hughes-Jones, N.C. and B. Gardner, The reaction between the complement subcomponent C1q, IgG complexes and polyionic molecules. Immunology, 1978. 34(3): p. 459-63.
105. Sledge, C.R. and D.H. Bing, Purification of the human complement protein C1q by affinity chromatography. J Immunol, 1973. 111(3): p. 661-6.
106. Trendelenburg, M., et al., Lack of occurrence of severe lupus nephritis among anti-C1q autoantibody-negative patients. Arthritis Rheum, 1999. 42(1): p. 187-8.
107. Borsos, T. and H.J. Rapp, Complement fixation on cell surfaces by 19S and 7S antibodies. Science, 1965. 150(3695): p. 505-6.
108. Burton, D.R., Antibody: the flexible adaptor molecule. Trends Biochem Sci, 1990. 15(2): p. 64-9.
109. Beurskens, F.J., R.A. van Schaarenburg, and L.A. Trouw, C1q, antibodies and anti-C1q autoantibodies. Mol Immunol, 2015.
110. Daha, N.A., et al., Complement activation by (auto-) antibodies. Mol Immunol, 2011. 48(14): p. 1656-65.
111. Van Schravendijk, M.R. and R.A. Dwek, Interaction of C1q with DNA. Mol Immunol, 1982. 19(9): p. 1179-87.
112. Jiang, H.X., J.N. Siegel, and H. Gewurz, Binding and complement activation by C-reactive protein via the collagen-like region of C1q and inhibition of these reactions by monoclonal antibodies to C-reactive protein and C1q. J Immunol, 1991. 146(7): p. 2324-30.
113. Krumdieck, R., et al., The proteoglycan decorin binds C1q and inhibits the activity of the C1 complex. J Immunol, 1992. 149(11): p. 3695-701.

114. Sjoberg, A., et al., The extracellular matrix and inflammation: fibromodulin activates the classical pathway of complement by directly binding C1q. J Biol Chem, 2005. 280(37): p. 32301-8.
115. Manderson, A.P., M. Botto, and M.J. Walport, The role of complement in the development of systemic lupus erythematosus. Annu Rev Immunol, 2004. 22: p. 431-56.
116. Korb, L.C. and J.M. Ahearn, C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited. J Immunol, 1997. 158(10): p. 4525-8.
117. Nauta, A.J., et al., Direct binding of C1q to apoptotic cells and cell blebs induces complement activation. Eur J Immunol, 2002. 32(6): p. 1726-36.
118. Taylor, P.R., et al., A hierarchical role for classical pathway complement proteins in the clearance of apoptotic cells in vivo. J Exp Med, 2000. 192(3): p. 359-66.
119. Nauta, A.J., et al., Opsonization with C1q and mannose-binding lectin targets apoptotic cells to dendritic cells. J Immunol, 2004. 173(5): p. 3044-50.
120. Clarke, E.V., et al., Complement protein C1q bound to apoptotic cells suppresses human macrophage and dendritic cell-mediated Th17 and Th1 T cell subset proliferation. J Leukoc Biol, 2015. 97(1): p. 147-60.
121. Trouw, L.A., et al., C4b-binding protein and factor H compensate for the loss of membrane-bound complement inhibitors to protect apoptotic cells against excessive complement attack. J Biol Chem, 2007. 282(39): p. 28540-8.
122. Trouw, L.A., A.M. Blom, and P. Gasque, Role of complement and complement regulators in the removal of apoptotic cells. Mol Immunol, 2008. 45(5): p. 1199-207.
123. Nauta, A.J., et al., Mannose-binding lectin engagement with late apoptotic and necrotic cells. Eur J Immunol, 2003. 33(10): p. 2853-63.
124. Xu, W., et al., Properdin binds to late apoptotic and necrotic cells independently of C3b and regulates alternative pathway complement activation. J Immunol, 2008. 180(11): p. 7613-21.
125. Baruah, P., et al., C1q enhances IFN-gamma production by antigen-specific T cells via the CD40 costimulatory pathway on dendritic cells. Blood, 2009. 113(15): p. 3485-93.
126. Stevens, B., et al., The classical complement cascade mediates CNS synapse elimination. Cell, 2007. 131(6): p. 1164-78.
127. Naito, A.T., et al., Complement C1q activates canonical Wnt signaling and promotes aging-related phenotypes. Cell, 2012. 149(6): p. 1298-313.
128. Sumida, T., et al., Complement C1q-induced activation of beta-catenin signalling causes hypertensive arterial remodelling. Nat Commun, 2015. 6: p. 6241.
129. Bossi, F., et al., C1q as a unique player in angiogenesis with therapeutic implication in wound healing. Proc Natl Acad Sci U S A, 2014. 111(11): p. 4209-14.
130. Peerschke, E.I. and B. Ghebrehiwet, cC1qR/CR and gC1qR/p33: observations in cancer. Mol Immunol, 2014. 61(2): p. 100-9.
131. Son, M., et al., C1q limits dendritic cell differentiation and activation by engaging LAIR-1. Proc Natl Acad Sci U S A, 2012. 109(46): p. E3160-7.
132. Walport, M.J., et al., Complement deficiency and autoimmunity. Ann.N.Y.Acad.Sci., 1997. 815: p. 267-281.
133. Carroll, M.C., The lupus paradox. Nat Genet, 1998. 19(1): p. 3-4.
134. Botto, M., et al., Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. Nat.Genet., 1998. 19(1): p. 56-59.
135. Mitchell, D.A., et al., C1q deficiency and autoimmunity: the effects of genetic background on disease expression. J Immunol, 2002. 168(5): p. 2538-43.
136. Racila, E., et al., A polymorphism in the complement component C1qA correlates with prolonged

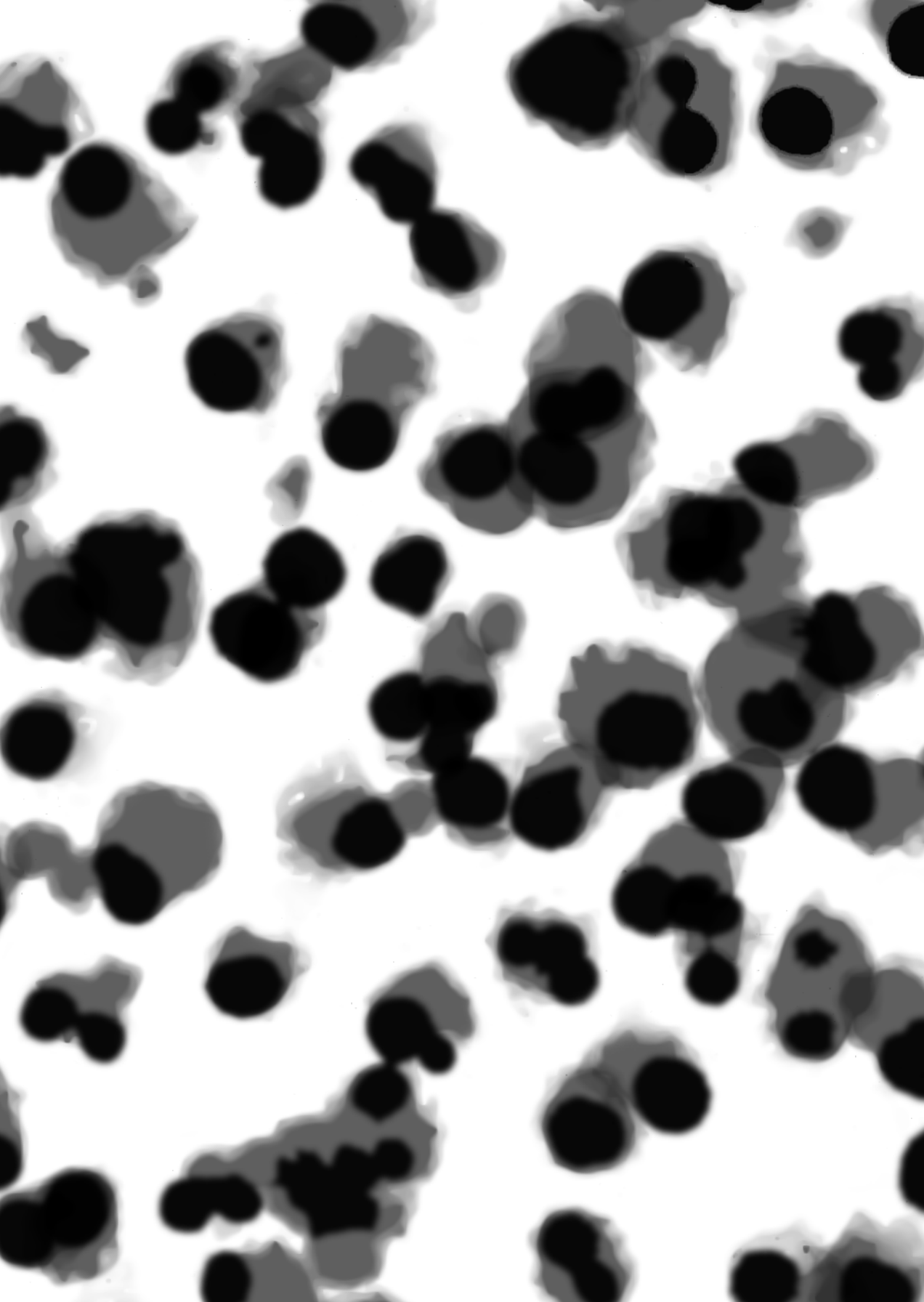
response following rituximab therapy of follicular lymphoma. Clin Cancer Res, 2008. 14(20): p. 6697-703.
137. Racila, E., et al., The pattern of clinical breast cancer metastasis correlates with a single nucleotide polymorphism in the C1qA component of complement. Immunogenetics, 2006. 58(1): p. 1-8.
138. Martens, H.A., et al., Analysis of C1q polymorphisms suggests association with systemic lupus erythematosus, serum C1q and CH50 levels and disease severity. Ann Rheum Dis, 2009. 68(5): p. 715-20.
139. Namjou, B., et al., Evaluation of C1q genomic region in minority racial groups of lupus. Genes Immun., 2009. 10(5): p. 517-524.
140. Racila, D.M., et al., Homozygous single nucleotide polymorphism of the complement C1QA gene is associated with decreased levels of C1q in patients with subacute cutaneous lupus erythematosus. Lupus, 2003. 12(2): p. 124-32.
141. Radanova, M., et al., Association of rs172378 C1q gene cluster polymorphism with lupus nephritis in Bulgarian patients. Lupus, 2015. 24(3): p. 280-9.
142. Rafiq, S., et al., Assessing association of common variation in the C1Q gene cluster with systemic lupus erythematosus. Clin Exp Immunol, 2010. 161(2): p. 284-9.
143. Zervou, M.I., et al., TRAF1/C5, eNOS, C1q, but not STAT4 and PTPN22 gene polymorphisms are associated with genetic susceptibility to systemic lupus erythematosus in Turkey. Hum Immunol, 2011. 72(12): p. 1210-3.
144. Trendelenburg, M., Antibodies against C1q in patients with systemic lupus erythematosus. Springer Semin Immunopathol, 2005. 27(3): p. 276-85.
145. Wener, M.H., S. Uwatoko, and M. Mannik, Antibodies to the collagen-like region of C1q in sera of patients with autoimmune rheumatic diseases. Arthritis Rheum, 1989. 32(5): p. 544-51.
146. Siegert, C.E., et al., IgG and IgA autoantibodies to C1q in systemic and renal diseases. Clin Exp Rheumatol, 1992. 10(1): p. 19-23.
147. Horvath, L., et al., Levels of antibodies against C1q and 60 kDa family of heat shock proteins in the sera of patients with various autoimmune diseases. Immunol Lett, 2001. 75(2): p. 103-9.
148. Potlukova, E., et al., Autoantibodies against complement C1q correlate with the thyroid function in patients with autoimmune thyroid disease. Clin Exp Immunol, 2008. 153(1): p. 96-101.
149. 149. Siegert, C.E., et al., The relationship between serum titers of autoantibodies to C1q and age in the general population and in patients with systemic lupus erythematosus. Clin Immunol Immunopathol, 1993. 67(3 Pt 1): p. 204-9.
150. Wisnieski, J.J. and S.M. Jones, Comparison of autoantibodies to the collagen-like region of C1q in hypocomplementemic urticarial vasculitis syndrome and systemic lupus erythematosus. J Immunol, 1992. 148(5): p. 1396-403.
151. Sinico, R.A., et al., Anti-C1q autoantibodies in lupus nephritis. Ann N Y Acad Sci, 2009. 1173: p. 47-51.
152. Hunnangkul, S., et al., Familial clustering of non-nuclear autoantibodies and C3 and C4 complement components in systemic lupus erythematosus. Arthritis Rheum, 2008. 58(4): p. 1116-24.
153. Prohaszka, Z., et al., C1q autoantibodies in HIV infection: correlation to elevated levels of autoantibodies against 60-kDa heat-shock proteins. Clin Immunol, 1999. 90(2): p. 247-55.
154. Saadoun, D., et al., Anti-C1q antibodies in hepatitis C virus infection. Clin Exp Immunol, 2006. 145(2): p. 308-12.
155. Seelen, M.A., L.A. Trouw, and M.R. Daha, Diagnostic and prognostic significance of anti-C1q antibodies in systemic lupus erythematosus. Curr Opin Nephrol Hypertens, 2003. 12(6): p. 619-24.
156. Mok, C.C., et al., Performance of anti-C1q, antinucleosome, and anti-dsDNA antibodies for detecting concurrent disease activity of systemic lupus erythematosus. Transl Res, 2010. 156(6): p. 320-5.

157. Akhter, E., et al., Anti-C1q antibodies have higher correlation with flares of lupus nephritis than other serum markers. Lupus, 2011. 20(12): p. 1267-74.
158. Matrat, A., et al., Simultaneous detection of anti-C1q and anti-double stranded DNA autoantibodies in lupus nephritis: predictive value for renal flares. Lupus, 2011. 20(1): p. 28-34.
159. Julkunen, H., S. Ekblom-Kullberg, and A. Miettinen, Nonrenal and renal activity of systemic lupus erythematosus: a comparison of two anti-C1q and five anti-dsDNA assays and complement C3 and C4. Rheumatol Int, 2012. 32(8): p. 2445-51.
160. Yang, X.W., et al., Combination of anti-C1q and anti-dsDNA antibodies is associated with higher renal disease activity and predicts renal prognosis of patients with lupus nephritis. Nephrol Dial Transplant, 2012. 27(9): p. 3552-9.
161. Trendelenburg, M., et al., High prevalence of anti-C1q antibodies in biopsy-proven active lupus nephritis. Nephrol Dial Transplant, 2006. 21(11): p. 3115-21.
162. Meyer, O.C., et al., Anti-C1q antibodies antedate patent active glomerulonephritis in patients with systemic lupus erythematosus. Arthritis Res Ther, 2009. 11(3): p. R87.
163. Moura, C.G., et al., Negative anti-C1q antibody titers may influence therapeutic decisions and reduce the number of renal biopsies in systemic lupus erythematosus. Nephron Clin Pract, 2011. 118(4): p. c355-60.
164. Zhang, C.Q., et al., Anti-C1q antibodies are associated with systemic lupus erythematosus disease activity and lupus nephritis in northeast of China. Clin Rheumatol, 2011. 30(7): p. 967-73.
165. Pradhan, V., et al., Anti-C1q antibodies and their association with complement components in Indian systemic lupus erythematosus patients. Indian J Nephrol, 2012. 22(5): p. 353-7.
166. ElGendi, S.S. and W.T. El-Sherif, Anti-C1q antibodies, sCD40L, TWEAK and CD4/CD8 ratio in systemic lupus erythematosus and their relations to disease activity and renal involvement. Egypt J Immunol, 2009. 16(1): p. 135-48.
167. Moroni, G., et al., Anti-C1q antibodies may help in diagnosing a renal flare in lupus nephritis. Am J Kidney Dis, 2001. 37(3): p. 490-8.
168. Yin, Y., et al., Diagnostic value of serum anti-C1q antibodies in patients with lupus nephritis: a meta-analysis. Lupus, 2012. 21(10): p. 1088-97.
169. Grootscholten, C., et al., A prospective study of anti-chromatin and anti-C1q autoantibodies in patients with proliferative lupus nephritis treated with cyclophosphamide pulses or azathioprine/methylprednisolone. Ann Rheum Dis, 2007. 66(5): p. 693-6.
170. Kozyro, I., et al., Autoantibodies against complement C1q in acute post-streptococcal glomerulonephritis. Clin Immunol, 2008. 128(3): p. 409-14.
171. Hogarth, M.B., et al., Autoantibodies to the collagenous region of C1q occur in three strains of lupus-prone mice. Clin Exp Immunol, 1996. 104(2): p. 241-6.
172. Trouw, L.A., et al., Glomerular deposition of C1q and anti-C1q antibodies in mice following injection of antimouse C1q antibodies. Clin Exp Immunol, 2003. 132(1): p. 32-9.
173. Trouw, L.A., et al., Anti-C1q autoantibodies deposit in glomeruli but are only pathogenic in combination with glomerular C1q-containing immune complexes. J Clin Invest, 2004. 114(5): p. 679-88.
174. Trouw, L.A., et al., Anti-C1q autoantibodies in murine lupus nephritis. Clin Exp Immunol, 2004. 135(1): p. 41-8.
175. Hiepe, F., et al., C1q: a multifunctional ligand for a new immunoadsorption treatment. Ther Apher, 1999. 3(3): p. 246-51.
176. Berner, B., et al., Rapid improvement of SLE-specific cutaneous lesions by C1q immunoadsorption. Ann Rheum Dis, 2001. 60(9): p. 898-9.
177. Pfueller, B., et al., Successful treatment of patients with systemic lupus erythematosus by

immunoadsorption with a C1q column: a pilot study. Arthritis Rheum, 2001. 44(8): p. 1962-3.
178. Kohro-Kawata, J., M.H. Wener, and M. Mannik, The effect of high salt concentration on detection of serum immune complexes and autoantibodies to C1q in patients with systemic lupus erythematosus. J Rheumatol, 2002. 29(1): p. 84-9.
179. ntes, U., H.P. Heinz, and M. Loos, Evidence for the presence of autoantibodies to the collagen-like portion of C1q in systemic lupus erythematosus. Arthritis Rheum, 1988. 31(4): p. 457-64.
180. Golan, M.D., R. Burger, and M. Loos, Conformational changes in C1q after binding to immune complexes: detection of neoantigens with monoclonal antibodies. J Immunol, 1982. 129(2): p. 445-7.
181. Schaller, M., et al., Autoantibodies against C1q in systemic lupus erythematosus are antigen-driven. J Immunol, 2009. 183(12): p. 8225-31.
182. Vanhecke, D., et al., Identification of a major linear C1q epitope allows detection of systemic lupus erythematosus anti-C1q antibodies by a specific peptide-based enzyme-linked immunosorbent assay. Arthritis Rheum, 2012. 64(11): p. 3706-14.
183. Tsacheva, I., et al., Detection of autoantibodies against the globular domain of human C1q in the sera of systemic lupus erythematosus patients. Mol Immunol, 2007. 44(8): p. 2147-51.
184. He, S. and Y.L. Lin, In vitro stimulation of C1s proteolytic activities by C1s-presenting autoantibodies from patients with systemic lupus erythematosus. J Immunol, 1998. 160(9): p. 4641-7.
185. Agnello, V., et al., C1q PRECIPITINS IN THE SERA OF PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS AND OTHER HYPOCOMPLEMENTEMIC STATES: CHARACTERIZATION OF HIGH AND LOW MOLECULAR WEIGHT TYPES. J Exp Med, 1971. 134(3): p. 228-41.
186. Uwatoko, S., et al., Characterization of C1q-binding IgG complexes in systemic lupus erythematosus. Clin Immunol Immunopathol, 1984. 30(1): p. 104-16.
187. Uwatoko, S., et al., C1q solid-phase radioimmunoassay: evidence for detection of antibody directed against the collagen-like region of C1q in sera from patients with systemic lupus erythematosus. Clin Exp Immunol, 1987. 69(1): p. 98-106.
188. Wener, M.H., et al., Relationship between renal pathology and the size of circulating immune complexes in patients with systemic lupus erythematosus. Medicine (Baltimore), 1987. 66(2): p. 85-97.

Part I

The clinical association of C1q and anti-C1q



Chapter 2

Identification of a novel non-coding mutation in C1qB in a Dutch child with C1q deficiency associated with recurrent infections

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Abstract

C1q deficiency is a rare genetic disorder that is strongly associated with development of Systemic Lupus Erythematosus (SLE). Several mutations in the coding regions of the C1q genes have been described that result in stop-codons or other genetic abnormalities ultimately leading to C1q deficiency. Here we report on a Dutch boy suffering from recurrent infections with a complete C1q deficiency, without any SLE symptoms.

The presence of C1q in serum was assessed using ELISA and hemolytic assay. By western blot we examined the different C1q chains in cell lysates. We identified the mutation using deep-sequencing. By qPCR we studied the mRNA expression of C1qA, C1qB and C1qC in the PBMCs of the patient.

Deep-sequencing revealed a homozygous mutation in the non-coding region of C1qB in the patient, whereas both parents were heterozygous. The mutation is located two nucleotides before the splice site of the second exon. In-silico analyses predict a complete abrogation of this natural splice site. Analyses of in vitro cultured cells from the patient revealed a lack of production of C1q and intracellular absence of C1qB in the presence of C1qA and C1qC peptides. Quantitative PCR analysis revealed total absence of C1qB mRNA, a reduced level of C1qA mRNA and normal levels of C1qC mRNA.

In this study we report a new mutation in the non-coding region of C1qB that is associated with C1q deficiency.

Introduction

C1q is the recognition molecule of the classical pathway of complement activation [1]. Next to its role in activation of the complement system, C1q has also been shown to bind to apoptotic and necrotic cells to facilitate their clearance [2, 3]. In the absence of C1q, accumulation of apoptotic material was noted, which led to the formulation of the 'waste disposal hypothesis'[4, 5]. Likewise, in the absence of C1q also immune complexes cannot be cleared effectively resulting in their accumulation. Over the recent years more insight has been generated in the role of C1q in modulating the adaptive immune response [6-8]. Collectively these data indicate that absence of C1q may not only impact on clearance mechanisms for e.g. apoptotic cells and immune complexes, but also on the adaptive immune responses [9]. In most C1q deficient individuals the balance is shifted towards autoimmunity and the development of Systemic Lupus Erythematosus (SLE). Complete genetic

deficiency of C1q is strongly associated with development of SLE, but in some individuals the disease mainly presents with recurrent infections or, in exceptional cases, remains largely unnoticed [10-12].
The C1 complex is composed of 3 different proteins; one C1q molecule, two C1r molecules and two C1s molecules. C1q is the recognition molecule of the C1 complex and is composed of 18 polypeptide chains: 6 C1qA, 6 C1qB and 6 C1qC. Each chain has a collagen-like region (N-terminal region) and a globular head region. The C1qA chains associate with the C1qB chains as heterodimers. C1qC chains first will form homodimers and finally associate with the A-B heterodimers to eventually form a tulip-like structure [13]. The genes encoding these three subunits are located on chromosome 1 within a genomic region of ~25kb and have an ACB orientation [14]. C1q is thought to be produced predominantly by immature dendritic cells and macrophages [15, 16]. C1q production can be upregulated via IFN-γ stimulation in which the expression of the three chains is suggested to be synchronized via transcription factors PU.1 and IRF8 [17].
Nowadays around 65 cases of C1q deficiencies have been reported [18]. These deficiencies are mostly caused by homozygous mutations in one of the chains. The most common mutation is in the A-chain caused by a nonsense mutation whereby a transition of C to T occurs in a codon for Glu-186 [13]. Next to mutations causing C1q deficiency also several genetic variations in C1q have been associated with increased risk for developing autoimmunity [19, 20]. To which extent mutations in different C1q chains affect the clinical presentation, e.g. infection versus SLE, is currently unclear.
Here we report on a non-coding homozygous mutation in an RNA splice site that leads to complete lack of expression of C1qB and hence lack of secretion of C1q.

Materials and Methods

Patient and controls

We have analyzed in detail one C1q-deficient Caucasian patient and studied materials obtained from both his parents as controls as well as a reference panel of 48 healthy adult controls collected in the LUMC. Informed consent was obtained from the parents and the controls in compliance with the Helsinki declaration.

Samples

Blood was collected from the patient and both parents in order to obtain serum and to isolate DNA. From the patient also PBMCs were collected using Ficoll-Paque

density gradient centrifugation. Control serum samples were chosen matched by age of the parents.

Functional reconstitution complement activity assays

Using an erythrocyte lysis assay the complement-mediated hemolytic activity was measured. Rabbit antibody coated sheep erythrocytes were used and different dilutions of serum of the patient or normal human sera (NHS) were added to C1q-, C2- or C4-depleted serum. Hemolysis was assessed by measuring OD at 414 nm, and specific complement-mediated lysis was calculated as described before [21]. To exclude the possibility that next to C1q deficient the patients sample would also be deficient for C1r or C1s we performed assays to measure activation of the classical pathway of the patient serum by reconstitution of purified C1q. Plates coated with human IgG were incubated with 1% serum of the patient (diluted in GVB++; 0.1 % gelatin, 5 mM Veronal, 145 mM NaCl, 0.025 % NaN3, 0.15 mM calcium chloride, 0.5 mM magnesium chloride, pH 7.3) with or without addition of purified C1q (Quidel) in different concentrations. As a read-out C4 deposition was measured.

Cell stimulation

PBMCs (1 x 106 cells/ml) were stimulated in a 48 wells plate (Corning Inc.) for 72 hours using RPMI (Gibco) supplemented with IFN-γ (200 U/ml, Peprotech) and 10 μM dexamethasone (Pharmacy LUMC, Leiden, The Netherlands) as described before to increase the C1q secretion (Walker, 1998;Moosig et al., 2006;Kaul & Loos, 2001). After 72 hours the supernatants and the cells were collected. The supernatants were used for ELISA and the cells for extraction of mRNA for qPCR and for western blot analysis of cell lysates.

Detection of C1q by ELISA

C1q levels in serum and culture supernatants were analyzed by ELISA. Maxisorp plates (Nunc) were coated with rabbit anti-human C1q (DAKO) in coating buffer (0.1 M NA2CO3, 0.1 M NaHCO3, pH 9.6) overnight at 4°C. Plates were washed in PBS/0.05% Tween (PBS-T, Sigma). Then the wells were blocked with PBS/1% BSA for 1 hour at room temperature. After washing, the standard of C1q (Quidel), applied in a two-fold dilution series starting from 150 ng/ml, control samples and patient samples in a 1:8000 dilution in PBS/1% BSA/0.05% Tween (Sigma) were added to the wells. The standard and the serum samples were incubated for 1 hour at 37°C. After incubation the plates were washed and rabbit anti-C1q DIG (Nephrology, LUMC) was added to the wells and the plate was incubated for 1

hour at 37°C. As detection antibody sheep anti-DIG HRP (Roche diagnostics) was added for 1 hour at 37°C, following final washing a substrate reaction was performed using ABTS (Sigma). C1q levels in the cell supernatants were measured with minor adjustments.

Detection of C1q by western blot

The presence of C1q in serum and in lysates of cultured PBMCs was analyzed by western blot. Serum samples of the patient and his parents were analyzed using reducing conditions at a dilution of 1:100 of the samples. Cell lysates were generated from PBMCs after 72 hours of stimulation. Cells were lysed on ice in lysis buffer (Roche Diagnostics) supplemented with a protease inhibitor (Sigma) for 30 minutes on ice. To avoid contamination of cell debris, cell lysates were centrifuged. The cell lysates were diluted 4x with Laemmli sample buffer (Bio-Rad)/ 5% ß-mercaptoethanol (Merck) and boiled for 5 minutes on 95°C to reduce the samples. Proteins were separated by SDS/PAGE on a mini-protean TGX precast gel (4-15%, Bio-Rad) followed by protein transfer on a Trans-Blot Turbo Transfer pack: mini, 0.2 µM PVDF (Bio-Rad). The membrane was blocked one hour in PBS containing 0.05% Tween and 3% skimmed milk (Fluka) on room temperature. Next, the blot was incubated with rabbit anti-human C1q (DAKO) overnight on 4°C. After washing with PBS/0.1% Tween the membrane was incubated with goat anti-rabbit HRP (DAKO) for 1 hour at room temperature. Then the blot was washed and C1q was visualized using ECL Western Blotting Analysis system (GE Healthcare).

Sequencing

Genomic DNA was extracted from blood collected with tubes supplemented with EDTA. Sequencing of the complete C1q genes (C1qA, C1qB and C1qC), of both introns and exons was performed. Deep-sequencing was performed using the 454 NGS Roche GS FLX Titanium platform. Data were compared to internal controls and to Human Genome build 19 as well as Human_v37_2 de dbSNP database v132 using the NextGENe software package for Next Generation Sequence Analysis (NGS) from Softgenetics. The effect of the mutation on splicing was in-silico analyzed using the NetGene2 Server http://www.cbs.dtu.dk/services/NetGene2/. To confirm the mutation Sanger sequencing was performed on C1qB using standard protocol.

qPCR

RNA was isolated from the cultured cells using the mirVana RNA isolation kit (Life Technologies) and analyzed with the NanoDrop (NanoDrop Technologies), followed by DNase treatment (Invitrogen) and cDNA synthesis with superscript III (200U/µl,

Invitrogen). Real time SYBR Green I qPCR was performed with primers specific for C1q genes C1qA/B/C separately (see table 1 for primer sequences) to analyze expression on transcriptional level. ß-actin was used as reference gene, CD14 gene expression was used as control for cellular input. The cDNA was diluted 1:50 and qPCR was performed using SensiFast Sybr (Bio-line) and primers with a start concentration of 10 pmol. The qPCR was performed on the real time PCR system (BioRad CFX-384) with an activation step of 3 min on 95°C (hot start polymerase activation), a melting temperature of 95°C for 5 seconds and an annealing temperature of 60.5 °C for 5 seconds and an elongation step for 20 seconds on 72°C for 40 cycles. At the end of the protocol melting curves were performed from 65°C to 95°C to test specific binding of SensiFast Sybr. The qPCR products were analyzed on a 2% agarose gel and visualized with nancy 520 (Sigma).

Gene	5' Forward	3' Reverse
ß-actin	GCAATGAGCGGTTCCGCTGC	CGATCCACACGGAGTACTTG
C1qA exon 3	TGGAGTTGACAACAGGAGGC	CGATATGGCCAGCACACAGA
C1qB exon 1 - 2	GACCGAGGGCAGTAGGCTC	TCATCATACTGTGTCAGACGCC
C1qC exon 2 - 3	AAGGARGGGTACGACGGACT	GTAAGCCGGGTTCTCCCTTC
CD14	GCCGCTGTGTAGGAAAGAAG	AGGTTCGGAGAAGTTGCAGA

Table 1. Primers used in the qPCR analysis

Statistical analysis

Statistical analysis on the qPCR data was performed using paired sample t-test. P-value's <0.05 were considered significant (Graphpad Prism software version 5.01).

Results

Patient

Here we describe a Dutch boy born from two Caucasian, healthy, non-consanguineous parents. During the first years of life he has suffered from recurrent upper airway infections. At the age of three he developed redness and swelling of the left ankle combined with fever (39°C) after an injury. Initially a cellulitis was suspected and treated with flucloxacillin. Due to persistent complaints he was admitted to a local hospital. A subsequent skeletal scintigraphy yielded a hotspot of the distal tibia compatible with the diagnosis osteomyelitis. Blood cultures remained negative. After intravenous antibiotics he recovered completely.

At the age of 5, he was admitted due to seizures and high fever. The analysis of cerebrospinal fluid yielded 470 x10E6 leukocytes/liter and both blood and CSF cultures showed a pneumococcal infection. Treatment with cephalosporin and dexamethasone resulted in complete recovery.
Examination of his medical history revealed that he had frequent upper airway infections and analysis for immune deficiencies showed a low classical pathway activity of 2% (reference range > 74 %) and a low level of IgG4 (0.007 g/l) (reference range 0.017 – 1.58 g/l), whereas C3 (1.1 mg/L, reference range: 0.9-2.0mg/L) and C4 (267 mg/L, reference range: 95-415 mg/L) were in the normal range. The alternative pathway (74%, reference range: >39%) showed no abnormalities and no antinuclear antibodies and rheumatoid factor were detected. Using an activity assay we investigated which complement protein from the classical pathway was defective. Complete absence of functional complement protein C1q was detected by reconstitution experiments in a C1q-specific hemolytic assay (Figure 1A). Similar reconstitution experiments revealed that the patient has a normal C2 and C4 activity (Figure 1B-C). With a reconstitution assay we could rescue the activity of the classical pathway by adding purified C1q to the patient serum (Figure 1D). At follow-up at the age of 8 years he was healthy without infectious problems or signs of autoimmunity.

Complete absence of C1q in the serum

Using ELISA we confirmed a complete C1q deficiency in the patient. Sera of both parents were also analyzed and their level of circulating C1q was in the same range as values obtained in a set of healthy adult controls (Figure 2A). In addition we have used western blot to analyze the presence of C1q in sera of the patient, parents and controls, which also confirmed the complete absence of circulating C1q in the patient, with normal levels in the parents (Figure 2B).

Identification of a splice site mutation in C1qB

Deep sequencing of the C1q genes (C1qA,B,C), revealed a mutation in homozygous state in the patient and in heterozygous state in both parents (data not shown). This mutation is located 2 nucleotides before the mRNA splice site of the second exon of C1qB (g.6251A>C). In-silico prediction programs indicate a complete absence of splicing when the mutant form is present. As a confirmation also Sanger sequencing was performed on the C1qB region identified by deep-sequencing. Using this technology, it was confirmed that the patient has a nucleotide change replacing adenine into a cytosine (Figure 3).

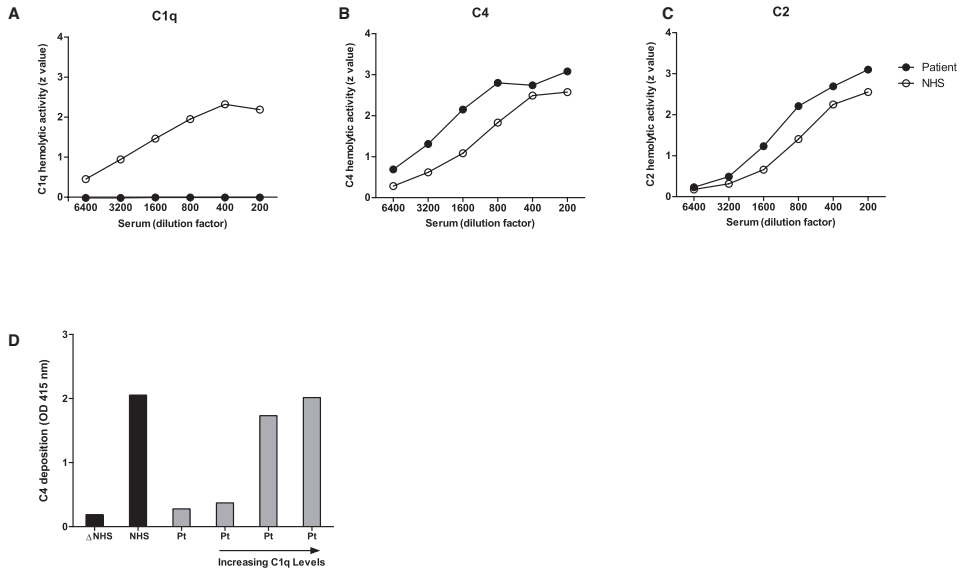

Figure 1. Functional reconstitution complement activity assays.

A. The addition of patient serum or NHS in a dilution range to C1q depleted serum. The total lysis is used as read-out. As positive control (100% lysis) distilled water was used. **B.** The addition of patient or NHS to C2 depleted serum. **C.** The addition of patient or NHS to C4 depleted serum. **D.** Reconstitution of the classical pathway by adding 0.05 µg/ml, 0.5 µg/ml and 2.5 µg/ml purified C1q to the patient serum. As a positive control normal human serum was used (NHS) and as a negative control heat inactivated NHS (ΔNHS) was used.

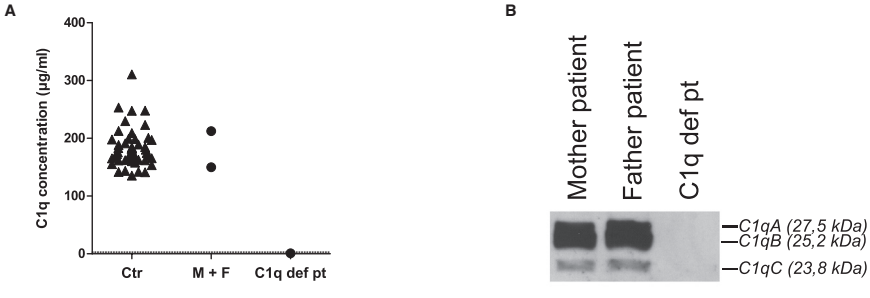

Figure 2. ELISA and western blot analysis of patient serum.

A. The presence of C1q in the serum was analyzed by ELISA. Diluted serum from the C1q-deficient patient (C1q def pt), mother and father (M + F) were compared with healthy controls (n=48) (Ctr). Dashed horizontal line indicates the lower detection limit of the assay, which was 0.01 µg/ml. **B.** Serum samples from the C1q-deficient patient together with the mother and father were analyzed by western blotting for the presence of the peptides C1qA, C1qB and C1qC in the serum using an antibody staining for C1q.

No secretion of C1q by PBMCs of the patient in-vitro

With western blot we confirmed that C1q is present in serum from both parents whereas the child is completely C1q-deficient (Figure 2B). As absence of circulating C1q in serum could be the result of either lack of production or consumption, we studied the secretion and intracellular accumulation of C1q using cells of the patient and control. Analysis of culture supernatant from PBMC's of the patient collected after 72 hours of stimulation with IFN-γ and dexamethasone revealed no secretion of C1q, whereas significant amounts of C1q were present in the supernatant of control PBMC in unstimulated and in the stimulated conditions (Figure 4A). Lack of secretion of C1q can be the result of intracellular accumulation of C1q. Therefore we also investigated the intracellular presence of C1q, using western blot analysis on cell lysates. Following 72 hours stimulation with IFN-γ and dexamethasone the three peptides of C1q were clearly detectable in the lysates of the control PBMC's. However, in the lysates of PBMC's of the patient only expression of C1qA and C1qC was detected, but no C1qB peptide (Figure 4B). Taken together, the results show that C1qA and C1qC peptides are transcribed and translated and that the C1q deficiency is due to the absence of the C1qB peptide.

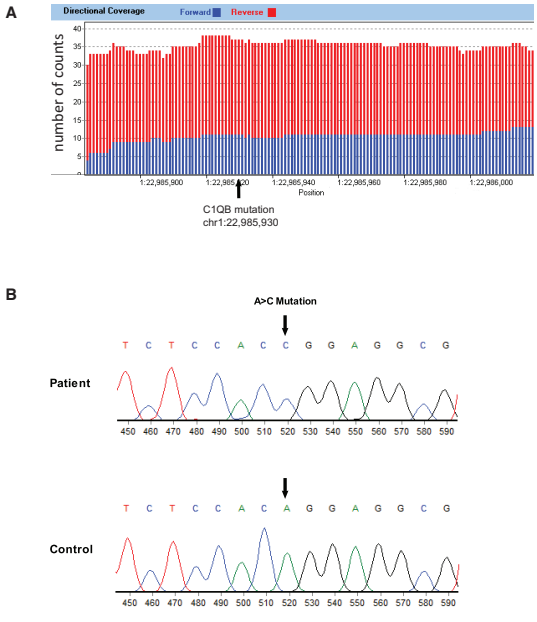

Figure 3. DNA sequencing data from the C1q-deficient patient.

A. Data obtained from deep sequencing showing a C1qB mutation on location chr1:22,985,930, g.6251A > C. **B.** As confirmation Sanger sequencing is performed indicating a nucleotide change in the intron before exon 2 of the C1qB gene. The C1q deficient patient is compared to a healthy donor.

qPCR analysis of expression of C1qA, C1qB and C1qC

A set of intron spanning primers was designed (Table 1) to analyze the expression levels of C1qA, C1qB and C1qC relative to the expression of the reference gene ß-actin. RNA isolated from the in-vitro stimulated PBMCs of patient and control was used as input material. We observed, as expected, complete lack of expression of C1qB in the patient (Figure 5), but also a partial reduction in the expression of C1qA by qPCR. The expression level of C1qC was not different between patient and control (Figure 5A). With the agarose gel we confirm that the primers for the qPCR are specific (Figure 5B). Expression levels of CD14, as a control for the input of monocytes, were similar in both control and patient (data not shown).

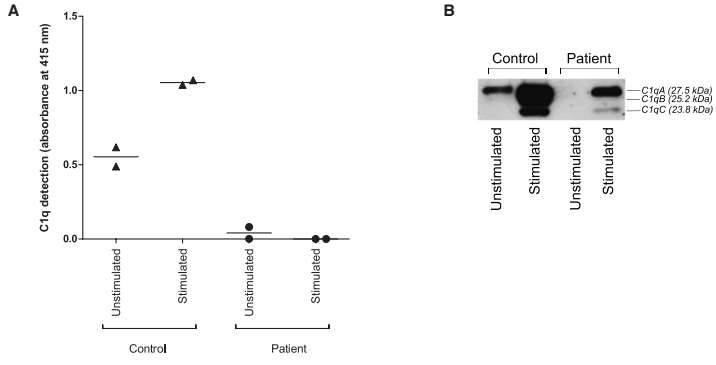

Figure 4. ELISA and western blot of stimulated cells from the C1q-deficient patient.

A. Undiluted cell supernatants were analyzed in duplicate by ELISA for the presence of C1q with or without stimulation of PBMC by IFN-γ (200 U/ml) together with DXM (10 μM). PBMCs of the C1q-deficient patient were stimulated for 72 hour to increase the C1q production. PBMCs from a healthy person were used as a control. **B.** After stimulation, the presence of intracellular C1qA, C1qB and C1qC peptides were examined in reduced cell lysates. The three peptides were detected closely together: C1qA at 27.5 kDa, C1qB at 25.2 kDa and C1qC at 23.8 kDa.

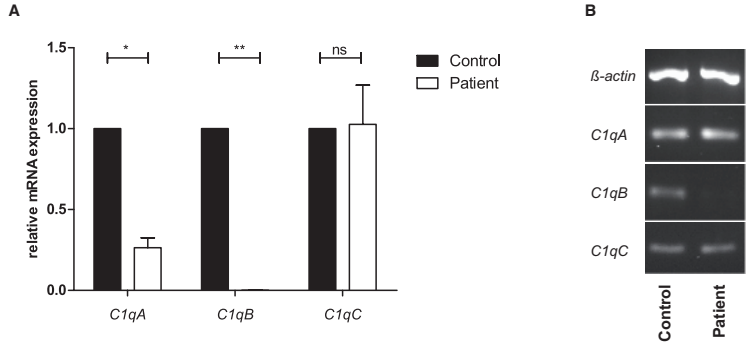

Figure 5. Expression levels of C1qA, C1qB and C1qC

A. Relative mRNA expression of the three C1q chains after stimulation with IFN-γ and DXM for 72 hours. As reference gene ß-actin was used. **P < 0.01, *** P < 0.001. **B.** qPCR products from control PBMCs and the C1q-deficient patient were analyzed on a 2% agarose gel.

Discussion

C1q deficiency is a rare hereditary condition associated with a high prevalence of SLE. The combination of reduced clearance of dying cells and immune complexes and an altered balance in the adaptive immune system likely increases the chance to develop the autoimmune disease SLE [7, 22, 23]. Globally around 65 patients have been described with a C1q deficiency [10]. Interestingly these patients display substantial variation in their clinical presentation, disease progression and outcome [24].

The patient described in this manuscript showed a clear absence of circulating C1q, but is not suffering from lupus, as evidenced by a lack of antinuclear antibodies and rheumatoid factor, but rather from recurrent infections. Although the patient is currently not suffering from lupus, the patient may develop lupus in the future. The IgG4 levels were lower compared to normal levels in this age category. This is often observed in patients with a complement deficiency in the classical pathway and may correspond with the recurrent infections of the upper airways [25].

A common phenomenon in C1q deficiency is that the parents are consanguineous, but the parents of this patient reported not to be related to each other (although not confirmed by genetic analyses). In the vast majority of C1q-deficient patients the mutation is located in the coding region of one of the chains, with only one report on a non-coding mutation [10, 26]. The current patient also harbors a mutation in a non-coding region. The mutation, located in C1qB, results in the abolition of a splice site with as a consequence complete C1q deficiency. This splice site in intron 1 in front of exon 2, which influences the splicing of intron 1. Intron 1 has a size of 6kb, which is too large to be quantified by qPCR and therefore the product of C1qB will conceivably be too large for efficient transcription or an unstable protein will be translated. By qPCR the C1qA and C1qC chains are still detected. This suggests that in the absence of C1qB the transcription of C1qA and C1qC will remain.

By western blot we could not detect any peptide of C1q in serum, but in cell lysates of activated PBMC's, C1qA and C1qC chains, but not C1qB chains could be detected. The protein levels for the C1qA and C1qC chains were lower compared to the control. Because the C1qB chain is not produced, a complete C1q protein cannot be generated, possibly followed by degradation of C1qA and C1qC. This is confirmed by ELISA, where no secreted C1q could be detected after stimulation of PBMCs of the patient.

In conclusion, we have identified a novel non-coding mutation in a splice site for C1qB that is associated with complete C1q deficiency. The patient showed several infectious problems but is currently doing well and shows no signs of autoimmunity.

He is receiving prophylactic antibiotics to protect him from severe infections and will stay under close control to detect possible development of autoimmunity in an early stage.

Acknowledgements

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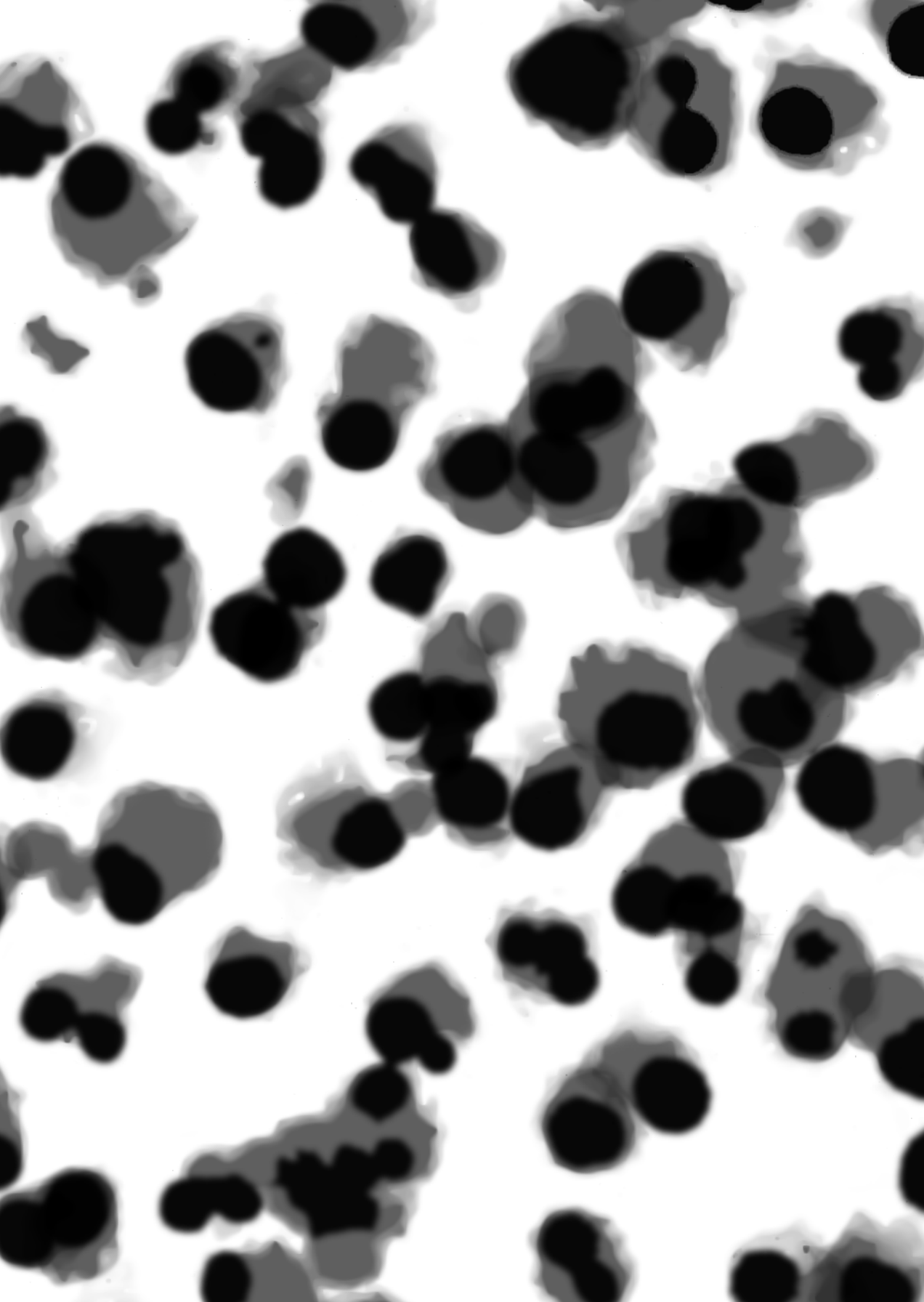
Reference list

1. Daha, N.A., et al., Complement activation by (auto-) antibodies. Mol.Immunol., 2011. 48(14): p. 1656-1665.
2. Korb, L.C. and J.M. Ahearn, C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited. J.Immunol., 1997. 158(10): p. 4525-4528.
3. Nauta, A.J., et al., Direct binding of C1q to apoptotic cells and cell blebs induces complement activation. Eur.J.Immunol., 2002. 32(6): p. 1726-1736.
4. Trouw, L.A., A.M. Blom, and P. Gasque, Role of complement and complement regulators in the removal of apoptotic cells. Mol.Immunol., 2008. 45(5): p. 1199-1207.
5. Walport, M.J., Complement. Second of two parts. N.Engl.J.Med., 2001. 344(15): p. 1140-1144.
6. Fossati-Jimack, L., et al., C1q deficiency promotes the production of transgenic-derived IgM and IgG3 autoantibodies in anti-DNA knock-in transgenic mice. Mol.Immunol., 2008. 45(3): p. 787-795.
7. Baruah, P., et al., C1q enhances IFN-gamma production by antigen-specific T cells via the CD40 costimulatory pathway on dendritic cells. Blood, 2009. 113(15): p. 3485-3493.
8. Jiang, K., et al., T cell activation by soluble C1q-bearing immune complexes: implications for the pathogenesis of rheumatoid arthritis. Clin.Exp.Immunol., 2003. 131(1): p. 61-67.
9. Santer, D.M., et al., C1q deficiency leads to the defective suppression of IFN-alpha in response to nucleoprotein containing immune complexes. J.Immunol., 2010. 185(8): p. 4738-4749.
10. Schejbel, L., et al., Combined IL-12 receptor and IgA deficiency in an adult man intestinally infested by an unknown, non-cultivable mycobacterium. Scand.J.Immunol., 2011. 74(6): p. 548-553.
11. Vassallo, G., et al., Clinical variability and characteristic autoantibody profile in primary C1q complement deficiency. Rheumatology.(Oxford), 2007. 46(10): p. 1612-1614.
12. Walport, M.J., K.A. Davies, and M. Botto, C1q and systemic lupus erythematosus. Immunobiology, 1998. 199(2): p. 265-285.
13. Petry, F., Molecular basis of hereditary C1q deficiency. Immunobiology, 1998. 199(2): p. 286-294.
14. Sellar, G.C., D.J. Blake, and K.B. Reid, Characterization and organization of the genes encoding the A-, B- and C-chains of human complement subcomponent C1q. The complete derived amino acid sequence of human C1q. Biochem.J., 1991. 274 (Pt 2): p. 481-490.
15. Loos, M., H. Martin, and F. Petry, The biosynthesis of C1q, the collagen-like and Fc-recognizing molecule of the complement system. Behring Inst.Mitt., 1989(84): p. 32-41.
16. Castellano, G., et al., Maturation of dendritic cells abrogates C1q production in vivo and in vitro. Blood, 2004. 103(10): p. 3813-20.
17. Chen, G., et al., Molecular mechanisms for synchronized transcription of three complement C1q subunit genes in dendritic cells and macrophages. J.Biol.Chem., 2011. 286(40): p. 34941-34950.
18. Schejbel, L., et al., Molecular basis of hereditary C1q deficiency--revisited: identification of several novel disease-causing mutations. Genes Immun., 2011. 12(8): p. 626-634.
19. Martens, H.A., et al., Analysis of C1q polymorphisms suggests association with systemic lupus erythematosus, serum C1q and CH50 levels and disease severity. Ann Rheum Dis, 2009. 68(5): p. 715-20.
20. Trouw, L.A., et al., Genetic variants in the region of the C1q genes are associated with Rheumatoid Arthritis. Clin.Exp.Immunol., 2013.
21. Roos, A., et al., Specific inhibition of the classical complement pathway by C1q-binding peptides. J.Immunol., 2001. 167(12): p. 7052-7059.
22. Trendelenburg, M., et al., Monocytosis and accelerated activation of lymphocytes in C1q-deficient

autoimmune-prone mice. Immunology, 2004. 113(1): p. 80-88.
23. Gullstrand, B., et al., Complement classical pathway components are all important in clearance of apoptotic and secondary necrotic cells. Clin.Exp.Immunol., 2009. 156(2): p. 303-311.
24. Al-Mayouf, S.M., H. Abanomi, and A. Eldali, Impact of C1q deficiency on the severity and outcome of childhood systemic lupus erythematosus. Int.J.Rheum.Dis., 2011. 14(1): p. 81-85.
25. Bird, P. and P.J. Lachmann, The regulation of IgG subclass production in man: low serum IgG4 in inherited deficiencies of the classical pathway of C3 activation. Eur.J.Immunol., 1988. 18(8): p. 1217-1222.
26. Higuchi, Y., et al., The identification of a novel splicing mutation in C1qB in a Japanese family with C1q deficiency: a case report. Pediatr.Rheumatol.Online.J., 2013. 11(1): p. 41.



Chapter 3

Marked variability in clinical presentation and outcome of patients with C1q immunodeficiency

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Abstract

Globally approximately 60 cases of C1q deficiency have been described with a high prevalence of Systemic Lupus Erythematosus (SLE). So far treatment has been guided by the clinical presentation rather than the underlying C1q deficiency. Recently, it was shown that C1q production can be restored by allogeneic haematopoietic stem cell transplantation. Current literature lacks information on disease progression and quality of life of C1q deficient persons which is of major importance to guide clinicians taking care of patients with this rare disease.
We performed an international survey, of clinicians treating C1q deficient patients. A high response rate of >70% of the contacted clinicians yielded information on 45 patients with C1q deficiency of which 25 are published.
Follow-up data of 45 patients from 31 families was obtained for a median of 11 years after diagnosis. Of these patients 36 (80%) suffer from SLE, of which 16 suffer from SLE and infections, 5 (11%) suffer from infections only and 4 (9%) have no symptoms. In total 9 (20%) of the C1q deficient individuals had died. All except for one died before the age of 20 years. Estimated survival times suggest 20% case-fatality before the age of 20, and at least 50% of patients are expected to reach their middle ages.
Here we report the largest phenotypic data set on C1q deficiency to date, revealing high variance; with high mortality but also a subset of patients with an excellent prognosis. Management of C1q deficiency requires a personalized approach.

Introduction

C1q deficiency is a rare hereditary disorder, which is strongly associated with development of Systemic Lupus Erythematosus (SLE)[1, 2]. The first C1q deficient patient was reported in 1979 [3]. To date more than 60 cases of C1q deficiency have been published with various mutations [4-8]. C1q deficiency has been observed in persons from several ethnic backgrounds [1].
C1q is the recognition molecule of the classical pathway of the complement system and together with C1r and C1s it forms the C1 complex. This complex is important for recognizing e.g. immune complexes and to activate the complement system. C1q is mainly produced by macrophages and immature dendritic cells and has several ligands including bound IgM, complexed IgG but also DNA and CRP [9-11] In the context of autoimmunity another important ligand for C1q is present on apoptotic and necrotic cells [12-14]. Hence, C1q is important to clear necrotic

cells or apoptotic blebs from the circulation as described as the “waste disposal hypothesis” [15]. When the “waste disposal” is disturbed, apoptotic and necrotic material containing autoantigens accumulates resulting in a state that could predispose to development of autoimmunity like in SLE[16]. In addition to a role in the waste disposal process C1q has also been implicated in modulating the adaptive immune response[17-19]. Collectively these data indicate that absence of C1q may not only predispose to infections but also predispose to autoimmunity because of defective clearance of autoantigens and an altered adaptive immune response [20]. In most identified C1q deficient individuals the clinical presentation is towards autoimmunity and the development of SLE, whereas in some individuals the disease mainly presents in the form of recurrent infections e.g. meningitis and in exceptional cases remains largely unnoticed [5, 21].

Until now 16 nonsense and missense mutations have been described which are present in 1 of the 3 chains of C1q (chromosomal location: 1p34-1p36.3) [5, 22-25]. Mutations causing C1q deficiency are in most cases present in homozygous form and the parents often report a degree of consanguinity [5].

The treatment of C1q deficient patients has until recently mainly been aimed at the symptoms, rather than reversing the underlying C1q deficiency. The exception in the past has been the infusion of fresh frozen plasma containing C1q in a subset of the patients. This treatment has been well tolerated, led to substantial clinical improvements and did not lead to overt induction of anti-C1q antibody formation [23, 26]. Based on the observation that C1q levels could be restored by bone marrow transplantation in C1q deficient mice [27, 28], now Haematopoietic Stem Cells Transplantations (HSCT) have been performed in two C1q deficient individuals in Sweden and one in the United Kingdom. In all three cases the transplantation led to restoration of circulating C1q levels and an improvement in clinical symptoms [29-31]. During follow-up two patients did well, whereas the other passed away due to intracerebral hemorrage and multi-organ failure. The risk of HSCT related morbidity and mortality has to be weighed against its potential benefits. HSCT related risk is increased in patients with advanced autoimmune disease, or organ damage caused by recurrent infections. Therefore, insight into the natural history of C1q deficiency is crucial to develop a therapeutic algorithm. Most current C1q deficiency literature reports on the identification of new mutations, in young children, but there is no data available on clinical follow up. In this study, we have conducted a survey by contacting clinicians who are currently treating C1q deficient patients.

The aim of this study was to obtain insight into the prognosis of C1q deficient individuals.

Methods

Questionnaire

To study the clinical follow up of C1q deficient individuals, we designed a questionnaire (Table 1). This was sent by email to the corresponding authors of several case- and concise reports as well as to clinicians treating C1q deficient patients. From the 45 individuals, 25 individuals are published in literature and 20 are undescribed.

The questionnaire:
• What is the age, gender and country of origin?
• Are the parents consanguineous?
• At which age was the C1q deficiency established?
• Was the C1q deficiency confirmed by genetic tests?
• What was the clinical diagnosis at the moment of establishing C1q deficiency (Infection/SLE/other)?
• What was the age of first symptoms?
• What were the first symptoms (Infection/SLE/other)?
• What treatment options were applied and what was the response?
• Were there any severe infections? Which type?
• What is the frequency of mild infections? Otitis media / Upper respiratory tract infections / Mild GI infections / Unexplained fever above 38°C (never / 1-2 x per year / 3-5 x per year / > 5 times per year)?
• Had there been any other significant clinical problems presentations after diagnosis?
• Has the patient been successfully vaccinated? Has the patient received plasma, and was this successful?
• Was stem cell transplantation considered? Why (not)?
• Is the patient still alive? Yes (current age) No (age of death and cause of death)
• What is your impression of the overall quality of life of the patient (grade from 1-10 with 1 being very poor and 10 being great)
• Please provide information of affected and unaffected relatives.

Table 1. The questionnaire that was sent to all clinicians treating C1q deficient patients

Statistical analysis

The data from the completed questionnaires was analyzed using IBM SPSS Statistics Data Editor Version 20. The odds ratios are reported with 95% confidence interval and a p value. P-values <0.05 were considered significant. The differences between the quality of life in living patients and deceased patients were studied using a nonparametric t-test.

Results

Patient Cohort

We received completed questionnaires of 45 C1q deficient individuals from 31 different families originating from 14 countries (Table 1). Although most of the cases were from countries in the Middle East, we also observed cases of native Dutch and Swedish origin. No sex bias was found for C1q deficiency (male 49% - female 51%) or for SLE among the C1q deficient patients (male 42% - female 58%) (Table 3). The median time from diagnosis to completion of the questionnaire was 6 years (range:0-34 years). The deficiency for C1q was mostly identified using hemolytic complement assays (CH50). In 60% of the described C1q deficient patients genetic analysis was also performed to identify the mutation associated with the C1q deficiency. Half of the patients have a mutation that has been previously reported in the literature. These mutations are most commonly in the C1qA and C1qC chains.

Country of origin	*Number of patients*
Australia	1
Greenland	3
Iraq	1
Kosovo	1
Netherlands	7
Pakistan	7
Saudia Arabia	9
Spain	1
Sweden	4
Sudan	2
Tunisia	2
Turkey	4
United Kingdom	2
USA	1

Table 2. Overview of the country of origin of patients with C1q deficiency

Onset of disease

The median age at the time of the diagnosis of C1q deficiency was 9 years, but a wide age range from newborn until the sixth decade of life was observed (Figure 1). Most of the C1q deficiencies were identified as part of the routine work up for patients suffering from SLE or from unexplained recurrent infections. In the asymptomatic or less affected C1q deficient family members the diagnosis of C1q deficiency was often made during family screening. At the time of diagnosis 80% of the patients were suffering from SLE, 11% had only experienced recurrent infections and no

SLE while 36% displayed SLE as well as recurrent infections. Cutaneous, discoid lupus was the most common presentation and related symptoms included a malar rash, oral ulcers, recurrent fever and vasculitis. SLE involving the central nervous system was found in one patient. In our series 7% of the individuals, all male, were asymptomatic when C1q deficiency was diagnosed (Table 3). Overall 9 individuals (20%) died, all except for one before the age of 20 years (alive: N= 36, mean 20.0 years ± 11.5 vs deceased: N = 9, mean 12.6 years ±11.7) (Figure 1).

Among C1q deficient siblings one may present with SLE and/or infections, whereas the other is asymptomatic as previously observed in one Maroccan and one Turkish family [1, 25, 32]. In our series, one female from Sweden, one male from the Netherlands, and 4 affected brothers from the United Kingdom, presenting clinically with SLE and/or infections, had a C1q deficient brother that were completely asymptomatic at an age of 8 to 31 years.

C1q deficient individuals	Number of cases	Percentage of cases
Sex M/F	22/23	49/51
Deceased Y/N	9/36	20/80
Deceased Males	3	14
Deceased Females	6	26
Clinical presentation		
SLE Y/N	36/9	80/20
Only SLE	20	44
Only Infections	6	13
Both SLE + Infections	16	36
No symptoms	3	7
Therapy		
FFP given	14	31
HSCT performed	3	7
HSCT considered	10	22

Table 3. Overview of the questionnaires about the C1q deficient individuals

Infectious diseases

The first symptoms of disease reported for most C1q deficient patients were bacterial infections. The most common bacterial infections reported as first symptoms were recurrent otitis, meningitis, gingivostomatitis and urinary tract infection. In our questionnaire we asked the frequency of milder infections per year. We specifically asked for the more common mild infections such as otitis media, upper respiratory tract infections, mild gastrointestinal infections or unexplained fever (Figure 2). In 11 patients (24%) these recurrent infections did not occur at all, whereas in 34

patients (76%) this was a frequent process typically involving multiple sites of infection. From this group thirteen patients had only suffered from mild infections (29%), three patients had only severe infections (7%) and eighteen patients that had both (40%). Patients suffering from severe infections often also suffered more from milder infections (OR: 5.1, 95% CI: 1.2-21.9, p = 0.029).

Severe infections occurred in 47% of the C1q deficient patients with SLE as well as in 53% of the C1q deficient patients without SLE (OR: 1.1, 95% CI: 0.3-4.9, p = 0.881).

All patients had been vaccinated against the regular childhood diseases such as measles, diphtheria, tetanus and poliomyelitis. One patient did receive vaccinations, but no live-attenuated vaccines. Patients with C1q deficiency are vulnerable for infectious diseases and although it would be obvious to provide additional vaccinations, not all individuals received such vaccinations. From the 45 C1q deficient individuals 30 individuals received additional vaccinations. The additional vaccinations of some patients were against Pneumococcus, Meningococcus, Hepatitis B and the seasonal influenza vaccinations [32].

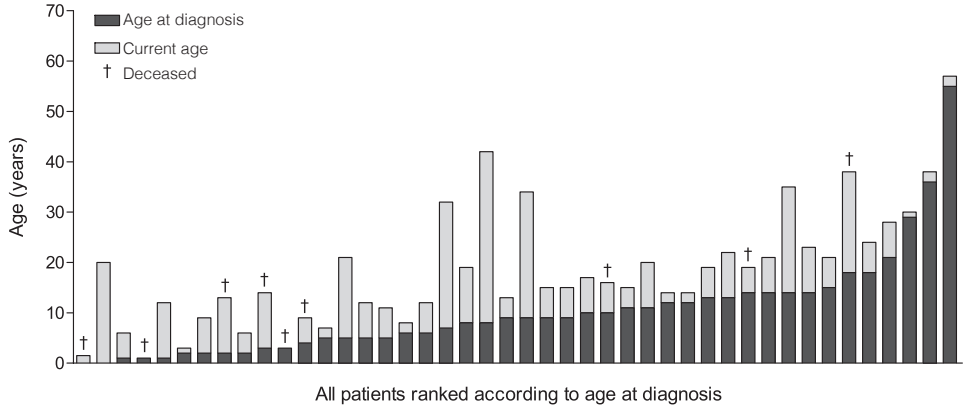


Figure 1. Overview of the age at diagnosis vs. current age (N = 45)

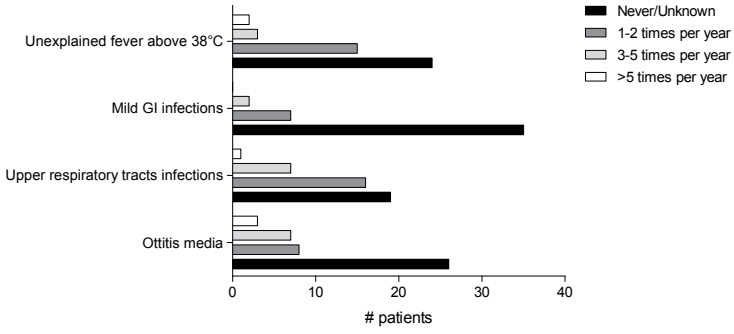


Figure 2. Frequency of 4 milder infections in times per year.

Treatment and outcome

The treatment of C1q deficient patients consisted so far mainly of immunosuppressive therapy such as corticosteroids for SLE or (prophylactic) antibiotic therapy for infections. Other frequently used drugs were chloroquine and hydroxychloroquine as maintenance therapy, while major flares have been treated with rituximab and cyclophosphamide.

The lack of C1q itself has been reversed using fresh frozen plasma (FFP) in 13 out of 45 patients (Table 2). In most patients the FFP is given at weekly intervals, but C1q levels peak early and then decline fast after an infusion [26].

HSCT has been attempted in three C1q deficient patients [29-31]. The allogeneic HSCT has been performed to restore C1q production and mainly treat SLE, but also the immunodeficiency, in C1q deficient children. In all patients the HSCT procedure was successful and normal C1q levels in plasma were obtained. Currently two patients are doing well whereas the other one died of a intracerebral hemorrhage. The latter child was already in a relatively poor condition prior to HSCT. Despite major progress in the field of HSCT, this procedure still has a significant risk profile. From the questionnaire it became clear that several clinicians considered HSCT for their patient but they considered the severity of the clinical symptoms not sufficient to proceed or they reported that no sufficiently matching donor was available to perform HSCT.

In total 9 (20%) patients died during follow-up. The cause of death of 5 C1q deficient patients was septic shock. Other causes of death were bacterial meningitis, gastrointestinal bleeding, Pneumocystis jirovecii pneumonia and cerebral haemorrhage. It is important to note that except for one patient all fatalities occurred in the age group below 20 years (Figure 1). However, the limited follow-up time of the patients in this cohort as a whole does not yet allow strong conclusions.

Estimates of the quality of life

In the questionnaire we asked the treating clinicians to give an estimation of the quality of life of the C1q deficient patient(s). Due to the variability of the clinical presentation of C1q deficiency the estimates of the quality of life varied widely. Remarkably most of the C1q deficient patients are doing well and on a scale from 1-10 (1 very poor – 10 great) the median score was 7 (Figure 3A).

A total of 80% of the C1q deficient patients in this study were still alive with a median age of 19 years and a median quality of life of 7, range (1-10). The 20% of the C1q deficient patients who had died had had a low quality of life (2.44, range (1-6), p = 0.013) and suffered of recurrent infections. In order to have a reference group for the quality of life analyses we asked the clinicians to score also the quality of life of the two SLE patients they most recently examined in their clinic. Also for this group we obtained a median score of 7 (N=7).

The estimated overall survival time (Kaplan-Meier) of the reported C1q deficient patients was evaluated (Figure 3B). In children (less than 20 years old) mortality was estimated to 20%. Moreover, the estimated median survival time seems to be at least 50 years of age although the estimation may not be reliable due to the rather low number of cases.

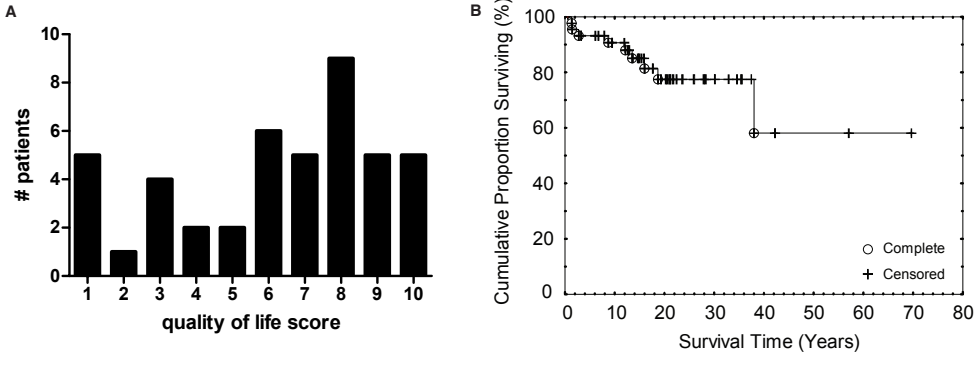


Figure 3. Estimated quality of life and survival analysis.

A. Estimated quality of life, as reported by the treating physicians. The median quality of life is 7.

B. Estimated life expectancy (Kaplan-Meier survival curve) of C1q deficient patients. O = Complete (deceased), + = Censored (at last date of follow up).

Discussion

With this survey we have collected data on the current age, clinical manifestations and quality of life of patients suffering from C1q deficiency. Surprisingly we noticed clear differences. C1q deficiency is associated with a high case-fatality and with early onset of lupus-like disease or full blown SLE in the majority of cases. However, there are also individuals who only suffer from infections without signs of autoimmune disease as well as a sizable group, who are relatively or completely free from symptoms with an excellent quality of life. Until now no data were available on life expectancy and quality of life of individuals with C1q deficiency. It has been described that the course of C1q deficiency is variable [21]. By sending questionnaires to the clinicians who are currently treating C1q deficient patients or have treated deceased patients, we now have a first impression on life expectancy, cause of death, quality of life and treatment regimens. In this study there are some limitations in the use of the questionnaires. We received completed questionnaires covering 45 C1q deficient individuals. Although this is more than 70% of the published cases (as is common for questionnaire based studies [33]), it could reflect a selection bias. Even though this is an international study it may not be a completely worldwide study as C1q deficient individuals in many countries may have been missed. The cases for which we did not receive a response were not restricted to a certain geographical region.

Although C1q deficiency has been reported to occur in many countries around the world we noticed that most patients in this study had their origin from the Middle East, which may reflect to a higher frequency of consanguineous unions in this area [3, 34].

The mutations associated with C1q deficiency include deletions; changes of amino acids or changes in intron-exon splice sites [5, 7, 22]. As most of the mutations result in a condition where no C1q protein is secreted there does not seem to be an obvious relationship between the mutation involved and the clinical outcome of C1q deficiency [4]. Especially since within one family the clinical presentation can vary significantly among individuals homogenous for the same mutation. Differences in phenotype of patients illustrate that other unidentified (epi)-genetic and environmental influences are also important in the overall clinical picture [35, 36]. Understanding what factors determine that some C1q deficient individuals remain asymptomatic whereas others develop SLE, infections or both, will be an important, yet difficult, focus of future studies.

The use of fresh frozen plasma as a treatment option is well described and was applied in 14 patients[23, 26]. In mouse studies bone marrow transplantation in

C1q deficient mice showed positive results and was suggested as a therapeutic option in patients suffering from severe disease [27, 28].

From the questionnaires it became clear that in a substantial number of cases clinicians have considered stem cell transplantation. On the one hand the HSCT should be performed in a patient that is not marked by the underlying SLE or infections, whereas on the other hand performing a HSCT in a patient that is (still) doing fine may not counterbalance the potential risks involved in HSCT. This survey shows that there is a substantial percentage of C1q deficient individuals without any clinical or serological markers of disease. Whether HSCT or gene therapy could be a good option for individuals who have not yet suffered major health problems depends on better insight into the prognosis and on the improvement of these treatments in the future.

Our data show that even during follow up there is enormous diversity in the clinical presentation and severity of symptoms in persons that are deficient for C1q. Even though this case series comprised 45 individuals (comprising the majority of cases known to date) there is no clear algorithm to describe how to manage C1q deficiency. From the data it seems that once the C1q deficient patients reach adulthood that then the chance of fatal infections is reduced. However, follow up data from a longer period is needed to address this question. Remarkably, patients with C1q deficiency showed also other complications. In the questionnaires we received information on two C1q deficient patients who were diagnosed with Moyamoya disease and another patient with signs of Rothmund-Thomson syndrome [24]. The relation between C1q deficiency and to these unexpected complications is not known, but this will likely contribute to a reduction of the life expectancy of these patients.

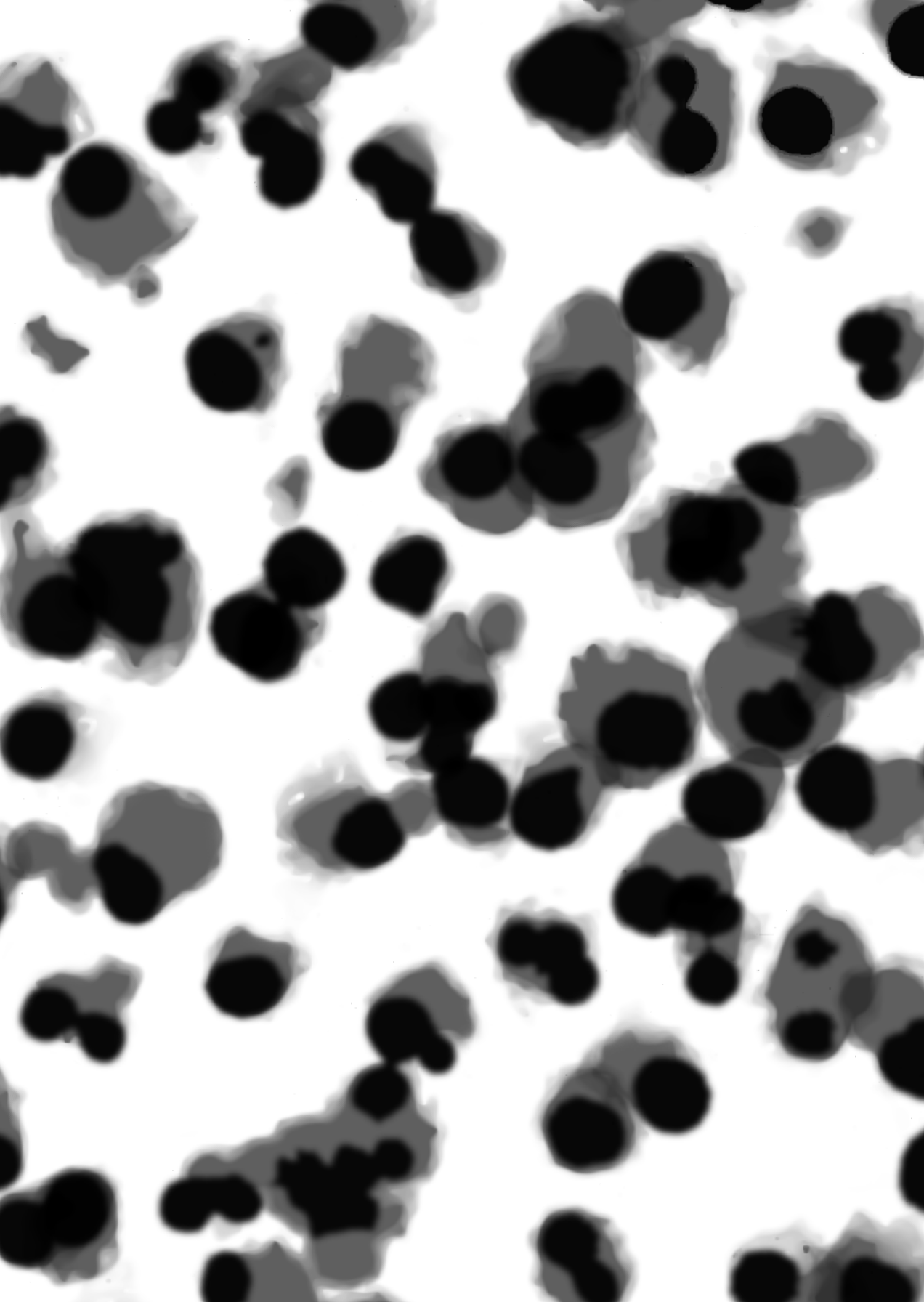
Conclusion

With this overview we aimed to bring together the currently available information on whether, when and which clinical manifestations occur in C1q deficient patients to be able to make the best possible estimation regarding current treatment options like immunosuppressive drugs, FFP or HSCT. From this survey it became clear that there is enormous diversity in the clinical presentation and severity of symptoms in persons that are deficient for C1q.

References

1. Walport, M.J., K.A. Davies, and M. Botto, C1q and systemic lupus erythematosus. Immunobiology, 1998. 199(2): p. 265-285.
2. Walport, M.J., et al., Complement deficiency and autoimmunity. Ann.N.Y.Acad.Sci., 1997. 815: p. 267-281.
3. McAdam, R.A., D. Goundis, and K.B. Reid, A homozygous point mutation results in a stop codon in the C1q B-chain of a C1q-deficient individual. Immunogenetics, 1988. 27(4): p. 259-64.
4. Schejbel, L., et al., Molecular basis of hereditary C1q deficiency--revisited: identification of several novel disease-causing mutations. Genes Immun., 2011. 12(8): p. 626-634.
5. Pickering, M.C., et al., Systemic lupus erythematosus, complement deficiency, and apoptosis. Adv Immunol, 2000. 76: p. 227-324.
6. van Schaarenburg, R.A., et al., Identification of a novel non-coding mutation in C1qB in a Dutch child with C1q deficiency associated with recurrent infections. Immunobiology, 2014.
7. Jlajla, H., et al., New C1q mutation in a Tunisian family. Immunobiology, 2014. 219(3): p. 241-6.
8. Daha, N.A., et al., Complement activation by (auto-) antibodies. Mol.Immunol., 2011. 48(14): p. 1656-1665.
9. Van Schravendijk, M.R. and R.A. Dwek, Interaction of C1q with DNA. Mol Immunol, 1982. 19(9): p. 1179-87.
10. Jiang, H.X., J.N. Siegel, and H. Gewurz, Binding and complement activation by C-reactive protein via the collagen-like region of C1q and inhibition of these reactions by monoclonal antibodies to C-reactive protein and C1q. J Immunol, 1991. 146(7): p. 2324-30.
11. Korb, L.C. and J.M. Ahearn, C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited. J.Immunol., 1997. 158(10): p. 4525-4528.
12. Trouw, L.A., A.M. Blom, and P. Gasque, Role of complement and complement regulators in the removal of apoptotic cells. Mol.Immunol., 2008. 45(5): p. 1199-1207.
13. Nauta, A.J., et al., Direct binding of C1q to apoptotic cells and cell blebs induces complement activation. Eur.J.Immunol., 2002. 32(6): p. 1726-1736.
14. Walport, M.J., Complement. Second of two parts. N.Engl.J.Med., 2001. 344(15): p. 1140-1144.
15. Taylor, P.R., et al., A hierarchical role for classical pathway complement proteins in the clearance of apoptotic cells in vivo. J Exp Med, 2000. 192(3): p. 359-66.
16. Fossati-Jimack, L., et al., C1q deficiency promotes the production of transgenic-derived IgM and IgG3 autoantibodies in anti-DNA knock-in transgenic mice. Mol.Immunol., 2008. 45(3): p. 787-795.
17. Baruah, P., et al., C1q enhances IFN-gamma production by antigen-specific T cells via the CD40 costimulatory pathway on dendritic cells. Blood, 2009. 113(15): p. 3485-3493.
18. Jiang, K., et al., T cell activation by soluble C1q-bearing immune complexes: implications for the pathogenesis of rheumatoid arthritis. Clin.Exp.Immunol., 2003. 131(1): p. 61-67.
19. Santer, D.M., et al., C1q deficiency leads to the defective suppression of IFN-alpha in response to nucleoprotein containing immune complexes. J.Immunol., 2010. 185(8): p. 4738-4749.
20. Vassallo, G., et al., Clinical variability and characteristic autoantibody profile in primary C1q complement deficiency. Rheumatology.(Oxford), 2007. 46(10): p. 1612-1614.
21. Higuchi, Y., et al., The identification of a novel splicing mutation in C1qB in a Japanese family with C1q deficiency: a case report. Pediatr.Rheumatol.Online.J., 2013. 11(1): p. 41.
22. Topaloglu, R., et al., C1q deficiency: identification of a novel missense mutation and treatment with fresh frozen plasma. Clin.Rheumatol., 2012. 31(7): p. 1123-1126.

23. Lopez-Lera, A., et al., Rothmund-Thomson Syndrome and Glomerulonephritis in a Homozygous C1q-Deficient Patient Due to a Gly164Ser C1qC Mutation. J.Invest Dermatol., 2014. 134(4): p. 1152-1154.
24. Troedson, C., et al., Systemic lupus erythematosus due to C1q deficiency with progressive encephalopathy, intracranial calcification and acquired moyamoya cerebral vasculopathy. Lupus, 2013. 22(6): p. 639-643.
25. Mehta, P., et al., SLE with C1q deficiency treated with fresh frozen plasma: a 10-year experience. Rheumatology.(Oxford), 2010. 49(4): p. 823-824.
26. Cortes-Hernandez, J., et al., Restoration of C1q levels by bone marrow transplantation attenuates autoimmune disease associated with C1q deficiency in mice. Eur.J.Immunol., 2004. 34(12): p. 3713-3722.
27. Petry, F., et al., Reconstitution of the complement function in C1q-deficient (C1qa-/-) mice with wild-type bone marrow cells. J.Immunol., 2001. 167(7): p. 4033-4037.
28. Olsson R, H.S., Ringden O, Truedsson L, Åhlin A., Allogeneic haematopoietic stem cell transplantation restores complement function in human hereditary C1q deficiency. Bone marrow transplantation, 2013. 48: p. S338.
29. Arkwright, P.D., et al., Successful cure of C1q deficiency in human subjects treated with hematopoietic stem cell transplantation. J Allergy Clin Immunol, 2014. 133(1): p. 265-7.
30. Topaloglu, R., et al., Molecular basis of hereditary C1q deficiency associated with SLE and IgA nephropathy in a Turkish family. Kidney Int., 1996. 50(2): p. 635-642.
31. Berkel, A.I., et al., Clinical and immunological studies in a case of selective complete C1q deficiency. Clin Exp Immunol, 1979. 38(1): p. 52-63.
32. Berkel, A.I., et al., Molecular, genetic and epidemiologic studies on selective complete C1q deficiency in Turkey. Immunobiology, 2000. 201(3-4): p. 347-55.



Chapter 4

C1q deficiency and neuropsychiatric systemic lupus erythematosus

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Abstract

C1q deficiency is a rare immunodeficiency, which is strongly associated with the development of systemic lupus erythematosus (SLE). A mutation in one of the C1q genes can either lead to complete deficiency or to low C1q levels with C1q polypeptide in the form of low-molecular weight (LMW) C1q. Patients with C1q deficiency mainly present with cutaneous and renal involvement. Although less frequent, neuropsychiatric (NP) involvement has also been reported in 20% of the C1q-deficient patients. This involvement appears to be absent in other deficiencies of early components of the complement classical pathway (C1r/C1s, C2 or C4 deficiencies). We describe a new case with C1q deficiency with a homozygous G34R mutation in C1qC producing LMW-C1q presenting with a severe SLE flare with NP involvement. The serum of this patient contained very low levels of a LMW variant of C1q polypeptides. Cell lysates contained the three chains of C1q but no intact C1q was detected, consistent with the hypothesis of the existence of a LMW-C1q. Furthermore we provide a literature overview of NP-SLE in C1q deficiency and hypothesise about the potential role of C1q in the pathogenesis of NP involvement in these patients. The onset of NP-SLE in C1q deficient individuals is more severe when compared with complement competent NP-SLE patients. An important number of cases present with seizures and the most frequent findings in neuroimaging are changes in basal ganglia and cerebral vasculitis.
A defective classical pathway, because of non-functional C1q, does not protect against NP involvement in SLE. The absence of C1q and subsequently some of its biological functions may be associated with more severe NP-SLE.

Introduction

C1q-deficiency is a rare autosomal recessive inherited defect of the complement system caused by mutations occurring in one of the three C1q genes (C1qA; C1qB; C1qC). [1] Up to date, three different categories of mutations according to C1q level have been described. Apart from nonsense mutations and missense mutations leading to absence of C1q in serum, a missense mutation with detectable C1q levels has been described. [2] In the last case, some authors have demonstrated a low gradient density of C1q compared with healthy controls and is therefore called low molecular weight C1q (LMW-C1q). [3, 4] Until now, a total of 77 C1q-deficiency patients in 49 families have been described. [5-7] An important variability in clinical presentation and outcome of these patients has been observed, ranging

from asymptomatic patients to life-threatening encapsulated bacterial infections. [7-9] C1q-deficiency is also strongly related to systemic lupus erythematosus (SLE), being so far the most penetrant genetic factor predisposing to this disease. From all patients described, a total of 85% presented SLE-like symptoms while around 50% have been addressed as SLE according to the American College of Rheumatology diagnostic criteria. [1, 3, 4, 7, 8] Cutaneous involvement, oral ulcers and renal involvement are the most consistent manifestations. Although nervous system involvement is less frequent, with only 15 patients described, it can lead to severe neuropsychiatric (NP) symptoms.

Several reports, based on mouse models and/or in-vitro experiments describe that C1q plays a role in the brain during different developmental stages. C1q can be neuroprotective in the context of neurotoxicity induced by beta-amyloid, [10, 11] but it is also reported to be involved in damage in the context of Alzheimer's disease [12]. It remains to be established to what extent C1q is involved in cognitive (dys) function in humans and how and in which stages of development C1q is protective or damaging to brain tissue.

In this report we describe a new C1q deficient patient with a G34R mutation in the C1qC chain leading to severe NP-SLE and review 15 SLE cases with C1q deficiency and NP involvement in the literature. Furthermore we analyse the biochemical structure of LMW-C1q in serum and in cell lysates.

Patient and methods

Clinical presentation of the C1q deficient patient

A 24-year-old Dutch man was admitted to our hospital with a 2-day history of progressive weakness and sensory loss of the left arm, visual field loss on the left side and subjective cognitive complaints with regard to concentration and memory. He had been diagnosed with a SLE-like illness associated with C1q deficiency at the age of 10 months when he presented a butterfly rash and antinuclear antibodies (ANAs) positivity. The C1q deficiency was caused by a homozygous g.5499G>A mutation at the C1qC gene, resulting in a G34R change in the C1q protein. Consanguinity was not reported.

At the age of three he developed polyarthritis, which was successfully treated with naproxen. At the age of seven he was admitted due to a relapsing polyarthritis and subacute cutaneous lupus, fever, aphthous ulcers, sunlight hypersensitivity, malaise and positive antibodies including ANAs, anti-Ro, anti-RNP70 and Sm. SLE was diagnosed and hydroxychloroquine 200 mg was started. Examination of the

past medical history also included frequent upper airway and ear infections during the first 3 years of his life, Pertussis infection at the age of four, relapsing impetigo with a Staphylococcus aureus septicemia at the age of 19 years and relapsing virus varicella zoster infection after the age of 20.

On the current admission, the patient's body temperature was 37.7°C and blood pressure was 100/60 mmHg. Physical examination was remarkable with a butterfly rash (figure 1A), severe sensory loss of the left arm, hyperesthesia of the left hand and homonymous hemianopsia of the left side. Laboratory tests revealed increased ESR (63 mm/h; normal <15) and CRP (13.7 mg/L; normal <5), a normal haemoglobin and complete blood count. Except for a reduced serum albumin level (31 g/L; normal 34-48), electrolytes, serum cholesterol, renal and liver testing were normal. Analysis of the urine was normal without casts or dysmorphic red cells. Protein excretion was 9.87 g/24h. The antibody profile was positive for ANAs, anti-Ro (>240 U/mL, normal <7), anti-RNP70 (79 U/mL, normal <5) and anti-Sm antibodies (>120 U/mL, normal <5). Anti-double-stranded DNA, anticardiolipin antibodies, Beta-2-GP1 antibodies, lupus anticoagulant, anti-phospholipase-A2-Receptor (PLA2R) and Anti-C1q autoantibodies were negative. At this time analysis of complement showed a classical pathway activity of 0% (normal > 74%), a low alternative pathway activity (22%, normal >39%), a low level of C1q (21 mg/L, normal 102–171 mg/L), whereas C3 (1.4 g/L, normal 0.9–2.0 g/L) and C4 (396 mg/L, normal 95–415 mg/L) were in the normal range. Blood and urine cultures were negative. Findings from the renal biopsy were compatible with a class V lupus nephritis, with a 'nearly full house' immunostaining showing a strong granular staining for IgG and a moderate granular staining for C3, both along the glomerular basement membrane; a slight granular staining for IgA and IgM, and kappa and lambda light chains, sometimes also in mesangial areas, but no staining for C1q (Figure 1C). Electron microscopy revealed subendothelial, subepithelial and mesangial deposits (Figure 1D and E). A low Minimental State Examination for the age and education of the patient (24, range 0-30) was found. A brain computed-tomography (CT) scan demonstrated a hyperdensity at the right frontal and parietal lobes and a contrast enhanced CT showed a bilateral filling defect in the transverse sigmoid sinus. A Magnetic Resonance Imaging (MRI) showed multifocal diffuse grey matter hyperintensities located in the fronto-temporal right lobe and high-intensity area on T2 in multiple regions of the right frontal and parietal lobes with high-intensities on the diffusion weighted imaging study (Figure 1B). A CT-angiography showed no signs of cerebral vasculitis. A diagnosis of lupus nephritis type V and NP-SLE with both inflammatory and ischemic phenotype were established. The patient was treated with daily clopidrogrel 75 mg and intravenous methylprednisolone 1 gr 3

days plus oral prednisone 1 mg/kg/d in a tapering dose, and monthly intravenous cyclophosphamide 1 gm/m2 for six months. Proteinuria improved dramatically in the first week and homonymous hemianopsia and cognitive dysfunction resolved after 2 weeks. After 3 months the patient still presented a mild sensory loss of the left arm. Both the patient and his parents provided informed consent for the studies.

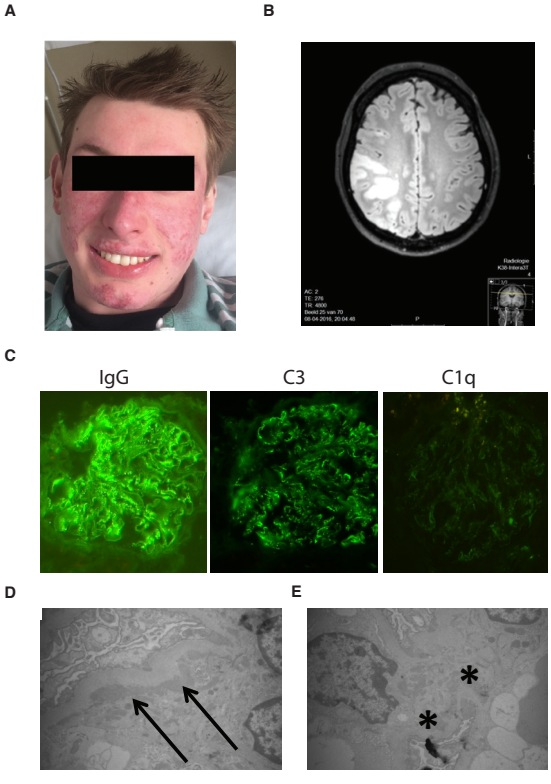

Figure 1. Clinical presentation of the C1q deficient patient. A. Malar rash and discoid lupus leading to mild scarring and atrophy **B.** 3-Tesla MRI brain (FLAIR image): multifocal diffuse grey matter hyperintensities located in the fronto-temporal right lobe and high-intensity area in multiple regions of the right frontal and parietal lobes **C.** Immunofluorescence staining of IgG deposition, C3 deposition and C1q deposition on the kidney. **D.** Electron micrograph of the sub-endothelial deposition (arrows) of electron dense material. **E.** Electron micrograph of mesangial deposition (stars) of electron dense material..

Samples

Serum and PBMCs, isolated by Ficoll-Paque density gradient centrifugation were collected from the patient and an age matched control. During the admission a kidney biopsy was performed.

Microscopy

Slides for light microscopy evaluation were stained by hematoxylin and eosin, PAS and silver staining. Immunofluorescent stainings on cryostat sections were performed for IgA, IgG, IgM, C3, c1q and kappa and lambda light chains. Part of the renal specimen was used for electron microscopy. Pictures were taken with a JEM-1011 electron microscope (JEOL USA, Inc.) at various magnifications.

Gel filtration

Gel filtration experiments were carried out using the Äktaprime plus system (GE Healthcare, 11001313). 500 ul of filtered serum sample, either the healthy control serum or serum from the C1q deficient patient, was run through a Hiload Superdex Prep grade 200 16/600 column (GE Healthcare), using PBS as the running buffer. Fractions of 1ml were collected starting after half an hour for the duration of approximately 50 fractions. The protein levels in the fractions were analysed using a Piercetm BCA Protein Assay Kit (ThermoFisher Scientific).

C1q ELISA

The levels of C1q in serum and supernatants were measured using an in-house developed ELISA. Maxisorp plates (Nunc) were coated with mouse anti-human C1q (Department of Nephrology, LUMC) in coating buffer (0.1 M NA2CO3, 0.1 M NaHCO3, pH 9.6) overnight at 4°C. Plates were washed in PBS/0.05% Tween (PBS-T, Sigma). Then the wells were blocked with PBS/1% BSA for 1 hour at room temperature. After washing, the patient serum and control serum were added to the wells in a two-fold dilution series starting from 1:100 diluted in PBS/1% BSA/0.05% Tween (Sigma). After incubation for 1 hour at 37°C, the plates were incubated with rabbit anti-human C1q (DAKO) for 1 hour at 37°C and as detection antibody goat anti-rabbit HRP (DAKO) was used. Finally the substrate was added using ABTS (sigma). The C1q levels were measured at an absorbance level of 415 nm.

Western blot

Using western blot the composition of C1q was examined by detection of the three chains of the C1q protein. Due to the low amount of C1q present in the serum of the patient, we applied ten times more serum of the patient than the healthy donor. Cell lysates and supernatants of stimulated and unstimulated PBMCs of the healthy control and the patient were used in the same amount in reduced and non-reduced SDS conditions. The western blot was performed using previously described methods. [9]

Reconstitution complement activity assay

To exclude the possibility that next to C1q deficient the patients sample would also be deficient for C1r or C1s we performed assays to measure activation of the classical pathway of the patient serum by reconstitution of purified C1q. Plates coated with human IgG were incubated with 1% serum of the patient (diluted in GVB++; 0.1 % gelatin, 5 mM Veronal, 145 mM NaCl, 0.025 % NaN3, 0.15 mM CaCl, 0.5 mM MgCl, pH 7.3) with or without addition of purified C1q (Quidel) in different

concentrations. As a read-out C4 deposition was measured.

Sequencing

Genomic DNA was extracted from blood collected with tubes supplemented with EDTA. Sequencing of the complete C1q genes (C1qA, C1qB and C1qC), of both introns and exons was performed as before. [9] Deep-sequencing was performed using the 454 NGS Roche GS FLX Titanium platform. Data were compared to internal controls and to Human Genome build 19 as well as Human_v37_2 de dbSNP database v132 using the NextGENe software package for Next Generation Sequence Analysis (NGS) from Softgenetics. The effect of the mutation on splicing was in-silico analysed using the NetGene2 Server, http://www.cbs.dtu.dk/services/NetGene2/.

Results

Detection of LMW-C1q in serum

With deep sequencing we identified a homozygous g.5499G>A mutation in the C1qC gene, resulting in a change in the C1qC chain where glycine was changed into an arginine at position 34 (G34A), while both parents show a heterozygous state of the mutation (Figure 2A). The routine diagnostics laboratory reported the patient to be completely lacking classical pathway activity (Figure 2B). This is compatible with a C1q deficiency, but to exclude that next to C1q also other factors would be deficient in the patient we performed a reconstitution assay where we add purified C1q to the serum of the patient and analyse C4 deposition. To compare the activity we performed the same analysis with C1q depleted serum. After adding purified C1q we were able to detect C4 deposition at a similar range as C1q depleted serum reconstituted with pC1q (Figure 2C). This indicated that the patient was able to produce C1r and C1s, C2 and C4 and together with purified C1q was able to activate the classical pathway. Furthermore, we were also able to measure C5b9 and C3c deposition. This implied that there were no other complement deficiencies downstream in the complement system (Figure 2D&E).

Using ELISA we could detect a decreased amount of C1q in the patient compared to the control samples (Figure 3A). We used western blot to examine the molecular structure of C1q in the patient serum. In reducing conditions all the three chains of the correct size are detected. However, using non-reducing conditions the dimers of C1q (2 x A-B and 1 x C-C) show an abnormal pattern. Using non-reducing/non-denaturing conditions we were able to detect high molecular weight C1q in

the healthy control but not in the patient, suggesting that the C1q of the patient is of a LMW species (Figure 3B). With the usage of gel filtration the serum samples of the healthy donor and the patient were fractionated on size and with a BCA the amount of protein was analysed. While the protein profiles of both gel filtrations are similar, the location of C1q in the elution profiles is clearly different (Figure 3C&D). Please note that since the serum of the patient was very low in C1q concentration we had to use different dilutions for the patient and the control in the ELISA to detect the presence of C1q in the fractions. These size-exclusion chromatography data confirm the LMW nature of C1q in the serum of the patient.

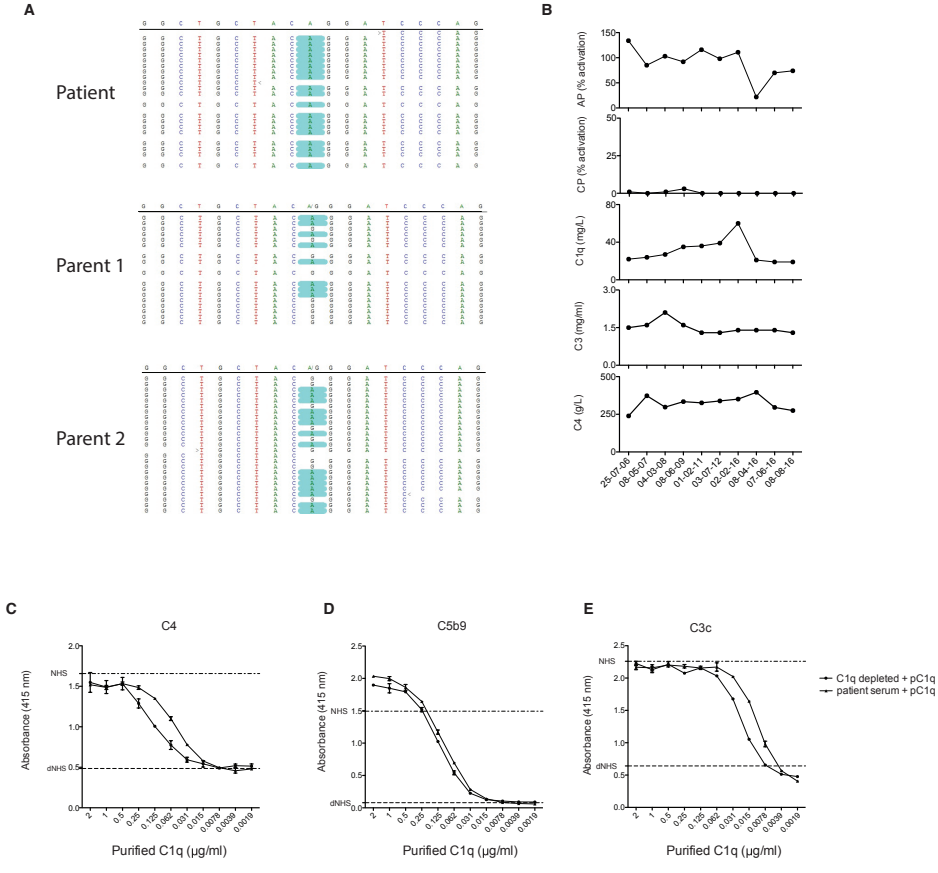

Figure 2. Genetic analysis of the patient and complement activation assays. A. Data obtained from deep sequencing show a G34R mutation in the C1qC chain. **B.** Measurement of the alternative pathway (AP) (Wieslab), classical pathway (CP) (Wieslab), C1q, C3 and C4 with nephelometer measurement in the diagnostic laboratory. **C.** Reconstitution of the classical pathway by adding different concentrations of purified C1q to the patient serum. As a positive control normal human serum was used (NHS) and as a negative control heat inactivated NHS (ΔNHS) was used. C4 deposition was used as detection antibody. **D.** C5b9 deposition after adding purified C1q to the patient serum and C1q depleted serum. **E.** C3c deposition.

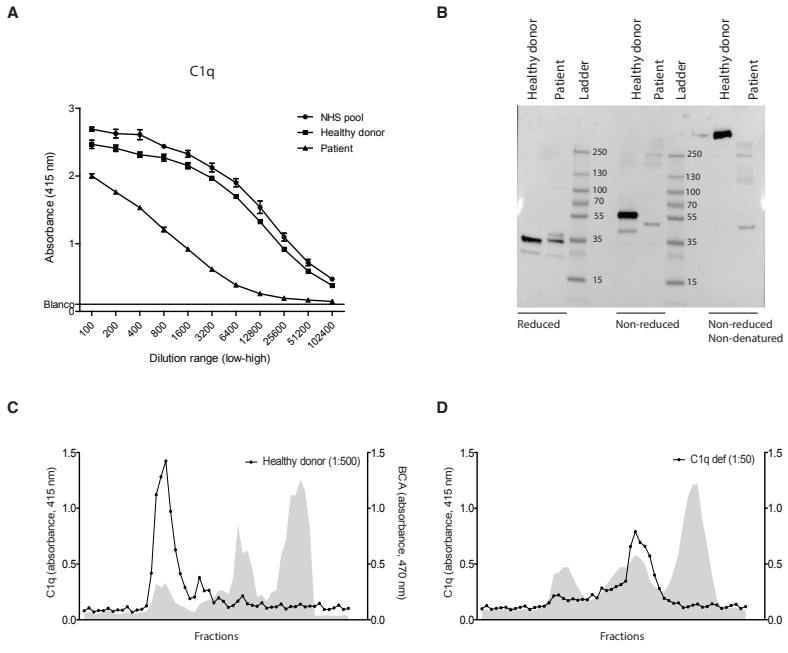

Figure 3. Detection of LMW-C1q in serum. A. C1q ELISA by using a dilution range of the serum of the C1q deficient patient (▲), age-matched control (■) and NHS (●) as extra control. **B.** Western blot analysis of the serum in reduced, non-reduced and non-reduced/non-denatured conditions. As positive control an age-matched control is used. Patient serum was diluted 50x and the healthy control 500x. **C.** Protein analysis using a BCA protocol and C1q ELISA of different fractions after gel filtration of the serum of a healthy donor. **D.** Protein and C1q analysis of the patient.

Composition of C1q in PBMC of the C1q deficient patient

To further examine the production of C1q by the cells of the patient by Western Blot, we stimulated PBMCs of the patient and the control with DXM and IFN-γ to upregulate the C1q production. Compared to the serum we loaded the same amount of lysate and supernatant to the lanes. In reducing conditions we see all the three C1q chains in the lysate of the PBMCs (Figure 4A). The dimers of C1q can also be detected in the lysates of the PBMCs from the patient. However, in non-reducing non-denaturing conditions, the dimers of C1q are detected, while additional bands are seen in the PBMCs of the patients, which may indicate the presence of intracellular LMW-C1q (Figure 4B).

To examine the composition of secreted C1q, the supernatant of the PBMCs was analysed using western blot. The three chains of C1q were detected in the control supernatant as well as in the patient supernatant in reducing conditions. Surprisingly, the amount of C1q seems comparable between the patient and the control (Figure 4C). In non-reducing, non-denaturing conditions the high molecular

size of C1q (460 kDa) is detected only in a very low concentration compared to the supernatant of the healthy control (Figure 4D).

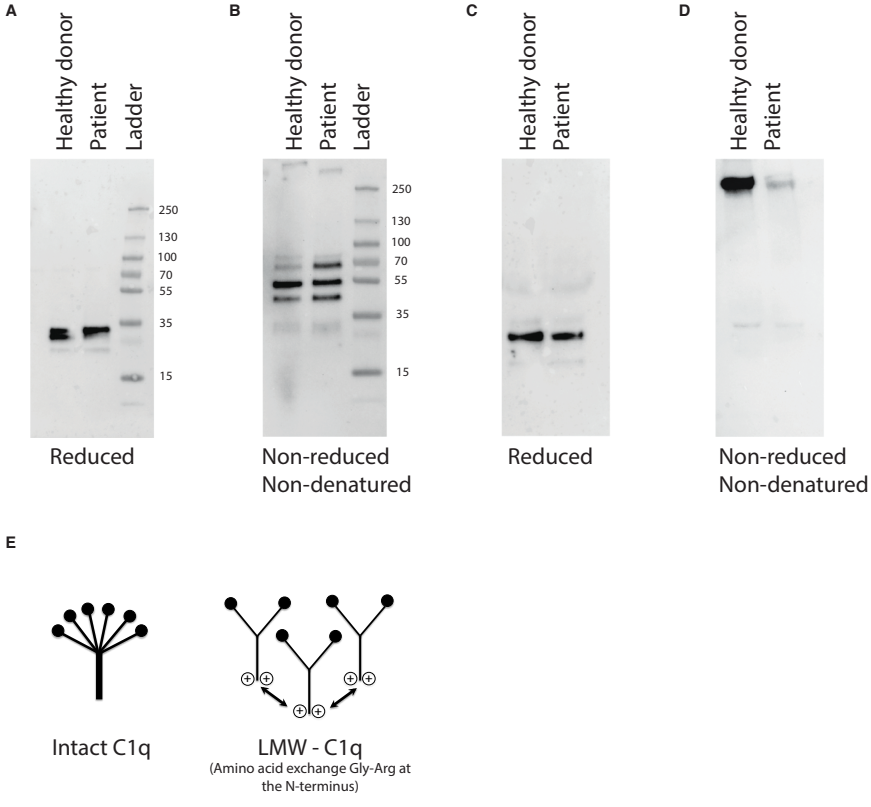

Figure 4. Analysis of stimulated cells from the C1q-deficient patient on the presence of C1q, A. Western blot analysis of cell lysates from stimulated PBMCs in reducing conditions, **B.** non-reducing and non-denaturing conditions. **C.** Western blot analysis of the supernatant of the PBMCs from the patient and the healthy donor (control) after 72h of culturing in reducing conditions. **D.** In non-reducing and non-denatured conditions. The cell lysates and supernatant were added in the same amount. **E.** A schematic representation of intact C1q and LMW-C1q. In LMW-C1q positive charges are introduced in the collagen-like tail due the amino acid exchange Gly-Arg at the N-terminal region (modified from [2]).

C1q deficiency and NP-SLE

We performed an extensive electronic literature search from 1980 to 2016 using online databases (PubMed, Embase, Medline). We found 15 C1q-deficient patients with NP-SLE. All these patients presented at least one major central nervous system (CNS) manifestation. Clinical and neuroimaging characteristics of these patients are summarized in Supplementary Table 1. Among all C1q-deficient patients

with NP-SLE described so far in the literature, seizures was the most frequent NP symptom presented (10 patients; 67%). [6, 13-20] Furthermore, five patients (33%) presented with a series of severe non-specific NP symptoms characterized by encephalopathy and difficulties to walk associated with cerebral infarcts and thought to be related with a cerebral vasculitis. [5, 13, 19-21] Transverse myelitis [6, 22] and psychosis [14, 22] were also present in 2 patients (13%). Neuroimaging of the brain showed as more frequent finding affection of basal ganglia (calcification or ischemic lesions) in 40 % of the cases [16, 17, 19-21, 23] followed by cerebral vasculitis (27%) [13, 15, 20, 21] and brain atrophy (20%) [6, 17, 24].

Discussion

The present study investigated an extremely rare case of C1q-deficiency due to non-functional LWM-C1q associated with a severe clinical phenotype presenting with membranous lupus nephritis and a mixed inflammatory and ischemic NP-SLE. C1q deficiency is a very strong susceptibility factor for the development of SLE where patients mainly present during childhood with skin or renal involvement and less frequently also with neuropsychiatric involvement. [7] Interestingly, although all the deficiencies of early components of the complement classical pathway are known to be a susceptibility factor for the development of SLE-like disease, neuropsychiatric involvement appears to be absent in C1r/C1s, C2 or C4 deficiencies. [24, 25] This makes us to speculate about the possible role of C1q in the underlying process leading to NP-SLE.

NP involvement in SLE-related C1q-deficiency presents with severe major CNS manifestations and its prevalence seems to be slightly higher than in complement competent NP-SLE patients (20% vs. <5%). [26] Seizures were the most common manifestation, presented in 60% of NP-SLE patients. In animal models, the production of C1q by neuronal cells was reported to lead to opsonisation of synapses in the developing postnatal CNS, that are next eliminated by microglia. [27] Several studies in murine models have described that C1q plays a role in the brain during different developmental stages. A neuroprotective role for C1q was reported in the context of beta-amyloid-induced neurotoxicity [10, 11] , while on the other hand, C1q is reported to be involved in damage in the context of Alzheimer's disease. [12] The complement system can hence facilitate normal neuronal development and protect against damage or contribute to neurodegenerative disease depending on yet to be identified triggers and timing. Currently it has not been formally studied whether C1q deficient patients have cognitive impairments. The neurological status

of the current case completely normalised after the successful treatment of the SLE flare with immunosuppression, without any residual cognitive impairment. Moreover, studies using C1q knockout mice have demonstrated how a defective neocortical pruning of excessive excitatory synapses in these animals results in spontaneous and evoked epileptiform activity and increased intracortical excitatory connectivity. [28, 29] This may explain the increased prevalence of seizures among these patients. Of note, neuroimaging demonstrated that a total of 40% of patients with C1q-deficiency presenting with NP-SLE showed involvement of the basal ganglia and in 27% of these patients findings were compatible with cerebral vasculitis. Neuroimaging changes in basal ganglia have been rarely reported in SLE patients. It has been suggested that these findings may represent vasogenic oedema and vascular changes occurring due to a vasculitic process localized in the basal ganglia probably due to immune-mediated underlying pathogenesis or effect of inflammation. Moreover, these MRI findings have been described to be reversible after starting immunosuppressive therapy. [30] SLE associated vasculitis may be associated with the deposition of immune complexes (ICs) in the endothelium. The deposition of these ICs may lead to endothelial cell activation and inflammatory cell infiltration. [31] Previous reports have proposed an important role of C1q in the clearance of apoptotic cells and circulating ICs. [32, 33] Non-cleared debris due to absence of C1q may lead to helper T cells stimulation and autoantibody production. [34, 35] Furthermore, in the last years C1q has been demonstrated to be of importance in vascular endothelial permeability and integrity. C1q and mannose binding lectin have been reported in in-vitro studies to help in the removal of atherogenic lipoproteins, which has been proposed as a link between C1q deficiency and cardiovascular disease in SLE, as seen in our patient. [36, 37]

Globally more than 60 patients are described with a C1q deficiency mostly due to a homozygous mutation. From these patients, 6 have the g.5499G>A mutation resulting in a G34A amino acid change and C1q deficiency. [4, 14, 16, 17, 20, 38] Previous case reports that described the G34R mutation suggested the development of LMW-C1q, which is known as a non-functional C1q. In this study we demonstrate a C1q deficient patient with a low level of circulating C1q and an absence of classical pathway activity recorded over a long time period. Using sequencing we confirmed a homozygous G34R mutation. As suggested in previous studies, we also observed that the C1q present in this patient is LMW-C1q. Using western blot and gel filtration of the patient serum we detected a different molecular size of C1q in the patient serum at low concentrations. When we analysed the production of C1q by PBMCs we could detect all three C1q chains at a same concentration intracellularly, but after analysing C1q in the supernatant in non-reducing and non-denaturing conditions

almost no fully folded C1q was detected. This confirms that the patient is able to produce all C1q chains but is unable to fold a complete functional C1q molecule. It is conceivable that the incorrectly folded C1q polypeptide chains have a strongly reduced half-life. Circulating C1q was completely absent after a flare of NPSLE. This may suggest that there is consumption of the little C1q polypeptide that the patient produces. However, in the renal biopsy no C1q was detected, which could also indicate that it is not consumption of LMW C1q but rather a reduced production at the time of flare. Although temporary expression of LMW-C1q has been reported to occur during SLE flares or even in healthy persons, this production is temporary and involves only part of the total C1q pool. [39, 40] In the current patient the production of LMW-C1q is genetically regulated and permanent and results in a completely defective classical pathway.

In conclusion, NP-SLE is a rare but severe complication in C1q-deficiency patients that must be diagnosed and treated promptly. The low level of LMW C1q observed in the patient did not allow any classical pathway activity, making the patient functionally C1q deficient. The role of C1q or its absence in the pathogenesis of NP-SLE merits further studies

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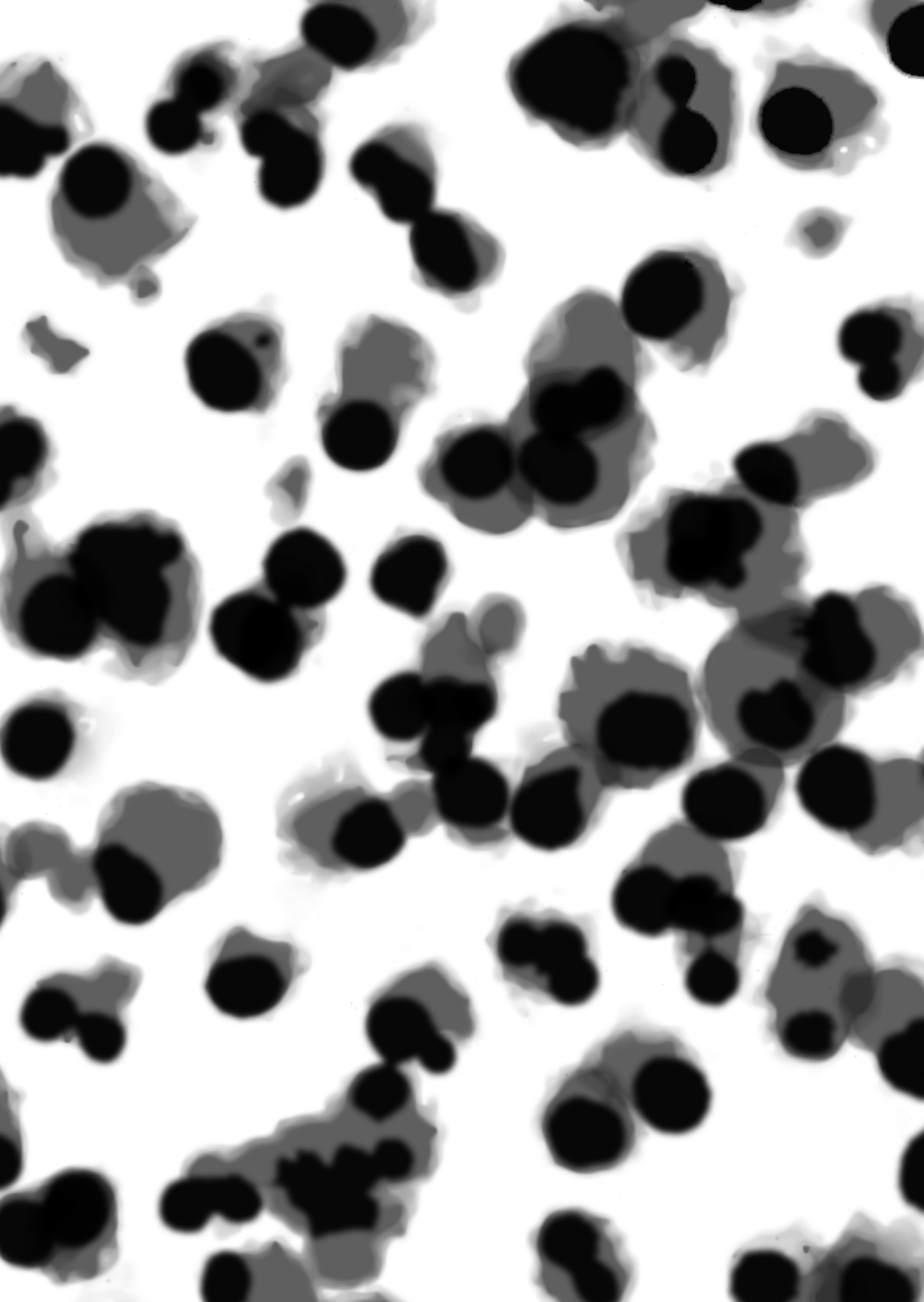
References

1. Schejbel, L., et al., Molecular basis of hereditary C1q deficiency--revisited: identification of several novel disease-causing mutations. Genes Immun, 2011. 12(8): p. 626-34.
2. Petry, F., et al., Non-sense and missense mutations in the structural genes of complement component C1q A and C chains are linked with two different types of complete selective C1q deficiencies. J Immunol, 1995. 155(10): p. 4734-8.
3. Jlajla, H., et al., New C1q mutation in a Tunisian family. Immunobiology, 2014. 219(3): p. 241-6.
4. Walport, M.J., K.A. Davies, and M. Botto, C1q and systemic lupus erythematosus. Immunobiology, 1998. 199(2): p. 265-85.
5. Olsson, R.F., et al., Allogeneic Hematopoietic Stem Cell Transplantation in the Treatment of Human C1q Deficiency: The Karolinska Experience. Transplantation, 2016. 100(6): p. 1356-62.
6. Roumenina, L.T., et al., Functional complement C1q abnormality leads to impaired immune complexes and apoptotic cell clearance. J Immunol, 2011. 187(8): p. 4369-73.
7. Stegert, M., M. Bock, and M. Trendelenburg, Clinical presentation of human C1q deficiency: How much of a lupus? Mol Immunol, 2015. 67(1): p. 3-11.
8. van Schaarenburg, R.A., et al., Marked variability in clinical presentation and outcome of patients with C1q immunodeficiency. J Autoimmun, 2015. 62: p. 39-44.
9. van Schaarenburg, R.A., et al., Identification of a novel non-coding mutation in C1qB in a Dutch child with C1q deficiency associated with recurrent infections. Immunobiology, 2015. 220(3): p. 422-7.
10. Benoit, M.E. and A.J. Tenner, Complement protein C1q-mediated neuroprotection is correlated with regulation of neuronal gene and microRNA expression. J Neurosci, 2011. 31(9): p. 3459-69.
11. Pisalyaput, K. and A.J. Tenner, Complement component C1q inhibits beta-amyloid- and serum amyloid P-induced neurotoxicity via caspase- and calpain-independent mechanisms. J Neurochem, 2008. 104(3): p. 696-707.
12. Hong, S., et al., Complement and microglia mediate early synapse loss in Alzheimer mouse models. Science, 2016. 352(6286): p. 712-6.
13. Hannema, A.J., et al., SLE like syndrome and functional deficiency of C1q in members of a large family. Clin Exp Immunol, 1984. 55(1): p. 106-14.
14. Kirschfink, M., et al., Complete functional C1q deficiency associated with systemic lupus erythematosus (SLE). Clin Exp Immunol, 1993. 94(2): p. 267-72.
15. Mehta, P., et al., SLE with C1q deficiency treated with fresh frozen plasma: a 10-year experience. Rheumatology (Oxford), 2010. 49(4): p. 823-4.
16. Orihara, T., et al., Selective C1q deficiency in a patient with systemic lupus erythematosus. Br J Dermatol, 1987. 117(2): p. 247-54.
17. Slingsby, J.H., et al., Homozygous hereditary C1q deficiency and systemic lupus erythematosus. A new family and the molecular basis of C1q deficiency in three families. Arthritis Rheum, 1996. 39(4): p. 663-70.
18. Steinsson, K., et al., Selective complete Clq deficiency associated with systemic lupus erythematosus. J Rheumatol, 1983. 10(4): p. 590-4.
19. Troedson, C., et al., Systemic lupus erythematosus due to C1q deficiency with progressive encephalopathy, intracranial calcification and acquired moyamoya cerebral vasculopathy. Lupus, 2013. 22(6): p. 639-43.
20. Tsuge, I., et al., Hyper IgM syndrome and complement Clq deficiency in an individual with systemic lupus erythematosus-like disease. Clin Exp Rheumatol, 2010. 28(4): p. 558-60.
21. Vassallo, G., et al., Clinical variability and characteristic autoantibody profile in primary C1q

complement deficiency. Rheumatology (Oxford), 2007. 46(10): p. 1612-4.
22. Jesus, A.A., et al., Complement and antibody primary immunodeficiency in juvenile systemic lupus erythematosus patients. Lupus, 2011. 20(12): p. 1275-84.
23. Marquart, H.V., et al., C1q deficiency in an Inuit family: identification of a new class of C1q disease-causing mutations. Clin Immunol, 2007. 124(1): p. 33-40.
24. Pickering, M.C., et al., Systemic lupus erythematosus, complement deficiency, and apoptosis. Adv Immunol, 2000. 76: p. 227-324.
25. Macedo, A.C. and L. Isaac, Systemic Lupus Erythematosus and Deficiencies of Early Components of the Complement Classical Pathway. Front Immunol, 2016. 7: p. 55.
26. Kampylafka, E.I., et al., Incidence and prevalence of major central nervous system involvement in systemic lupus erythematosus: a 3-year prospective study of 370 patients. PLoS One, 2013. 8(2): p. e55843.
27. Stevens, B., et al., The classical complement cascade mediates CNS synapse elimination. Cell, 2007. 131(6): p. 1164-78.
28. Chu, Y., et al., Enhanced synaptic connectivity and epilepsy in C1q knockout mice. Proc Natl Acad Sci U S A, 2010. 107(17): p. 7975-80.
29. Ma, Y., et al., Remodeling of dendrites and spines in the C1q knockout model of genetic epilepsy. Epilepsia, 2013. 54(7): p. 1232-9.
30. Sato, S., et al., Reversible basal ganglia lesions in neuropsychiatric lupus: a report of three pediatric cases. Int J Rheum Dis, 2014. 17(3): p. 274-9.
31. Sun, W., et al., Immune complexes activate human endothelium involving the cell-signaling HMGB1-RAGE axis in the pathogenesis of lupus vasculitis. Lab Invest, 2013. 93(6): p. 626-38.
32. Nauta, A.J., et al., Direct binding of C1q to apoptotic cells and cell blebs induces complement activation. Eur J Immunol, 2002. 32(6): p. 1726-36.
33. Santer, D.M., et al., C1q deficiency leads to the defective suppression of IFN-alpha in response to nucleoprotein containing immune complexes. J Immunol, 2010. 185(8): p. 4738-49.
34. Clarke, E.V., et al., Complement protein C1q bound to apoptotic cells suppresses human macrophage and dendritic cell-mediated Th17 and Th1 T cell subset proliferation. J Leukoc Biol, 2015. 97(1): p. 147-60.
35. Martin, M. and A.M. Blom, Complement in removal of the dead - balancing inflammation. Immunol Rev, 2016. 274(1): p. 218-232.
36. Fraser, D.A. and A.J. Tenner, Innate immune proteins C1q and mannan-binding lectin enhance clearance of atherogenic lipoproteins by human monocytes and macrophages. J Immunol, 2010. 185(7): p. 3932-9.
37. Prechl, J. and L. Czirjak, The endothelial deprotection hypothesis for lupus pathogenesis: the dual role of C1q as a mediator of clearance and regulator of endothelial permeability. F1000Res, 2015. 4: p. 24.
38. Pickering, M.C., et al., Complement C1q and C8beta deficiency in an individual with recurrent bacterial meningitis and adult-onset systemic lupus erythematosus-like illness. Rheumatology (Oxford), 2008. 47(10): p. 1588-9.
39. Hoekzema, R., et al., Biosynthesis of normal and low-molecular-mass complement component C1q by cultured human monocytes and macrophages. Biochem J, 1989. 257(2): p. 477-86.
40. Hoekzema, R., et al., Significance of low molecular weight C1q in systemic lupus erythematosus. Ann Rheum Dis, 1990. 49(9): p. 698-704.

Supplementary Table 1. Cases reported with C1q deficiency and neuropsychiatric systemic lupus erythematosus

Age at onset/Sex /Flare	Country	Clinical features	NPSLE manifestation	Notes	Immunological tests	Complement functional tests	Neuroimaging	Medication	M
1/M/ND	Yougoslavia	Malar rash, oral ulcers, photosensitivity, arthritis, LN (MPGN), NPSLE (Seizure)	Seizure	Recurrent infections Died at 13	ANA + DNA+ SSA + Sm+ RNP+	C1q=0	ND	Corticoids, frozen plasma, plasmapheresis and IVIG	g.8
13/F/2	Saoudi Arabia	Malar rash, discoid rash, oral ulcers, arthritis, leukopenia, thrombopeina, alopecia	Seizures, mononeuritis multiplex	ND	ANA+ DNA- ENA-	C1q=0	ND	Corticoids, CYC	
7/F/7 and 20	Dutch	Malar rash, oral ulcers, LN, fever, alopecia, lymphadenopathy, myositis	Seizure, hemiplegia and lethargy. Probably cerebral vasculitis	Died at 20	ANA+ DNA- RNP+	C1q<0.1 C1r/C1s=0 C3/C4: N CH_{50}<1% C1inh= N	Brain scintigraphy: multiple spots with activity mainly right sided, probably due to vasculitis	Corticoids, CYC	
9/F/9	Japan	Malar rash, discoid rash, photosensitivity, oral ulcers, proteinuria (no biopsy), arthralgia	Seizure		ANA+ DNA – SSA + Sm+ RNP +	ND LMW C1q	Calcification of the basal ganglia and the temporal lobe (CT-scan)	Corticoids	g.5
6/F/18,24 and 29	Germany	Malar rash, oral ulcers, photosensitivity, leukopenia, pleuritis, arthritis, glomerulonephritis (Type V) and Libman-Sacks endocarditis, peritonitis	Seizure and psychosis	Renal and heart failure died at 29	ANA+ DNA+ Sm+	C1q=28% C1r/C1s=N C2-C4f =N C3/C4= N CH_{50}=0 AP_{50}=N C1inh =N	ND	Corticoids, plasmapheresis, chlorambucil, CYC, cyclosporin, IVIG	g.5
9/F/25	England	Malar rash, photosensivity, leukopenia, alopecia	Seizure and cognitive dysfunction	Recurrent infections Died at 28	ANA + DNA – SSA + Sm + RNP +	C1q=0 CH_{50}<5% AP_{50}= N	Periventricular and basal ganglia calcification, with severe cerebral atrophy	Corticoids, Azathioprine, frozen plasma and plasmapheresis	g.8
5/F/ND	Saudi Arabian	Discoid lupus, photosensitivity, lupus nephritis (non-specified), alopecia	CNS involvement with cerebral atrophy, non-specified		ANA + DNA - SSA + SSB + Sm +	ND	ND. Cerebral atrophy	Unknown	



Chapter 5

Complement levels and anti-C1q autoantibodies in patients with neuropsychiatric systemic lupus erythematosus

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Abstract

To analyse serum levels of anti-C1q, C1q circulating immune complexes (CIC), complement activation and complement components in systemic lupus erythematosus (SLE) patients during the first central nervous system neuropsychiatric event and to define the possible association between these results and clinical and laboratory characteristics.

A total of 280 patients suspected of having NP involvement due to SLE were recruited in the Leiden NPSLE-clinic. All SLE patients were classified according to the ACR 1982 revised criteria for the classification of SLE. The clinical disease activity was measured by the SLE Disease Activity Index 2000 (SLEDAI-2K) and NP diagnoses were classified according to the 1999 ACR case definitions for NPSLE. We measured in serum of all patients anti-C1q and C1q CIC levels, the activation capacity of complement (CH50 and AP50) and different complement components (C1q, C3, C4).

In 92 patients the symptoms were attributed to SLE. NPSLE patients consisted of 63 patients with focal NPSLE and 34 patients with diffuse NPSLE. Anti-C1q antibodies were significantly higher and CH50, AP50 and C3 were significantly lower in NPSLE patients compared with SLE patients without NPSLE. This association was specially marked for diffuse NPSLE while no differences were found for focal NPSLE. After using potential predictors, decreased C4 remained significantly associated with focal NPSLE, but only when antiphospholipid antibodies (aPL) were included in the model. C3 and AP50 were independently associated with diffuse NPSLE. When SLEDAI-2K was included in the model these two associations were lost. When individual NPSLE syndromes were analyzed, psychosis and cognitive dysfunction showed significantly lower values of complement activation capacity and all complement components. No significant associations were seen for other individual NPSLE syndromes.

The associations between diffuse NPSLE and anti-C1q, C3/AP50 and focal NPSLE and C4 may be explained by disease activity and the presence of aPL respectively. The role of complement activation and complement components in lupus psychosis and cognitive dysfunction merits further research.

Introduction

The complement system plays an important role in systemic lupus erythematosus (SLE). [1] Decreased levels of complement components, complement activation

and higher levels of antibodies against C1q (anti-C1q) are characteristic findings in active SLE. A correlation between renal involvement and circulating immune complexes (CIC), complement deposits and levels of anti-C1q has been found in SLE. [1-3] However, the pathogenic role of all these complement components in other organs, including the nervous system, is less clear.

Complement factors are known to contribute to the pathology of inflammatory central nervous system (CNS) and neurodegenerative diseases and they have been proposed as one of the multiple participants in the pathogenesis of neuropsychiatric systemic lupus erythematosus (NPSLE). [4-6] Data from human studies are scarce and contradictory. Although the exact underlying mechanism remains unknown, complement may collaborate in blood-brain barrier (BBB) alteration, brain cell dysfunction or vasculopathy and accelerated atherosclerosis. [5, 7, 8] Some authors have found an association between NPSLE and low serum levels of C3 and C4 complement components, while increased levels of these proteins and the soluble form of C5b-9 have been found in the cerebrospinal fluid (CSF) of SLE patients. [9-11] An enhance deposition of complement activation products on platelets has also been associated with the development of thrombosis in SLE, a process where antiphospholipid antibodies (aPL) have been reported to be collaborate notably. [12, 13]

In murine models, both deletion of factor B, a key alternative pathway protein, and inhibition of the classical and alternative complement cascade with the complement inhibitor Crry, demonstrated to alleviate experimental CNS lupus. [14, 15] In addition, selective inhibition of two complement receptors, C3aR and C5aR, reduced neuronal degeneration (apoptosis and gliosis) and alleviated CNS lupus respectively. [16, 17] C5 has also been reported to play a role in the maintenance of the BBB in a lupus rodent model. [18] Moreover, mice deficient in C3 and C5 components have also been reported to be resistant to enhanced thrombosis and endothelial cell activation induced by aPL antibodies, ameliorating the effect and pointing out the important role of alternative pathway complement activation on aPL-antibody mediated thrombogenesis. [19, 20]

Serum complement levels are an accessible and worldwide used biomarker of great value for monitoring SLE activity. Although several studies have pointed out the role of the complement system in different aspects of NPSLE pathogenesis, serum complement components (C1q, C3 and C4), the ability to activate the complement system (CH50, AP50), anti-C1q and C1q CIC have never been assessed in a large and well defined NPSLE cohort. The aim of the current study was to analyze serum complement levels and anti-C1q levels during the first neuropsychiatric (NP) event of patients included in the Leiden NPSLE-cohort, and to define the possible

association between these results and clinical (NPSLE syndromes, disease activity and damage) and laboratory characteristics.

Patients and methods

Patient selection and clinical evaluation

From September 2007 until September 2014, 280 consecutive patients suspected of having NP involvement due to SLE were referred to the Leiden NPSLE-clinic (Leiden University Medical Center, The Netherlands) for evaluation. All the subjects were admitted for 1-day and underwent multidisciplinary examination including neuropsychological testing, as well as extensive laboratory and radiological examination. A multidisciplinary consensus meeting took place soon after the evaluation of every patient. For further description of the multidisciplinary evaluation, please see reference [21]. All the patients were classified according to the American College of Rheumatology (ACR) 1982 revised criteria for the classification of SLE. [22, 23] The clinical disease activity was measured by the Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) patient. [24] For the better assessment of the effect of disease activity we decided to exclude the NP manifestations from the SLEDAI-2K. In the NPSLE group we included all patients having at least one NPSLE manifestation involving the CNS. NP diagnoses were classified according to the 1999 ACR case definitions for NPSLE syndromes and classified into focal and diffuse NPSLE according to these definitions. [21, 25] All patients with antiphospholipid syndrome (APS) had a history of anticardiolipin IgG or IgM (aCL), anti-beta2 glycoprotein 1 IgG or IgM (anti-β2GP1) and/or positive lupus anticoagulant (LAC) tests documented on two or more occasions at least 3 months apart. Furthermore, all these patients met the Sapporo clinical criteria. [26] In addition, 200 healthy controls (HC), aged between 20 and 70 years, were included in this study. All participants in the study provided informed consent and the study was approved by the local medical ethics committee.

Laboratory assessment

Serum samples of all patients were collected from each subject at 08:00 AM after overnight fasting. The functional capability of the complement components to activate the complement system of the classical pathway (CH50) and the alternative pathway (AP50) and levels of complement components (C1q, C3 and C4) were measured the same day of the blood extraction in the routine clinical laboratory at the Leiden University Medical Center (LUMC), The Netherlands. CH50 and AP50

were measured using functional assays. Levels of C1q, C3 and C4 in serum were measured using laser nephelometry. Based on the normal limits for our laboratory, CH50 level < 74%, AP50 < 39%, C1q < 102 mg/l, C3 < 0.9 g/l and C4 < 95 mg/l were defined as low. Plasma was also prepared by centrifugation and aliquoted (500 µl) into polypropylene tubes before freezing and stored at - 80°C. Patient´s sera were kept frozen until it was analyzed for the levels of anti-C1q and C1q CIC by enzyme-linked immunosorbent assay (ELISA). These laboratory determinations were performed at the Rheumatology Laboratory (LUMC, The Netherlands). Anti-C1q antibodies and C1q CIC in serum were measured by the QUANTA Lite ™ Anti-C1q ELISA and with the usage of the QUANTA Lite® C1q CIC ELISA (Inova Diagnostics, San Diego, CA, USA), following the protocol from the manufacturer. The reference intervals were defined as < 20 units/ml for anti-C1q and as < 4.4 µg Eq/ml for C1q CIC. These classifications were also used to classify the healthy subjects. Another set of blood samples was tested for aPL, anti-dsDNA, anti-Sm, anti-RNP, anti-SSA/Ro52 and anti-SSB/La antibodies in the routine clinical laboratory at the LUMC. IgG anti-dsDNA antibodies were detected using the Crithidia Luciliae indirect immune fluorescence technique (Immunoconcepts, Sacramento, USA). IgG antibodies against SS-A/Ro-52, SS-B/La, Sm, RNP and IgG and IgM anti-cardiolipine and anti-ß2-glycoproteine I antibodies were detected were determined using a Phadia® 250 EliA fluorescence enzyme immunoassay (FEIA) (Thermo Scientific, Freiburg, Germany). Lupus anticoagulans (LAC) was determined using STA-Rack en STA Evolution coagulation analysers (Stago, Parsippany, USA).

Statistical analysis

Patients with NPSLE and SLE patients were compared with respect to demographic characteristics, clinical manifestations, autoantibody profile and complement components using $\chi 2$ test or with Fisher's exact test and Mann-Whitney U-test when appropriate. Differences in anti-C1q and C1q CIC between HC, SLE and NPSLE or among NPSLE subgroups were analyzed by the Kruskal-Wallis test with the Dunn multiple comparison test or the Mann-Whitney U-test when needed. Differences in CH50 and AP50 between groups were compared by using one-way ANOVA test. $\chi 2$ test and Fisher exact test were used to compare between NPSLE subgroups (focal and diffuse NPSLE) and individual NPSLE syndromes and the complement components (C1q, C3 and C4). Odds ratios (OR) and 95% confidence intervals (CI) were also calculated. Five patients were included in both focal and diffuse NPSLE groups. We preferred this situation over leaving these patients out of the study completely or leave them in only one of the two groups. Binary logistic regression was used to ascertain the effects of age, disease activity measured by SLEDAI-

2K and different laboratory markers including antibodies and complement on the likelihood to have NPSLE, focal NPSLE or diffuse NPSLE. Laboratory variables judged to have clinical relevance based on a priori knowledge and previous univariate analysis were retained in the final models. Variables of interest were evaluated in two models, one with complement components (C1q, C3, C4) and other with complement activation (CH50 and AP50), independently added to individual antibodies of interest (LAC, aCL, anti-dsDNA, anti-Sm and anti-C1q antibodies) and SLEDAI-2K. $p \leq 0.05$ was considered statistically significant. Statistical analysis was performed with commercially available software (IBM SPSS statistics, version 20.0 for Windows; SPSS, Chicago, IL, USA). Figures were performed using GraphPad Prism 6 for Mac OS X ver. 6.0b, Graph-Pad Software, Inc., San Diego, CA, USA.

Results

Demographic data and clinical characteristics

A total of 280 patients were analyzed in our NPSLE clinic and 204 fulfilled the ACR classification criteria for SLE. [22, 23] In 112 SLE patients, the NP complaints were better explained by another cause. A NPSLE syndrome involving the CNS was diagnosed in 92 (45.1%) of the SLE patients. Among the patients diagnosed with CNS NPSLE, 144 different ACR NP syndromes were established. Thirty-four patients had at least one diffuse NPSLE syndrome while 63 patients were diagnosed with at least one focal NPSLE syndrome according to the ACR 1999 NPSLE definitions. [25] Five patients were diagnosed with both focal and diffuse symptoms. Patient demographics relevant to the present study are shown in Table 1. A description of all CNS syndromes included in the study is shown in Table 2.

Relationship of anti-C1q antibodies and C1q CIC and SLE and NPSLE

Using the recommended cut-off values by the manufacturer, the positivity rates of anti-C1q levels and C1q CIC in HC were 13.5% (27 of 200) and 19.5% (39 of 200), respectively. Prevalence of anti-C1q antibodies and C1q CIC in NPSLE and SLE patients is shown in Table 1. Levels of anti-C1q antibodies were higher in patients with NPSLE than in both SLE (median 16.9 versus 8.0; $P < 0.05$) and HC (16.9 versus 7.0; $P < 0,001$) (Figure 1A). The same trend was seen in the C1q CIC levels when SLE and NPSLE were compared with HC (Figure 1B).

As previously described by other authors , the prevalence of anti-C1q antibodies was significantly higher in SLE patients with renal involvement (OR=2.1, 95% CI 1.1–3.9, $P < 0.05$), positivity for anti-dsDNA (OR=5.1, 95% CI 2.6–9.7, $P < 0.001$), and

	SLE n = 112	NPSLE		
		Total n = 92	Focal * n = 63	Diffuse * n = 34
Age, mean ± SD years	44.01 ± 13:78	40 ± 13.68 [a]	43.23 ± 13.86	33.21 ± 10.19 [b,d]
Sex, no. female/male	99/13	82/10	55/8	32/2
Age at diagnosis SLE, mean ± SD years	35.4 ± 14.93	32.45 ± 14.8	35.01 ± 15.98	26.34 ± 10.05 [a,c]
SLE disease duration, mean ± SD years	8.61 ± 8.55	7. 83 ± 8.31	8.23 ± 8.7	7.57 ± 8.08
SLEDAI-2K	4 [0 – 19]	6 [0 – 22] [b]	6 [0 – 22] [b]	9 [0 – 22] [b,c]
ACR 1982 criteria for SLE †				
Malar Rash	54 (48.2)	34 (37)	21 (33.3)	15 (44.1)
Discoid rash	25 (22.3)	12 (13)	9 (14.3)	4 (11.8)
Photosensitivity	50 (44.6)	31 (33.7)	22 (34.9)	9 (26.5)
Oral ulcers	40 (35.7)	32 (34.8)	19 (30.2)	14 (41.2)
Arthritis	79 (70.5)	63 (68.5)	41 (65.1)	26 (76.5)
Serositis	30 (26.8)	30 (32.6)	22 (34.9)	11 (32.4)
Renal disorder	33 (29.5)	19 (20.7)	9 (14.3)	12 (35.3) [c]
Neurologic disorder	8 (7.1)	25 (27.2) [a]	14 (22.2)	12 (35.3)
Hematologic disorder	50 (44.6)	44 (47.8)	29 (46)	17 (50)
Immunologic disorder	78 (69.6)	71 (77.2)	49 (77.7)	26 (76.5)
Positive ANA	111 (99.1)	89 (96.7)	61 (96.8)	32 (94.1)
Autoantibodies and complement †				
aCL IgG	8 (7.1)	27 (29.3) [b]	21 (33.3) [b]	7 (20.6) [a]
aCL IgM	6 (5.4)	8 (8.7)	6 (9.5)	3 (8.8)
LAC	19 (17)	43 (46.7) [b]	35 (55.5) [b]	12 (35.3) [a,c]
Anti-β2GP1 IgG ††	6 (5.4)	17 (18.5) [a]	13 (20.6) [a]	5 (14.7)
Anti-β2GP1 IgM ††	2 (1.8)	5 (5.4)	5 (7.9)	1 (2.9)
Antinuclear antibody	75 (66)	78 (84.8) [a]	53 (84.1) [a]	29 (85.3) [a]
Anti-dsDNA	23 (20.5)	33 (35.9) [a]	22 (34.9) [a]	14 (41.2) [a]
ENA	66 (58.9)	48 (52.2)	32 (50.8)	20 (58.8)
Anti-SSA/Ro52	57 (50.9)	30 (32.6) [a]	21 (33.3) [a]	11 (32.4) [a]
Anti-SSB/La	19 (17)	8 (8.7)	5 (7.9)	4 (11.8)
Anti-RNP	12 (10.7)	18 (19.6)	11 (17.5)	8 (23.5)
Anti-Sm	4 (3.6)	12 (13) [a]	7 (11.1)	6 (17.6) [a]
C1q low	7 (6.3)	13 (14.1)	7 (11.1)	8 (23.5) [a]
C3 low	29 (25.9)	42 (45.7) [a]	24 (38.1)	22 (64.7) [b,c]
C4 low	27 (24.1)	30 (32.6)	14 (22.2)	18 (52.9) [a,c]
CH50	25 (22.3)	37 (40.2) [a]	19 (30.2)	19 (55.9) [b,c]
AP50	16 (14.3)	27 (29.3) [a]	14 (22.2)	16 (47.1) [b,c]
Anti-C1q high	34 (30.3)	41 (44.6) [a]	26 (41.3)	17 (50) [a]
C1q CIC high	43 (38.4)	40 (43.5)	27 (42.9)	15 (44.1)
Antiphospholipid syndrome				
APS diagnosis	4 (3.6)	22 (23.9) [b]	26 (41.3) [b]	6 (17.6) [a]
Arterial thrombosis ever	19 (17)	48 (52.2) [b]	43 (68.3) [b]	7 (20.6) [d]
Vascular thrombosis ever	6 (5.4)	15 (16.3) [a]	13 (20.6) [a]	3 (8.8)

aCL: anticardiolipin antibodies; ACR: American College of Rheumatology; ANA: antinuclear antibody; LAC: Lupus anticoagulant; NPSLE: neuropsychiatric SLE; SLE: systemic lupus erythematosus; SLEDAI-2K: Systemic Lupus Erythematosus Disease Activity Index 2000.
* 5 patients were included in both groups
† Number and percentage per group
†† Only available in 150 (69/81) patients
a. P < 0.05 when compared with SLE
b. P < 0.001 when compared with SLE
c. P < 0.05 when compared with focal SLE
d. P < 0.001 when compared with focal SLE

Table 1 Comparison clinical data SLE and NPSLE

anti-Sm antibodies (OR=5.9, 95% CI 1.8–19.2, P < 0.001). [3, 27, 28] We also found a higher prevalence of C1q CIC in SLE patients with renal involvement (OR=2.1, 95% CI 1.1–3.9, P < 0.05), positivity for anti-dsDNA (OR=3.8, 95% CI 2.1–7.4, P < 0.001), and anti-Sm antibodies (OR=4.9, 95% CI 1.5–15.9, P < 0.05). The titers of anti-C1q antibodies and C1q CIC were also correlated with the SLEDAI-2K scores (P < 0.001 and P < 0.05, respectively) (data not shown). Among NPSLE subsets, anti-C1q antibodies were significantly elevated only in diffuse NPSLE compared

with the rest of SLE patients (20.8 versus 8.7; P < 0.05) or HC (20.8 versus 7; P< 0.05). No differences in levels were found for C1q CIC when SLE and NPSLE patients where compared. Among the different NPSLE syndromes, only headache showed a significantly higher prevalence of anti-C1q antibodies (OR=4, 95% CI 1.1–14.6, P < 0.05). No significant associations were found between individual NPSLE syndromes and C1q CIC.

Central nervous system NPSLE syndromes	
Aseptic meningitis	1
Cerebrovascular disease	45
Demyelinating syndrome	1
Headache	11
Movement disorder	3
Transverse myelitis	6
Seizure disorder	12
Psychosis	11
Acute confusional state	3
Anxiety disorder	5
Cognitive disorder	28
Mood disorder	18
Diffuse vs. focal NPSLE syndromes	
Focal NPSLE *	63
Diffuse NPSLE **	34
Diffuse NPSLE without non-specific syndromes †	61
Focal NPSLE without non-specific syndromes ††	22

NPSLE: neuropsychiatric systemic lupus erythematosus; SLE: systemic lupus erythematosus.
a. Possible > 1 NPSLE syndrome per patient
* Focal neuropsychiatric-SLE: Aseptic meningitis, cerebrovascular disease, demyelinating syndrome, headache, movement disorder, transverse myelitis, seizure disorder.
** Diffuse neuropsychiatric-SLE: psychosis, acute confusional state, anxiety disorder, cognitive disorder, mood disorder.
† Non-specific diffuse NPSLE syndromes: mood disorder, anxiety and mild cognitive dysfunction.
†† Non-specific focal NPSLE syndromes: headache.

Table 2 Central nervous system NPSLE syndromesof patients included in the study (n=92)[a]

CH50 and AP50 and NPSLE

NPSLE patients showed significantly lower CH50 values (78.1 versus 89.8; P < 0.05) (Figure 2A) and AP50 (55.8 versus 69.8; P = 0.001) than SLE patients (Figure 2B). When the different NPSLE subgroups were analyzed, the levels of CH50 and AP50 were markedly lower in patients with diffuse NPSLE (both P < 0.001) when compared with SLE patients. No differences were found for focal NPSLE. We next examined the association between CH50 and AP50 with the different NPSLE syndromes. As shown in Figure 3, psychosis (OR=60, 95% CI 7.2–501, P < 0.001), headache (OR=5, 95% CI 1.4–18.3, P < 0.05), seizure (OR=6, 95% CI 1.7–20.9, P < 0.05) and cognitive dysfunction (OR=3.8, 95% CI 1.5–9.8, P < 0.05) had significantly

higher prevalence of low AP50 when compared with SLE, while psychosis (OR=9.2, 95% CI 2.2–37.6, P = 0.001), cognitive dysfunction (OR=3.4, 95% CI 1.5–8.2), P < 0.05) and mood disorder (OR=3.5, 95% CI 1.2–9.7, P < 0.05) showed a significantly higher prevalence of low CH50. No significant associations were seen with other individual NPSLE syndromes.

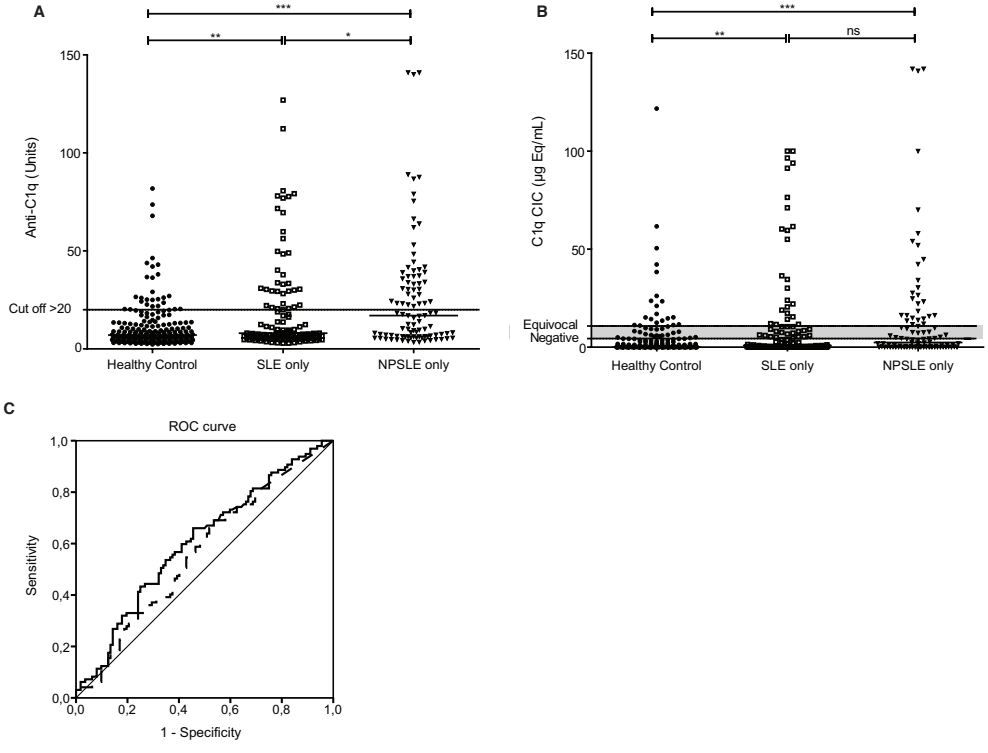

Figure 1. Serum titers of **A** anti-C1q antibodies and **B** C1q CIC in 92 consecutive patients with NPSLE, 112 patients with SLE and 200 HC. The titers of anti-C1q antibodies were significantly higher in patients with NPSLE than in the rest of SLE patients and HC (P < 0.05 and P < 0.001, respectively). For anti-C1q antibodies and C1q CIC we indicate with a broken line the cut-off value recommended by the manufacturer. Horizontal lines indicate median. **C.** Receiver-Operating-Characteristic (ROC) curves for the levels of anti-C1q and C1q-CIC in 112 patients with SLE and 92 patients with NPSLE. The mean (± SE) area under the curve for anti-C1q (continuous line) was 0.61 ± 0.04 and for C1q-CIC (dashed line) was 0.56 ± 0.04 for predicting NPSLE.

HC: healthy controls; NPSLE: neuropsychiatric systemic lupus erythematosus; SE: standard error; SLE: systemic lupus erythematosus.

Kruskal-Wallis test with Dunn's multiple comparison test and Mann-Whitney's U test, *P < 0.05, ** P < 0.01, *** P < 0.001

Circulating levels of C1q, C3 and C4 in relation to NPSLE

A significantly higher prevalence of low C3 was shown in NPSLE (OR=2.4, 95% CI 1.3–4.3, P < 0.05), and especially in diffuse NPSLE patients (OR= 5.2, 95% CI 2.3–11.9, P < 0.001), when compared with SLE patients (Figure 3). An association between NPSLE patients and lower values of C4 and C1q was not found; however low levels of these components were more prevalent in diffuse NPSLE (C4: OR= 3.5, 95% CI 1.5–7.8, P < 0.05; C1q: OR= 4.6, 95% CI 1.5–13.8, P < 0.05). No associations were found with focal NPSLE. Patients with lupus psychosis showed higher prevalence of low C1q (OR=5, 95% CI 1.5–15.8, P < 0.05), C3 (OR=28.6, 95% CI 3.5–230.4, P < 0.001) and C4 (OR=3.8, 95% CI 1.1–13.3, P < 0.05) when compared with SLE. Patients with cognitive dysfunction showed also higher prevalence of low C1q (OR=5, 95% CI 1.5–15.8, P < 0.05), C3 (OR=4.4, 95% CI 1.8–10.5, P < 0.001) and C4 (OR=3.6, 95% CI 1.5–8.6, P < 0.05) when compared with SLE. An association between headache and higher prevalence of low C4 (OR=3.7, 95% CI 1.1–13.3, P < 0.05) was also found. No significant associations were seen with other individual NPSLE syndromes.

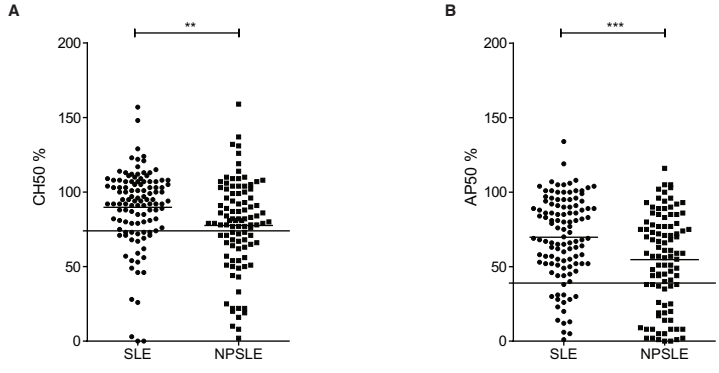

Figure 2. Measurement of the activation state of the **A** classical pathway (CH50) and **B** alternative pathway (AP50) in 92 consecutive patients with NPSLE and 112 patients with SLE. For CH50 and AP50 we indicate with a broken line the cut-off value used in our laboratory. The levels of both CH50 and AP50 were significantly lower in NPSLE patients than in SLE (P < 0.05 for CH50 and P < 0.001 for AP50). Horizontal lines indicate mean.

NPSLE: neuropsychiatric systemic lupus erythematosus; SLE: systemic lupus erythematosus.

One-way analysis of variance test, *P < 0.05, ** P < 0.01

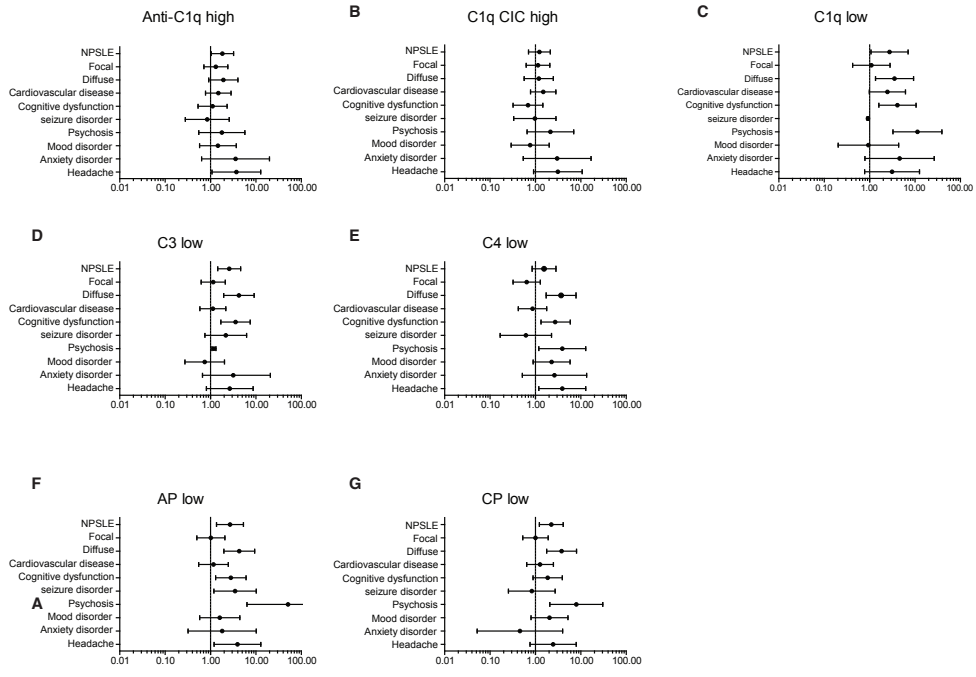

Figure 3. Odds ratios and 95% confidence interval analyzing the association of the more common NPSLE presentations in patients 204 SLE patients from the Leiden NPSLE-clinic. **A.** Anti-C1q high as considered by manufacturer (> 20 U/ml), **B.** C1q CIC high as considered by the manufacturer (> 4.4 µg Eq/ml), **C.** low C1q measured using laser nephelometry (< 102 mg/l), **D.** low C3 measured using laser nephelometry (< 0.9 g/l), **E.** low C4 measured using laser nephelometry (< 95 mg/l), **F.** low AP50 measured using functional assays (< 39%), and **G.** CH50 measured using functional assays (<74%).
NPSLE: neuropsychiatric systemic lupus erythematosus.
$\chi 2$ test and Fisher exact tests.

Complement activation and complement components as predictor of NPSLE

When possible complement activating factors were included in the model, NPSLE patients showed a positive significant association with aCL IgG (OR=3.126, 95% CI 1.2–7.8, $p < 0.05$), LAC (OR=3.233, 95% CI 1.6–6.5, $p = 0.001$) and AP50 (OR=0.985, 95% CI 0.975–0.996, $p < 0.05$) after controlling for age, anti-dsDNA, anti-Sm, anti-C1q and CP50. When complement components were included in the model aCL IgG and LAC remained significant. After using all the same potential predictors, only aPL IgG (OR=5.974, 95% CI 2.1–17.3, $P < 0.001$), LAC (OR=5.765, 95% CI 2.6–12.6, $P < 0.001$), and also C4 (OR=4.175, 95% CI 1.4–12.2, $P < 0.05$) remained significantly associated with focal NPSLE. After adjusting for above listed covariates, diffuse NPSLE was associated with a lower age ($P < 0.05$). When complement components were included in the model, C3 was significantly associated with diffuse NPSLE (OR=3.552, 95% CI 1.4–8.5, $P < 0.05$). Furthermore,

when complement activation instead of complement components were used in the model, AP50 was also significantly associated with diffuse NPSLE (OR=0.972, 95% CI 0.957–0.988, P < 0.001). When SLEDAI-2K was included in the model we missed these two associations.

Discussion

The pathogenic processes that lead to damage or dysfunction in the nervous system due to SLE remains poorly understood. Important associations have been reported between several autoantibodies and nervous system involvement in SLE, such as aPL and cerebrovascular disease and anti-ribosomal P and lupus psychosis. However, no specific autoantibodies have been identified and serological biomarkers for NPSLE are extremely needed. The role played for other elements beyond autoantibodies in the NPSLE pathogenesis remains unclear. [29, 30] This study analyzes for the first time the serum complement components (C1q, C3 and C4), complement activation (CH50 and AP50), anti-C1q and C1q CIC in a large and well defined cohort of NPSLE with CNS involvement. The results in the present study have disclosed that none of the complement elements studied is useful to differentiate between NPSLE and SLE, but that some of them may be associated with a certain subset of NPSLE patients.

We found an association between a low C4 and focal NPSLE. Complement activation is known as an important mechanism of tissue injury in cerebral ischemia. Platelets bearing the complement activation product C4d are a known link between cerebrovascular inflammation and thrombosis. Moreover, they have been proposed as a specific biomarker for SLE diagnosis, and a relation with NPSLE has also been suggested. [31, 32] An increase in deposition of complement activation products, such as C4d, on platelets is associated with the presence of LAC, aCL and anti-β2GP1 antibodies and it has been proposed as an essential mechanism in aPL-mediated thrombosis in SLE. [12, 13, 31, 32] Serum hypocomplementaemia is commonly seen in patients with primary APS, reflecting complement activation and consumption. [33] It has been suggested that aPL may activate monocytes and macrophages via anaphylatoxins produced in complement activation. [33] An increase in complement activation products in serum of aPL positive patients has been related with the development of transient ischemic events and stroke. [34] In our cohort, the focal NPSLE group was characterized by a higher prevalence of aPL and APS. [35] We have demonstrated that in this group the association with a low serum C4 was due to the association with the presence of LAC and aPL IgG. Serum C4 was not independently associated with focal NPSLE or with cerebrovascular

disease in SLE patients.

Diffuse NPSLE patients were associated with a markedly low AP50 and low C3. Furthermore, we have shown for the first time that higher levels of anti-C1q antibodies are significantly associated with this NPSLE subgroup when compared with SLE. Complement components C3 and C4 are recognized markers of global SLE activity and CH50 and AP50 are markedly reduced during SLE flares. [1, 36] As reported in previous reports, we also observed an association between anti-C1q antibodies and known markers of global SLE activity such as SLEDAI-2K, anti-dsDNA antibodies, C1q CIC, C3, C4 and CH50. [27, 37, 38] Furthermore, similarly to other authors, we also confirmed a relation between anti-C1q levels and lupus nephritis [39, 40] and younger age [28]. Whereas in murine studies the association between anti-C1q autoantibodies and lupus nepthritis has been wel established [41, 42], no such data is available to support the role of anti-C1q in other organ SLE manifestations. Diffuse NPSLE manifestations have been linked to higher global SLE activity. [7] In our cohort we corroborate this association. We also miss the association between AP50 and low C3 and diffuse NPSLE when SLEDAI-2K is included in the model. Since there is no gold-standard for NPSLE, we cannot exclude the possibility that the multidisciplinary team that attributed the NP complaints to SLE was influenced by hypocomplementaemia when taking into account disease activity, which may explain our results. However, in clinical practice only hypocomplementaemia and not the evaluation of individual complement components have been taken into account. The fact that only AP50 and C3 and not CP and C4 were related with diffuse NPSLE is intriguing and may be not biased by concomitant disease activity, leading us to make further interpretations. In murine models of lupus cerebritis, targeted and selective inhibition of the alternative complement pathway has been shown to be effective. [14-18] We could hypothesize that the complement alternative pathway may play a role in the pathogenesis of patients with diffuse NPSLE.

Among the NPSLE syndromes, patients with lupus psychosis had markedly higher complement activation and a higher prevalence of low serum C1q, C3 and C4. This association was especially marked for AP50 and C3. Lower serum C3 levels have been seen in corticosteroid-induced psychosis [43] and corticosteroid-induced psychiatric diseases [44] in SLE patients. In the last case, C1q and C4 were also seen to be lower, however only serum C3 level was an independent risk factor for new-onset of psychiatric disorder after corticosteroid therapy. [45] Interestingly, complement activation was increasingly linked to schizophrenia development and psychopathology. [46] Some authors have reported lower levels of serum C3 in schizophrenia patients when compared with HC [47] whereas others have observed higher levels of C3 in these patients. [48] Also at the molecular level, the gene

encoding C3, has been reported to be a genetic schizophrenia susceptibility region [49] , whereas others could not confirm this. [50] In SLE patients data is limited. Pego-Reigosa et al. reported low C3 levels in 4/10 patients with lupus psychosis and no other complement alterations were found. [51] Watanabe et al. reported lower serum C3 levels in NPSLE patients; however patients with lupus psychosis had higher serum C3 levels than other NPSLE patients. [52] Further research on the link between alternative pathway and psychosis in patients with and without SLE, taking into account other factors such as corticosteroid treatment, is warranted.

Complement components C1q and C3 have emerged in the last years as key mediators of synaptic elimination and connectivity during development, normal ageing and neurodegeneration. [53-55] Complement has been localized at synapses and mediates pruning of synapses through a C3-dependent microglial phagocytosis process. [56] Cognitive decline, mediated through synapse elimination, has become a recognized feature in several neurodegenerative diseases. [57] For example, recent data in multiple sclerosis, an immune mediated inflammatory disease characterized for demyelination and leading memory impairment in up to 65% of patients, support that in the hippocampus of these patients there were clear signs of activation of complement components C1q-C3. [58] This disease shares some similarities with NPSLE patients. [59] In our cohort, we found significantly lower levels of complement components, including C1q and C3, in patients with cognitive dysfunction due to SLE. The functional relationship between activation of complement components in brain pathology of NPSLE patients should be investigated.

Although measuring complement activation by evaluating consumption of serum C3 and C4 are regularly used to track disease activity in SLE, the interpretation of these levels is challenging. They are acute phase reactants that may not decrease until late in a SLE flare. [60] Alterations in several components of the complement system in human CSF in NPSLE patients have been scarcely studied. Higher levels of C3 and C4 have been reported in CSF when compared with controls. It has been proposed that this may reflect an intrathecal compensatory production [9]. Intrathecal activation of terminal complement by measuring SC5b-9 in NPSLE patients has also been seen. [11] Recent studies have demonstrated that several complement components are synthesized in the CNS [61] and also in human neuronal cells in vitro. [62] Autoantibodies in SLE are supposed to form immune complex with complement [63] and induce neuroinflammation, but how this process occurs is far from clear.

Our study has notable limitations. Complement split products, which may reflect more accurately complement activation, were not evaluated. Furthermore, since

lumbar puncture is not routinely performed in all the patients included in the NPSLE-cohort, we lack the results of complement components in CSF. Determination of complement split products and parallel analysis of CSF must be included in future studies. Another limitation of our study is the retrospective design. On the other hand, all NPSLE patients were unselected, consecutive patients, diagnosed in the same institution and in the same standardized multidisciplinary procedure. Our future work is aimed at prospectively finding associations between complement activation and components. Due to referral nuances, immunosuppressive therapy, including in some cases methylprednisolone, was already started in a few patients with diffuse NPSLE patients. The effect of the therapy, mainly methylprednisolone, on complement component levels was not investigated. The small number of NPSLE patients per syndrome may affect the power in this study and must be mentioned as a limitation. Definite conclusions concerning the relationship between complement components and NPSLE syndromes cannot be drawn.

To our knowledge, this study is the first to investigate associations between complement elements measured in serum and clinical and serologic parameters in a large NPSLE cohort. No association was found between anti-C1q or C1q CIC when all the NPSLE patients where compared with SLE. We found an association between diffuse NPSLE and anti-C1q, decreased C3 and AP50 and focal NPSLE and decreased C4. These associations found between certain NPSLE subgroups and several complement elements may be explained due to other factors such as aPL in the case of focal NPSLE and global disease activity in the case diffuse NPSLE. The roles of several complement aspects, especially alternative pathway activation and C3, in lupus psychosis and cognitive dysfunction merits further research.

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References

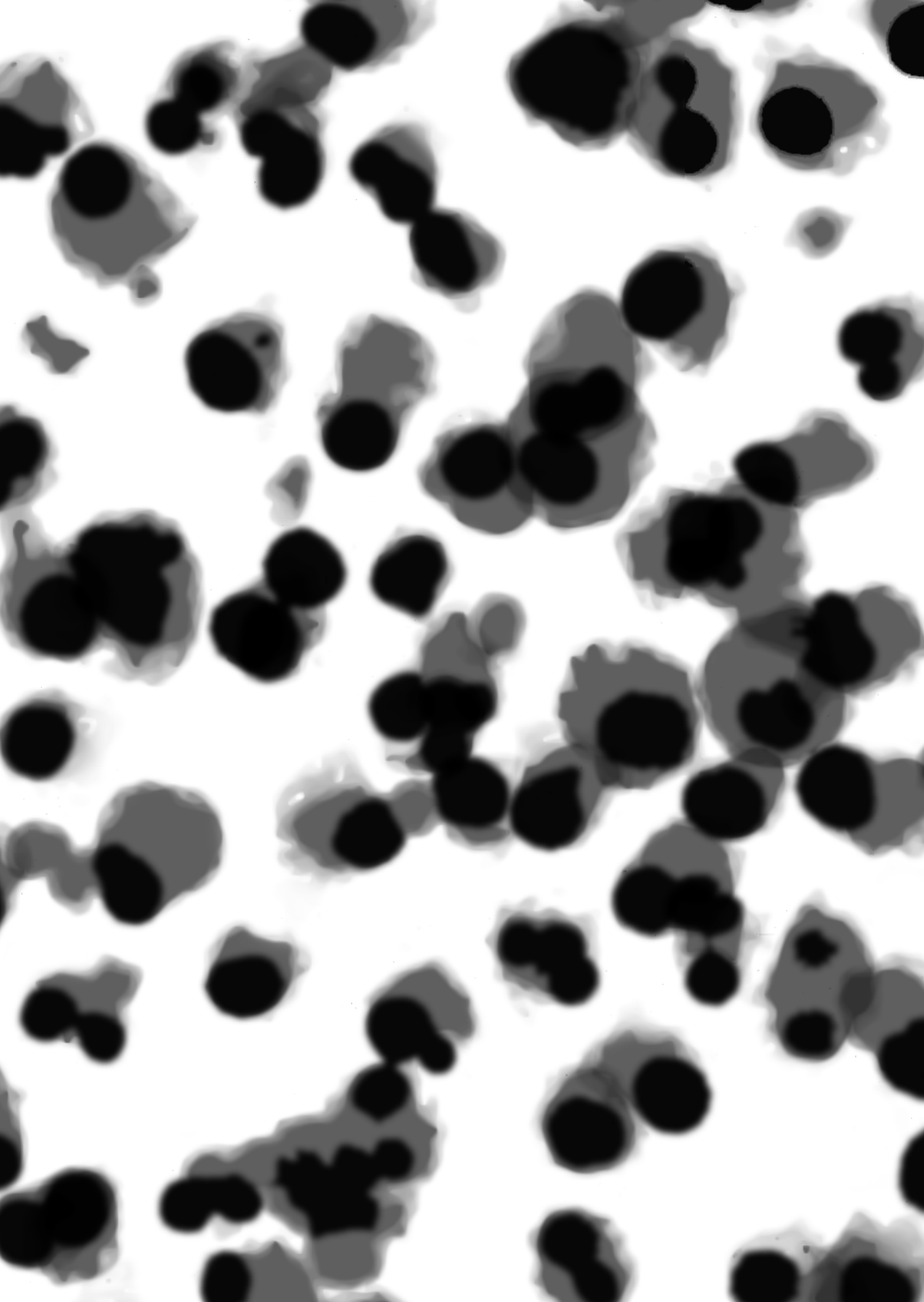
1. Leffler, J., A.A. Bengtsson, and A.M. Blom, The complement system in systemic lupus erythematosus: an update. Ann Rheum Dis, 2014. 73(9): p. 1601-6.
2. Sturfelt, G. and L. Truedsson, Complement and its breakdown products in SLE. Rheumatology (Oxford), 2005. 44(10): p. 1227-32.
3. Mahler, M., R.A. van Schaarenburg, and L.A. Trouw, Anti-C1q autoantibodies, novel tests, and clinical consequences. Front Immunol, 2013. 4: p. 117.
4. Alexander, J.J. and R.J. Quigg, Systemic lupus erythematosus and the brain: what mice are telling us. Neurochem Int, 2007. 50(1): p. 5-11.
5. Diamond, B., et al., Losing your nerves? Maybe it's the antibodies. Nat Rev Immunol, 2009. 9(6): p. 449-56.
6. Farber, K., et al., C1q, the recognition subcomponent of the classical pathway of complement, drives microglial activation. J Neurosci Res, 2009. 87(3): p. 644-52.
7. Bertsias, G.K. and D.T. Boumpas, Pathogenesis, diagnosis and management of neuropsychiatric SLE manifestations. Nat Rev Rheumatol, 2010. 6(6): p. 358-67.
8. Veerhuis, R., H.M. Nielsen, and A.J. Tenner, Complement in the brain. Mol Immunol, 2011. 48(14): p. 1592-603.
9. Jongen, P.J., et al., Cerebrospinal fluid C3 and C4 indexes in immunological disorders of the central nervous system. Acta Neurol Scand, 2000. 101(2): p. 116-21.
10. Karassa, F.B., et al., Risk factors for central nervous system involvement in systemic lupus erythematosus. Qjm, 2000. 93(3): p. 169-74.
11. Sanders, M.E., et al., Detection of activated terminal complement (C5b-9) in cerebrospinal fluid from patients with central nervous system involvement of primary Sjogren's syndrome or systemic lupus erythematosus. J Immunol, 1987. 138(7): p. 2095-9.
12. Peerschke, E.I., et al., Serum complement activation on heterologous platelets is associated with arterial thrombosis in patients with systemic lupus erythematosus and antiphospholipid antibodies. Lupus, 2009. 18(6): p. 530-8.
13. Lood, C., et al., Platelet activation and anti-phospholipid antibodies collaborate in the activation of the complement system on platelets in systemic lupus erythematosus. PLoS One, 2014. 9(6): p. e99386.
14. Alexander, J.J., et al., Administration of the soluble complement inhibitor, Crry-Ig, reduces inflammation and aquaporin 4 expression in lupus cerebritis. Biochim Biophys Acta, 2003. 1639(3): p. 169-76.
15. Alexander, J.J., et al., Absence of functional alternative complement pathway alleviates lupus cerebritis. Eur J Immunol, 2007. 37(6): p. 1691-701.
16. Jacob, A., et al., C3aR inhibition reduces neurodegeneration in experimental lupus. Lupus, 2010. 19(1): p. 73-82.
17. Jacob, A., et al., Inhibition of C5a receptor alleviates experimental CNS lupus. J Neuroimmunol, 2010. 221(1-2): p. 46-52.
18. Jacob, A., et al., C5a alters blood-brain barrier integrity in experimental lupus. Faseb j, 2010. 24(6): p. 1682-8.
19. Pierangeli, S.S., et al., Complement activation: a novel pathogenic mechanism in the antiphospholipid syndrome. Ann N Y Acad Sci, 2005. 1051: p. 413-20.
20. Thurman, J.M., et al., A novel inhibitor of the alternative complement pathway prevents antiphospholipid antibody-induced pregnancy loss in mice. Mol Immunol, 2005. 42(1): p. 87-97.
21. Zirkzee, E.J., et al., Prospective study of clinical phenotypes in neuropsychiatric systemic lupus

erythematosus; multidisciplinary approach to diagnosis and therapy. J Rheumatol, 2012. 39(11): p. 2118-26.
22. Hochberg, M.C., Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum, 1997. 40(9): p. 1725.
23. Tan, E.M., et al., The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum, 1982. 25(11): p. 1271-7.
24. Gladman, D.D., D. Ibanez, and M.B. Urowitz, Systemic lupus erythematosus disease activity index 2000. J Rheumatol, 2002. 29(2): p. 288-91.
25. The American College of Rheumatology nomenclature and case definitions for neuropsychiatric lupus syndromes. Arthritis Rheum, 1999. 42(4): p. 599-608.
26. Wilson, W.A., et al., International consensus statement on preliminary classification criteria for definite antiphospholipid syndrome: report of an international workshop. Arthritis Rheum, 1999. 42(7): p. 1309-11.
27. Katsumata, Y., et al., Anti-C1q antibodies are associated with systemic lupus erythematosus global activity but not specifically with nephritis: a controlled study of 126 consecutive patients. Arthritis Rheum, 2011. 63(8): p. 2436-44.
28. Orbai, A.M., et al., Anti-C1q antibodies in systemic lupus erythematosus. Lupus, 2015. 24(1): p. 42-9.
29. Sciascia, S., et al., Autoantibodies involved in neuropsychiatric manifestations associated with systemic lupus erythematosus: a systematic review. J Neurol, 2014. 261(9): p. 1706-14.
30. Zandman-Goddard, G., J. Chapman, and Y. Shoenfeld, Autoantibodies involved in neuropsychiatric SLE and antiphospholipid syndrome. Semin Arthritis Rheum, 2007. 36(5): p. 297-315.
31. Mehta, N., et al., Platelet C4d is associated with acute ischemic stroke and stroke severity. Stroke, 2008. 39(12): p. 3236-41.
32. Navratil, J.S., et al., Platelet C4d is highly specific for systemic lupus erythematosus. Arthritis Rheum, 2006. 54(2): p. 670-4.
33. Oku, K., et al., Complement activation in patients with primary antiphospholipid syndrome. Ann Rheum Dis, 2009. 68(6): p. 1030-5.
34. Davis, W.D. and R.L. Brey, Antiphospholipid antibodies and complement activation in patients with cerebral ischemia. Clin Exp Rheumatol, 1992. 10(5): p. 455-60.
35. Zirkzee, E.J., et al., Cluster analysis of an array of autoantibodies in neuropsychiatric systemic lupus erythematosus. J Rheumatol, 2014. 41(8): p. 1720-1.
36. Ceribelli, A., et al., Complement cascade in systemic lupus erythematosus: analyses of the three activation pathways. Ann N Y Acad Sci, 2009. 1173: p. 427-34.
37. Horak, P., et al., C1q complement component and -antibodies reflect SLE activity and kidney involvement. Clin Rheumatol, 2006. 25(4): p. 532-6.
38. Marto, N., et al., Anti-C1q antibodies in nephritis: correlation between titres and renal disease activity and positive predictive value in systemic lupus erythematosus. Ann Rheum Dis, 2005. 64(3): p. 444-8.
39. Moroni, G., et al., Are laboratory tests useful for monitoring the activity of lupus nephritis? A 6-year prospective study in a cohort of 228 patients with lupus nephritis. Ann Rheum Dis, 2009. 68(2): p. 234-7.
40. Yin, Y., et al., Diagnostic value of serum anti-C1q antibodies in patients with lupus nephritis: a meta-analysis. Lupus, 2012. 21(10): p. 1088-97.
41. Trouw, L.A., et al., Anti-C1q autoantibodies deposit in glomeruli but are only pathogenic in combination with glomerular C1q-containing immune complexes. J Clin Invest, 2004. 114(5): p. 679-88.

42. Trouw, L.A., et al., Anti-C1q autoantibodies in murine lupus nephritis. Clin Exp Immunol, 2004. 135(1): p. 41-8.
43. Chau, S.Y. and C.C. Mok, Factors predictive of corticosteroid psychosis in patients with systemic lupus erythematosus. Neurology, 2003. 61(1): p. 104-7.
44. Nishimura, K., et al., Blood-brain barrier damage as a risk factor for corticosteroid-induced psychiatric disorders in systemic lupus erythematosus. Psychoneuroendocrinology, 2008. 33(3): p. 395-403.
45. Nishimura, K., et al., New-onset psychiatric disorders after corticosteroid therapy in systemic lupus erythematosus: an observational case-series study. J Neurol, 2014. 261(11): p. 2150-8.
46. Mayilyan, K.R., D.R. Weinberger, and R.B. Sim, The complement system in schizophrenia. Drug News Perspect, 2008. 21(4): p. 200-10.
47. Wong, C.T., W.F. Tsoi, and N. Saha, Acute phase proteins in male Chinese schizophrenic patients in Singapore. Schizophr Res, 1996. 22(2): p. 165-71.
48. Hakobyan, S., A. Boyajyan, and R.B. Sim, Classical pathway complement activity in schizophrenia. Neurosci Lett, 2005. 374(1): p. 35-7.
49. Francks, C., et al., Population-based linkage analysis of schizophrenia and bipolar case-control cohorts identifies a potential susceptibility locus on 19q13. Mol Psychiatry, 2010. 15(3): p. 319-25.
50. Ni, J., et al., A Preliminary Genetic Analysis of Complement 3 Gene and Schizophrenia. PLoS One, 2015. 10(8): p. e0136372.
51. Pego-Reigosa, J.M. and D.A. Isenberg, Psychosis due to systemic lupus erythematosus: characteristics and long-term outcome of this rare manifestation of the disease. Rheumatology (Oxford), 2008. 47(10): p. 1498-502.
52. Watanabe, T., et al., Neuropsychiatric manifestations in patients with systemic lupus erythematosus: diagnostic and predictive value of longitudinal examination of anti-ribosomal P antibody. Lupus, 1996. 5(3): p. 178-83.
53. Howell, G.R., et al., Molecular clustering identifies complement and endothelin induction as early events in a mouse model of glaucoma. J Clin Invest, 2011. 121(4): p. 1429-44.
54. Rosen, A.M. and B. Stevens, The role of the classical complement cascade in synapse loss during development and glaucoma. Adv Exp Med Biol, 2010. 703: p. 75-93.
55. Stephan, A.H., et al., A dramatic increase of C1q protein in the CNS during normal aging. J Neurosci, 2013. 33(33): p. 13460-74.
56. Schafer, D.P., et al., Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. Neuron, 2012. 74(4): p. 691-705.
57. Selkoe, D.J., Alzheimer's disease is a synaptic failure. Science, 2002. 298(5594): p. 789-91.
58. Michailidou, I., et al., Complement C1q-C3-associated synaptic changes in multiple sclerosis hippocampus. Ann Neurol, 2015. 77(6): p. 1007-26.
59. Magro Checa, C., et al., Demyelinating disease in SLE: is it multiple sclerosis or lupus? Best Pract Res Clin Rheumatol, 2013. 27(3): p. 405-24.
60. Sturfelt, G. and A.G. Sjoholm, Complement components, complement activation, and acute phase response in systemic lupus erythematosus. Int Arch Allergy Appl Immunol, 1984. 75(1): p. 75-83.
61. Morgan, B.P., et al., The role of complement in disorders of the nervous system. Immunopharmacology, 1997. 38(1-2): p. 43-50.
62. Thomas, A., et al., Expression of a complete and functional complement system by human neuronal cells in vitro. Int Immunol, 2000. 12(7): p. 1015-23.
63. Daha, N.A., et al., Complement activation by (auto-) antibodies. Mol Immunol, 2011. 48(14): p. 1656-65.

Part II

The local production of C1q by immune and non-immune cells



Chapter 6

The production and secretion of complement component C1q by human mast cells

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Abstract

C1q is the initiation molecule of the classical pathway of the complement system and is produced by macrophages and immature dendritic cells. As mast cells share the same myeloid progenitor cells, we have studied whether also mast cells can produce and secrete C1q.
Mast cells were generated in vitro from CD34+ progenitor cells from buffy coats or cord blood. Fully differentiated mast cells were shown by both RNA sequencing and qPCR to express C1QA, C1QB and C1QC. C1q produced by mast cells has a similar molecular make-up as serum C1q. Reconstituting C1q depleted serum with mast cell supernatant in haemolytic assays, indicated that C1q secreted by mast cells is functionally active. The level of C1q in supernatants produced under basal conditions was considerably enhanced upon stimulation with LPS, dexamethasone in combination with IFN- γ or via FcεRI triggering. Mast cells in human tissues stained positive for C1q in both healthy and in inflamed tissue. Moreover, mast cells in healthy and diseased skin appear to be the predominant C1q positive cells.
Together, our data reveal that mast cells are able to produce and secrete functional active C1q and indicate mast cells as a local source of C1q in human tissue.

Introduction

Mast cells (MCs) are immune cells that are important in the first line of defence and are predominately present in tissues that are close to host/environment interfaces, like the skin and mucosal surfaces. MCs are important in host defence, innate and adaptive immunity, tissue homeostasis and immune regulation [1-3]. As effector cell in disease, MCs are well known for their anaphylactic effects in allergy. MCs are also described to play a role in other diseases like atherosclerosis, contact dermatitis, cancer and arthritis [4-7].
MCs can be activated via IgE receptor (FcεRI) cross-linking, resulting in degranulation of the MCs, but they can also be activated via other pathways. These pathways include activation via the C3a and C5a receptors resulting in degranulation, secretion of cytokines, chemokines and bioactive lipids [8, 9].
C1q is the first complement component in the classical pathway of the complement system [10]. Together with C1r and C1s it forms the C1 complex. C1q plays a prominent role in the clearance of immune complexes and by binding to apoptotic and necrotic cells and it can facilitate phagocytosis [11-13]. The interaction of C1q with immunoglobulins is of importance to protect against infections but it may also

contribute to tissue damage by targeting self-tissue [14].
The main producers of C1q have been reported to be macrophages and immature dendritic cells [15-18]. Also other studies have reported that other non-macrophage-like cells are able to produce C1q like trophoblast cells [19]. MCs are differentiated from the same myeloid precursor cells that can also give rise to macrophages and dendritic cells [20]. We have investigated whether also MCs can produce and secrete functionally active C1q as this would imply MCs to have a role in tissue homeostasis and innate immune defence via C1q.

Materials & Methods

MCs culture

CD34+ hematopoietic stem cells were isolated from either peripheral blood mononuclear cells or from mononuclear cells present in cord blood. Mononuclear cells were isolated from buffy coats (Sanquin, The Netherlands) or cord blood using Ficoll-Paque density gradient centrifugation. CD34+ hematopoietic stem cells were isolated from the PBMC's with CD34+ microbeads (Miltenyi Biotec, The Netherlands). The isolated CD34+ stem cells were differentiated into MCs by culturing the cells in culture medium as described before [21]. Heparinized cord blood was obtained through the department of Obstetrics of the Leiden University Medical Center (Leiden, The Netherlands), in accordance with the Declaration of Helsinki. CD34+ cells isolated from cord blood were cultured as described previously [22] [23]. After 8-9weeks the purity of the MCs was determined using Flow Cytometry, whereby CD117 (c-kit), FcεRI and CD203c were measured.

ELISA

MCs were cultured in a cell concentration of 2*106 cells/mL for 72h either in medium alone, or stimulated to increase C1q levels with LPS (Sigma, 200 ng/ml), Dexamethasone (Pharmacy LUMC, Leiden, The Netherlands, 10 μM) + IFN-γ (Peprotech, 200 U/mL). For FcεRI triggering, the MCs were sensitized with hybridoma IgE (non-immune, BPD-DIA-HE1-1, Enzo, Life Sciences, 0.1 μg/ml) overnight. Next, the MCs were washed to remove soluble IgE and activated using goat anti-human IgE (Nordic-MUbio, Susteren, The Netherlands, 10 μg/ml). Subsequently, culture supernatants were harvested and analysed by ELISA to determine C1q levels as described before [24].
IL-8 production was evaluated using the human IL-8 ready-set-Go!® (2nd generation) ELISA (eBioscience).

Haemolytic reconstitution assay

MCs were cultured for 72 with or without LPS (200 ng/ml). After 72h, The supernatant was harvested and used for a haemolytic reconstitution assay. Antibody opsonized sheep erythrocytes were prepared in sucrose containing veronal buffer and incubated with C1q-depleted serum (Quidel) diluted in veronal gelatin containing buffer and different dilutions of MC supernatant or different concentrations purified C1q (Quidel) as a positive control and as a negative control culture medium with or without LPS was used. After incubation the amount of lysis was measured compared to total lysis (sheep erythrocytes together with saponine and water) at 412 nm. The amount of lysis is corrected to medium control [25].

qPCR of C1qA, C1qB and C1qC

RNA was isolated from cultures mast cells after 7-8 weeks of cultured. The qPCR was performed as described previously [24]. Relative mRNA expression was calculated using the reference gene RPL5. The qPCR products were analyzed on a 2% agarose gel and visualized with Nancy 520 (Sigma).

Sequencing

RNA was isolated from cord blood derived mast cells (CBMC) from three independent donors. RNA was paired-end sequenced with Illumina Hiseq 2000, aligned to Hg38 human genome reference. Read were quantified per gene and RPKM (Reads Per Kilobase per Million) levels were calculated using EdgeR (as previously described Suurmond et al) [26].

Western blot analysis of C1q

Using western blot the composition of C1q was examined by detection of the three chains of the C1q protein. Supernatants of stimulated and unstimulated MCs were used in reduced and non-reduced SDS conditions. The western blot was performed using previously described methods [24].

ß-hexosaminidase release assay

In this assay the MCs were sensitized with IgE as described above in a cell concentration of 0.5 x 106 cells/ml in an 96-wells plate on 37°C/ 5% CO2. The next day, the cells were washed with Tyrode's buffer to clear unbound IgE and stimulated with mouse anti-human IgE or with compound 48/80 (Sigma, 50 µg/ml) for 15 minutes or with buffer only as a control. Triton-X100 (1%) was used as indicator of 100% degranulation. After incubation the supernatant was incubated at 37°C with substrate (2 mM 4-Nitrophenyl N-acetyl-b-D-glucosaminide, Sigma N9376). The reaction was stopped using stop solution (0.2 M Glycine, pH 10.7) after 1-hour incubation and extinction was measured at 405 nm.

Immunofluorescence staining on cytospins and human tissue

For the detection of C1q positive MCs in tissues, paraffin embedded skin tissue, psoriatic skin and synovial tissue of rheumatoid arthritis (RA) patients (5µm) was used. After treating the slides via standard methods to deparaffinise, tryptase and C1q were visualized using mouse anti-tryptase (Millipore) and rabbit anti-C1q (DAKO) or matching isotypes for 1 hour at RT. After washing the detection antibodies goat anti-rabbit ALEXA488 (Invitrogen) and donkey anti-mouse ALEXA568 (Invitrogen) were added to the slides for one hour at RT. After incubation the slides were washed and dried. Finally, the slides were covered with Vectashield containing DAPI (Vector laboratories) to stain nuclei and analysed on a Zeiss Axio ScopeA1 microscope and on a Confocal microscope (Leica SP8 confocal). Using cytospins, cultured MCs were pelleted onto poly-lysine coated microscope slides (Thermo scientific/Menzel-Glaser) in a cell concentration of 50.000 cells/slide and stained for immunofluorescence with minor adjustments. All participants in the study provided informed consent and the study was approved by the local medical ethics committee.

Stained sections were randomly analysed of three different slides per condition. The mean number and standard deviation in percentages of single- and double-positive cells in 10 high-power fields (magnification 400x) was scored blindly by three observers.

Statistical analysis

Statistical analysis on the ELISA data was performed using a Wilcoxon signed rank test. P-value's <0.05 were considered significant (Graphpad Prism software version 5.01).

Results

Cultured MCs spontaneously produce functionally active C1q

Mast cells were in-vitro differentiated from CD34-positive precursors. After 7-8 weeks of culture, the purity of the MCs population was determined by staining for CD117 (c-kit), FcεRI and CD203c, and was shown to range from 90% to 98% (Figure 1A). The expression of the mRNA's encoding the three C1q chains by unstimulated MCs was analysed by PCR. We could detect the expression of C1QA, C1QB and C1QC, all required to generate a functional C1q molecule (Figure 1B). With qPCR we analysed expression level of C1qA in PBMCs and MCs. Here we observe that PBMCs have a higher RNA expression level of C1qA compared to MCs (Figure 1C).

The expression of mRNA was confirmed using sequencing RNA extracted from CBMC. The RPKM values indicate that the expression of C1q genes in CBMCs are relatively high with an RPKM value >100 and only a 3 fold lower expression than the household gene RPL5. These data provide additional and independent evidence that at basal levels C1q is expressed by MCs (Figure 1D). Additionally, expression of C1q related genes were analysed on unstimulated CBMCs using RNA sequencing. This analysis demonstrates that MCs express several C1q receptors (Table 1). The C1q associated serine proteases C1r and C1s and also the inhibitor C1-INH show a low expression in CBMCs (Table 1).

To demonstrate that MCs are able to produce C1q, we performed a sandwich-ELISA for C1q using culture supernatant obtained from MCs cultures. In a time frame of 72 hours unstimulated MCs are able to secrete C1q as C1q was readily detectable in culture supernatants; mean 11.34 ng/ml; range 3.08-24.6 ng/ml (Figure 1E). To further confirm that mast cells produce C1q and to rule out the possibility that a few contaminating cells would be responsible for the C1q mRNA and C1q protein observed, we next performed a cytospin of cultured cells and analysed the presence of intracellular C1q. As depicted in figure 1E, all cells stained positive for tryptase (Figure 1F). In addition also a staining for C1q revealed that all cells stained positive for C1q compared to isotype controls (Figure 1F). This indicates that not contaminating cells but rather MCs are responsible for C1q production. Using western blot analysis we observed that C1q secreted from unstimulated MCs derived from either peripheral blood and cord blood contain all three C1q-polypeptides. These polypeptides display the same size as compared to C1q present in NHS (C1qA: 28 kDa, C1qB: 25 kDa, C1qC: 24 kDa). Likewise, the size of the secreted C1q protein is identical to C1q from NHS (Figure 1G). These results indicated that MCs are able to produce and secrete a complete C1q protein.

Next, we wished to examine whether C1q secreted by MCs is functionally active and is able to restore the activity of the classical pathway in C1q-depleted serum. Therefore we used a reconstitution experiment based on a haemolytic assay for the classical pathway. Antibody opsonized sheep erythrocytes were incubated with C1q depleted serum to which we added either buffer, unconditioned culture medium or culture medium in which mast cells had been cultured for three days. Whereas incubation with C1q depleted serum did not result in lysis of erythrocytes a limited degree of lysis was observed when culture supernatant of unstimulated mast cells was used. A substantial lysis was observed when culture supernatant of mast cells, stimulated with LPS to increase the C1q production, was used (Figure 1H). Overall these data demonstrate that C1q produced by mast cells is functionally active.

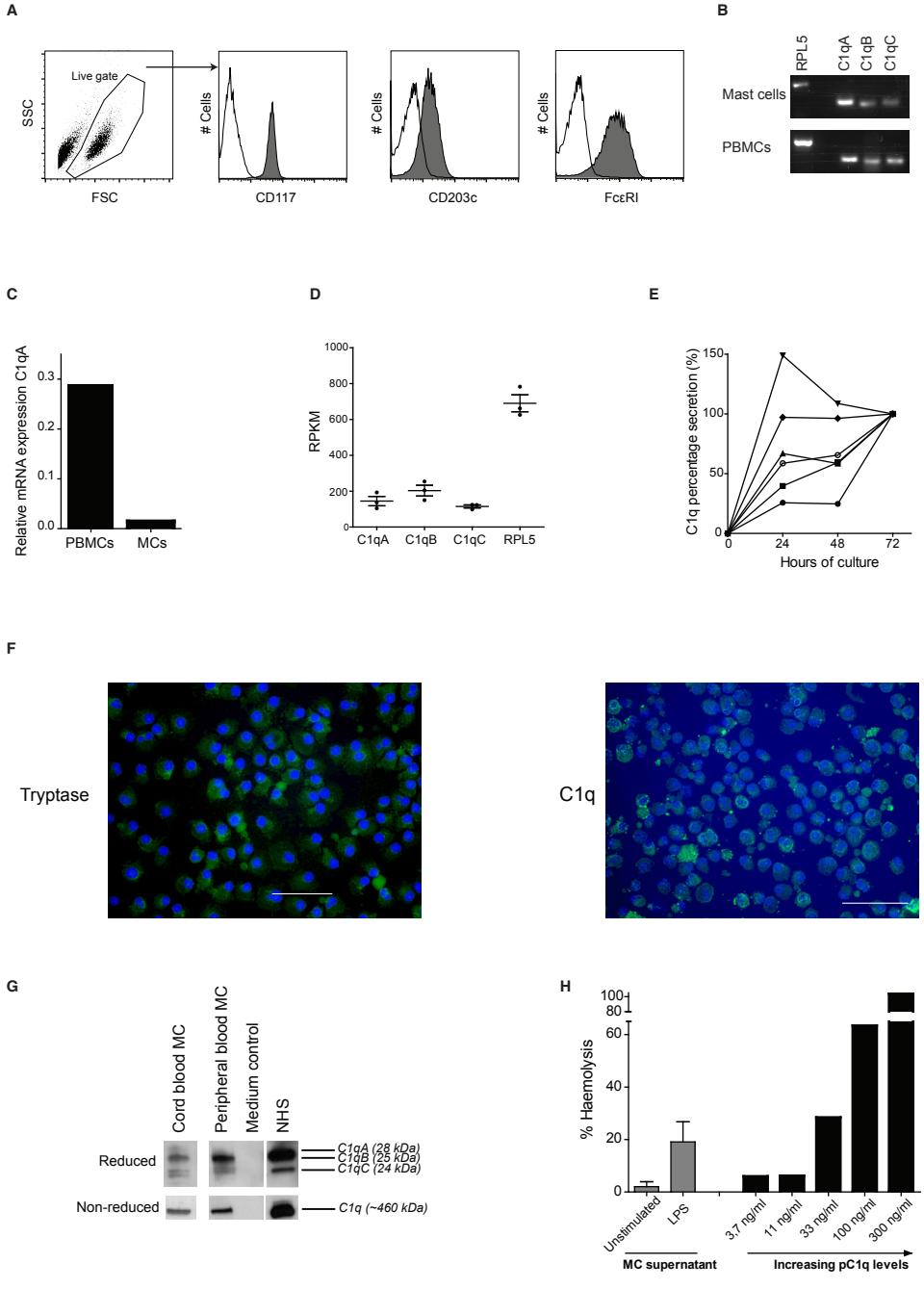

Figure 1. Production of C1q by cultured MCs.

A. Representative flow cytometric plots of characterization of MCs after 8-9 weeks of culture using CD117, CD203c and FcεRI staining on the cell surface. Histograms gated on live cells on forward scatter (FSC) and side scatter (SSC) Open histograms represents isotype controls, closed histograms represents the stainings. **B.** qPCR products from unstimulated MCs were analysed on a 2% agarose gel.

As positive control for C1qA, C1qB and C1qC DXM + IFN-γ stimulated PBMCs were used. RPL5 is used as housekeeping gene. **C.** Relative mRNA expression of C1qA in PBMCs and MCs. Data is relative to the house keeping gene RPL5 (N=1). **D.** Gene expression profile of C1q from three independent cord-blood derived mast cell donors. X axis depicts the three genes of C1q as well as RPL5. On the Y-axis, gene expression levels in reads per kilobase per million (RPKM) are depicted. **E.** C1q production during a time course of 72h (set at 100%) was analysed by ELISA (N = 6 different MCs donors). **F.** Cytospin staining of tryptase and DAPI together with matching isotype control of C1q (Rabbit Ig, ALEXA 568) on cultured peripheral MCs (400x magnification) and a of C1q and DAPI with matching isotype control of tryptase (Ms IgG1, ALEXA 568) on cytospins (400x magnification). Scale bars indicates 50 μm. **G.** Reduced and non-reduced supernatant of cultured MCs from peripheral blood and from cord blood was analysed on western blot. **H.** Haemolytic reconstitution assay of the classical pathway by adding MC supernatant to C1q depleted serum with the % heamolysis compared to a 100% lysis control as read-out (N=3) expressed as mean and standard deviation. As a positive control purified C1q ranging from 3,7 ng/ml to 300 ng/ml was used.

Gene	Conventional name	Mean RPKM	±SD
C1QA	C1qA	137.5	39.4
C1QB	C1qB	210.5	45.1
C1qC	C1qC	104.8	23.8
C1R	C1r	1.5	0.6
C1S	C1s	5	0.8
SERPING1	C1-INH	1.8	1
C1QBP	gC1qR	89.3	38.8
CALR	cC1qR	484	190.8
ITGB1	CD29	63.8	13.2
ITGA2	CD49B	4.5	1.7
LAIR1	CD305	15.5	7

Table 1. Gene expression levels of complement components in unstimulated CBMCs expressed in Reads Per Kilobase per Million (RPKM)

C1q production is upregulated after MC stimulation but does not correlate with IL-8 production or degranulation

We next wished to determine whether stimulation of MCs by triggers other than LPS could also alter C1q production by MCs. For this purpose, we stimulated MCs with LPS, DXM + IFN-γ or α-IgE for 72 hours. DXM in combination with IFN-γ are well-known stimulations to increase the C1q production in myeloid cells as described before for THP-1 derived macrophages [27]. All triggers enhanced C1q production as detected in culture supernatants harvested after 72 hours of stimulation (Figure 2A-C). Similar results were obtained using cord blood derived MCs (Figure 2D). However, IL-8 production, a prototype chemokine released by MCs was not increased upon triggering of MCs with DXM + IFN-γ (Figure 2E), indicating that C1q and IL-8 production are differentially regulated upon this stimulation.

MCs are well-known for their ability to release granules, containing different compounds e.g. ß-hexosaminidase, tryptase, chymase and histamine [28].

Therefore we were interested whether C1q is released after MC degranulation. To this end, we performed a degranulation assay, by stimulating the cells via the FcεRI or compound 48/80, two well-known triggers for degranulation [29, 30]. As a marker of degranulation we measured the release of ß-hexosaminidase next to C1q. Following FcεRI triggering for 15 minutes, ß-hexosaminidase was readily detected in the supernatants, whereas only a low level of C1q was present. (Figure 2F). However, after stimulation with compound 48/80 for 15 minutes a different pattern was noted as a secretion of 40% of C1q is seen (Figure 2G). These observations suggest that C1q is present in preformed vesicles of MCs and is released upon compound 48/80 triggering.

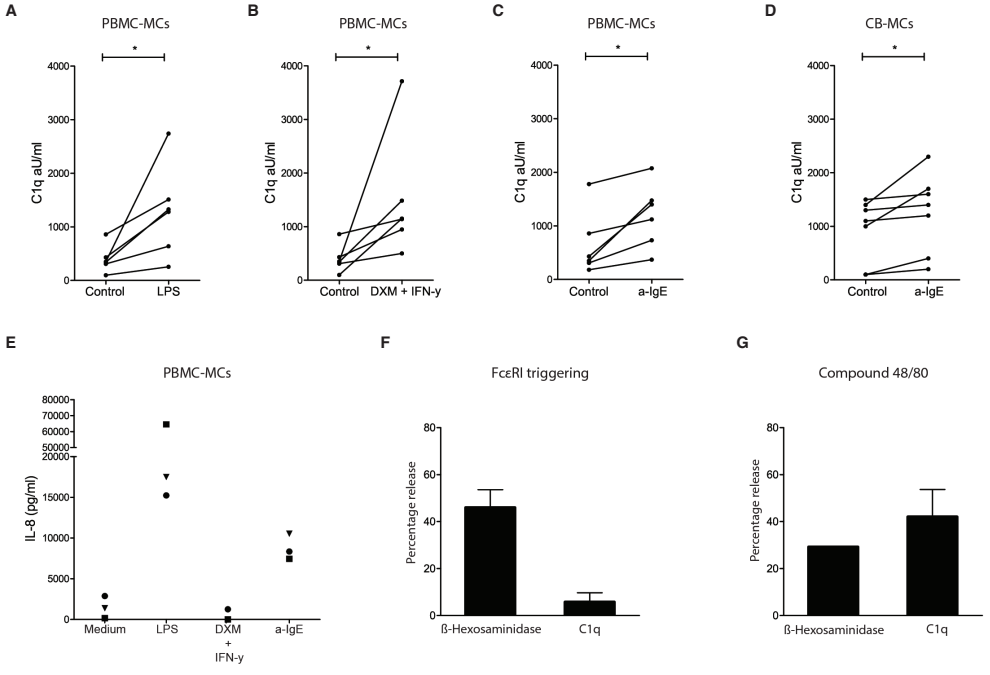

Figure 2. C1q production upon stimulation of MCs.

A. C1q production measured by ELISA in the supernatant of stimulated peripheral MCs for 72 h with LPS. **B.** DXM + IFN-γ. **C.** α-IgE. **D.** Stimulation of cord blood derived MCs with α-IgE. **E.** IL-8 ELISA on supernatant of peripheral MCs (N=3) after stimulation with LPS, DXM+IFN-γ and α-IgE. **F.** Percentages ß-hexosaminidase and C1q release assay after 15 min incubation of α-IgE defined by total release with Triton X100. **G.** Percentages ß-hexosaminidase and C1q release after 15 minutes of stimulation with compound 48/80 defined by total release with Triton X100.

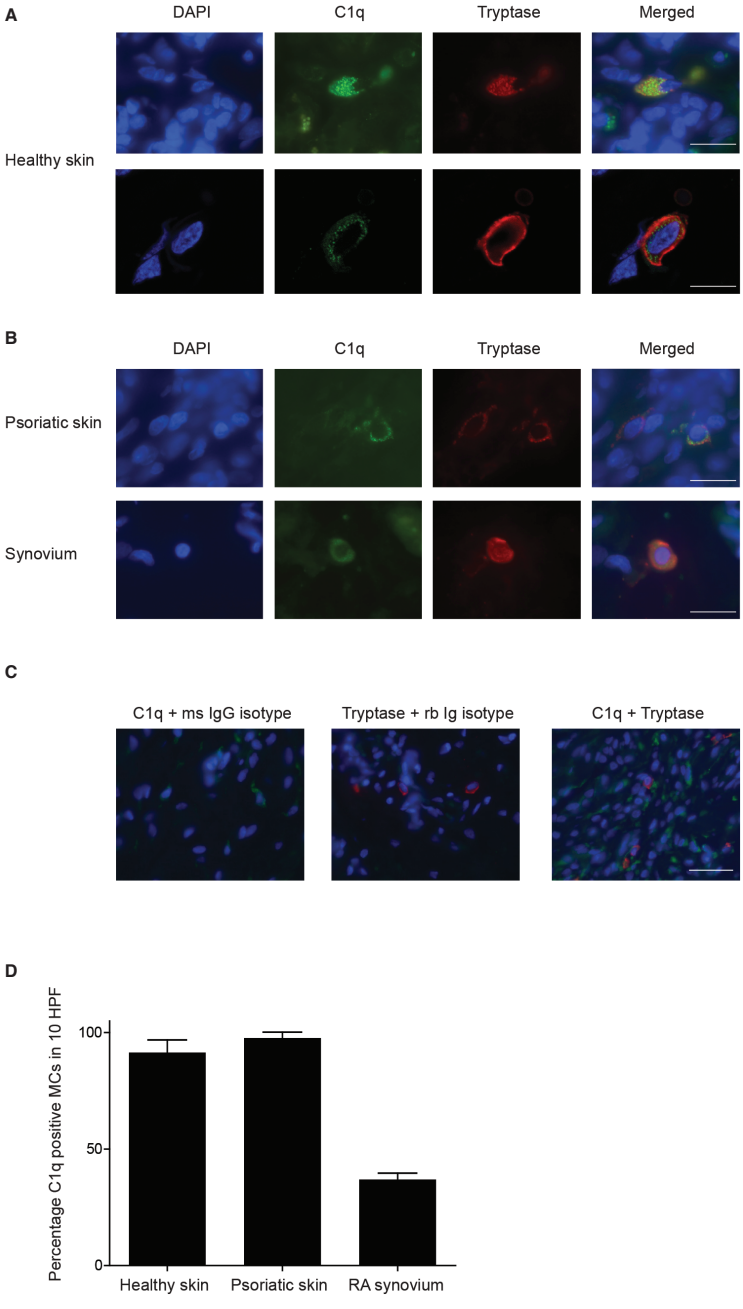

Figure 3. Distribution of C1q positive MCs in different tissue.

A. C1q and tryptase staining on healthy skin analysed on a conventional fluorescence microscope (upper images, 1000x magnification, scale bar is 10 µm) and on a confocal microscope (lower images, digital zoom 3000x magnification, scale bar is 3,3 µm). **B.** C1q and tryptase staining on psoriatic skin and on RA synovium (conventional microscope, 1000x magnification, scale bar is 10 µm). **C.** single staining C1q (green) with isotype control for tryptase (red), single staining tryptase (red) with isotype

control for C1q (green), double staining C1q (green), tryptase (red) on RA synovium. Conventional fluorescence microscope, 400x magnification. Scale bar is 50 µm **D.** Quantified percentages in 10 high power fields (HPF) of C1q positive mast cells in healthy skin (N=3), psoriatic skin (N=3) and RA synovium (N=3) expressed in mean and standard deviation.

C1q producing MCs are present in various tissues

ToW determine whether MCs present in tissue are positive for C1q we performed an immunofluoresence staining on healthy skin tissue using tryptase as a MC marker. As shown in figure 3A, tryptase positive MCs also express C1q as detected by immunofluorescence (Figure 3A), indicating that also tissue resident MCs express C1q (C1q positive MCs 91% ± 5%).

MCs have been indicated to play a role in several diseases. We wished to analyse whether MCs in diseased tissue express C1q. To this end, psoriatic skin and synovial tissue from RA patients was analysed for the presence of C1q positive MCs. As shown in figure 3B, all tryptase expressing cells in psoriatic skin are also positive for C1q (C1q positive MCs 98% ± 2%), resembling our observations made in the healthy skin. Likewise, in synovial tissue, tryptase positive MCs expressing C1q were present. However, in contrast to healthy and psoriatic skin, not all tryptase positive cells expressed C1q (C1q positive MCs 37% ± 3%). Moreover, also tryptase negative cells expressing C1q are observed in RA synovial tissue, indicating the presence of other C1q positive cells like macrophages and dendritic cells (Figure 3C).

As shown in figure 2G C1q is released via degranulation. As shown in Figure 3A-C, C1q is stained in a granular pattern in all C1q positive mast cells in different tissues. Together, these results indicate that tissue-resident mast cells, analysed directly ex vivo in healthy skin, psoriatic skin and synovial tissue, stain positive for C1q, and in particular in the skin, are the main cell C1q-positive cell subset.

Discussion

Macrophages and immature dendritic cells have been described as the main producers of C1q [15, 16]. Because MCs are derived from myeloid precursors cells, which also give rise to macrophages and dendritic cells, we wished to determine if MCs are able to produce C1q. Here, we demonstrate that unstimulated MCs produce and secrete functional C1q. Moreover, our data show that C1q production by mast cells can be modulated by several immune stimuli. The amount of C1q produced by mast cells (mean 11.34 ng/ml; range 3.08-24.6 ng/ml) is lower compared to published values of macrophages (64.6 ng/mL; range, 2-148 ng/

mL, after 48h culture) and dendritic cells (318.4 ng/mL; range, 100-679 ng/mL, after 48h culture) [15] and also as compared to our own analysis (data not shown). To investigate if the secretion of C1q is differently regulated from secretion of cytokines produced by MCs, we compared IL-8 secretion, a prototype cytokine produced by MCs, with C1q secretion after stimulation. We observed that stimulation of MCs by LPS and FcεRI cross-linking induced both C1q- and IL-8 release whereas stimulation by DXM + IFN-γ induced only C1q secretion. These results are intriguing as they suggest that C1q and IL-8 production are differentially regulated and that C1q production is also boosted in situations that MCs are not primed for IL-8 production. It is conceivable that this is important in the case of, for example, apoptotic cell clearance. C1q can bind to apoptotic cells and promote ingestion of apoptotic cells by macrophages [12, 13, 31]. The clearance of apoptotic cells is of particular importance in tissues, where apoptosis is abundant because of normal tissue turnover/homeostasis such as in the skin. In this process, infiltration of inflammatory cells would not be desirable. MCs producing C1q can contribute to this process to maintain tissue homeostasis by the production of C1q without the concomitant production of inflammatory cytokines. We did not observe a clear effect of C1q stimulating MCs (data not shown).

Another possible role of C1q is promoting wound healing. As shown in mouse studies, C1q can be deposited on endothelial cells, leading to the enhancement of permeability, tube formation and angiogenesis [32]. It is conceivable that also under these conditions, the release of inflammatory cytokines is not beneficial and that C1q produced by MCs contributes to angiogenesis and normal tissue repair in situations where the production of inflammatory cytokines is less desired.

In our ELISA analysis we observed a spontaneous production of C1q by unstimulated MCs. Likewise, in human tissue we observed a granular type of staining of C1q in MCs suggesting that C1q is also present in the granules of the MCs. Indeed, degranulation using compound 48/80 led to an increase in C1q release, which coincided with the release of ß-hexosaminidase in supernatants suggesting that C1q can also be produced upon granule release. Furthermore, we did not obtain any evidence that C1q is expressed on the cell surface of MCs (data not shown).

In the immunofluorescence staining of C1q positive mast cells is a granular pattern seen. In addition of production of C1q by mast cells, another feature what we have to take into account is the possibility of the uptake of C1q from the extracellular milieu and store them in granules [33].

A limitation of this study is the lack of an in vivo model to define the role of C1q produced by MCs in healthy and disease conditions as mouse MCs do not appear to produce C1q. Using an ELISA, we could not detect C1q in the supernatant of

bone marrow derived mouse MCs after the same stimulation as used for the human MCs. In the same assay, we did detect C1q in the supernatant of bone marrow derived macrophages and dendritic cells from the mouse (data not shown). It is therefore difficult to translate our results using human cells and tissue in an animal model for further studies aiming to analyse in vivo relevance.
Together, we show that MCs are able to produce and secrete functionally active C1q and that MCs in healthy and in diseased human tissue are positive for C1q. Our findings suggest that C1q from MCs could have a previously unrecognized contribution in several processes such as inflammation, clearing of immune complexes, apoptotic and necrotic cells. Especially in human skin, tryptase positive cells are the most abundant C1q expressing cells which makes it tempting to speculate that C1q produced by MCs is playing an important role in tissue homeostasis and defence.

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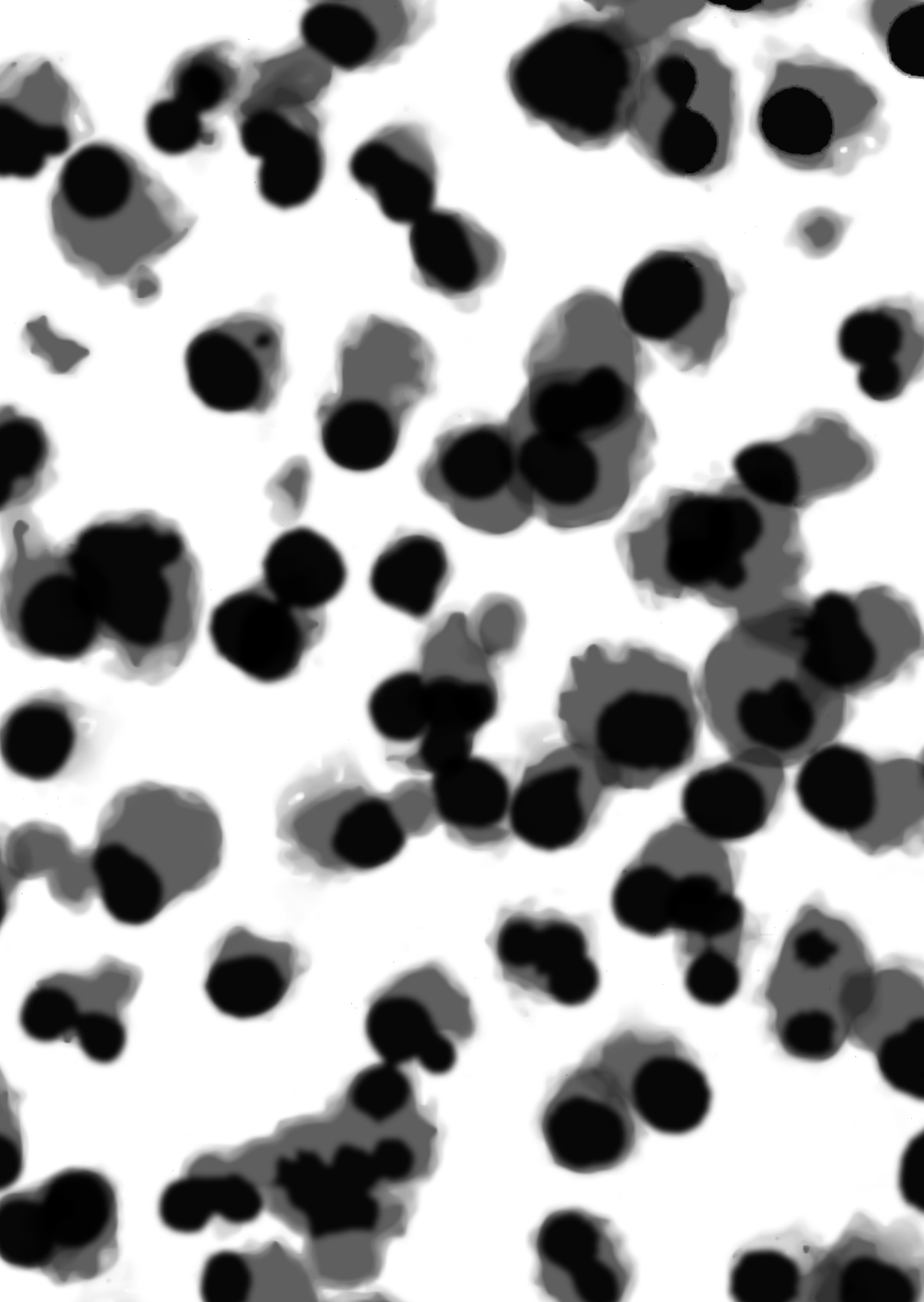
Reference list

1. Galli, S.J., et al., Mast cells as "tunable" effector and immunoregulatory cells: recent advances. Annu Rev Immunol, 2005. 23: p. 749-86.
2. Galli, S.J., S. Nakae, and M. Tsai, Mast cells in the development of adaptive immune responses. Nat Immunol, 2005. 6(2): p. 135-42.
3. Voehringer, D., Protective and pathological roles of mast cells and basophils. Nat Rev Immunol, 2013. 13(5): p. 362-75.
4. Bot, I. and E.A. Biessen, Mast cells in atherosclerosis. Thromb Haemost, 2011. 106(5): p. 820-6.
5. Ribatti, D. and E. Crivellato, Mast cells, angiogenesis and cancer. Adv Exp Med Biol, 2011. 716: p. 270-88.
6. Woolley, D.E., The mast cell in inflammatory arthritis. N Engl J Med, 2003. 348(17): p. 1709-11.
7. Vocanson, M., et al., Effector and regulatory mechanisms in allergic contact dermatitis. Allergy, 2009. 64(12): p. 1699-714.
8. Johnson, A.R., T.E. Hugli, and H.J. Muller-Eberhard, Release of histamine from rat mast cells by the complement peptides C3a and C5a. Immunology, 1975. 28(6): p. 1067-80.
9. Klos, A., et al., The role of the anaphylatoxins in health and disease. Mol Immunol, 2009. 46(14): p. 2753-66.
10. Daha, N.A., et al., Complement activation by (auto-) antibodies. Mol.Immunol., 2011. 48(14): p. 1656-1665.
11. Fraser, D.A., et al., C1q differentially modulates phagocytosis and cytokine responses during ingestion of apoptotic cells by human monocytes, macrophages, and dendritic cells. J.Immunol., 2009. 183(10): p. 6175-6185.
12. Korb, L.C. and J.M. Ahearn, C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited. J.Immunol., 1997. 158(10): p. 4525-4528.
13. Nauta, A.J., et al., Direct binding of C1q to apoptotic cells and cell blebs induces complement activation. Eur.J.Immunol., 2002. 32(6): p. 1726-1736.
14. Beurskens, F.J., R.A. van Schaarenburg, and L.A. Trouw, C1q, antibodies and anti-C1q autoantibodies. Mol Immunol, 2015.
15. Castellano, G., et al., Maturation of dendritic cells abrogates C1q production in vivo and in vitro. Blood, 2004. 103(10): p. 3813-20.
16. Loos, M., H. Martin, and F. Petry, The biosynthesis of C1q, the collagen-like and Fc-recognizing molecule of the complement system. Behring Inst.Mitt., 1989(84): p. 32-41.
17. Castellano, G., et al., Infiltrating dendritic cells contribute to local synthesis of C1q in murine and human lupus nephritis. Mol Immunol, 2010. 47(11-12): p. 2129-37.
18. Faust, D. and M. Loos, In vitro modulation of C1q mRNA expression and secretion by interleukin-1, interleukin-6, and interferon-gamma in resident and stimulated murine peritoneal macrophages. Immunobiology, 2002. 206(4): p. 368-76.
19. Agostinis, C., et al., An alternative role of C1q in cell migration and tissue remodeling: contribution to trophoblast invasion and placental development. J Immunol, 2010. 185(7): p. 4420-9.
20. Galli, S.J., N. Borregaard, and T.A. Wynn, Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils. Nat Immunol, 2011. 12(11): p. 1035-44.
21. Suurmond, J., et al., Communication between human mast cells and CD4(+) T cells through antigen-dependent interactions. Eur J Immunol, 2013. 43(7): p. 1758-68.
22. Radinger, M., et al., Generation, isolation, and maintenance of human mast cells and mast cell lines derived from peripheral blood or cord blood. Curr Protoc Immunol, 2010. Chapter 7: p. Unit 7 37.

23. Kirshenbaum, A.S., et al., Demonstration that human mast cells arise from a progenitor cell population that is CD34(+), c-kit(+), and expresses aminopeptidase N (CD13). Blood, 1999. 94(7): p. 2333-42.
24. van Schaarenburg, R.A., et al., Identification of a novel non-coding mutation in C1qB in a Dutch child with C1q deficiency associated with recurrent infections. Immunobiology, 2015. 220(3): p. 422-7.
25. Wouters, D., et al., Studies on the haemolytic activity of circulating C1q-C3/C4 complexes. Mol Immunol, 2008. 45(7): p. 1893-9.
26. Suurmond, J., et al., Repeated FcepsilonRI triggering reveals modified mast cell function related to chronic allergic responses in tissue. J Allergy Clin Immunol, 2016.
27. Walker, D.G., Expression and regulation of complement C1q by human THP-1-derived macrophages. Mol.Chem.Neuropathol., 1998. 34(2-3): p. 197-218.
28. Wernersson, S. and G. Pejler, Mast cell secretory granules: armed for battle. Nat Rev Immunol, 2014. 14(7): p. 478-94.
29. Kraft, S. and J.P. Kinet, New developments in FcepsilonRI regulation, function and inhibition. Nat Rev Immunol, 2007. 7(5): p. 365-78.
30. Yu, Y., et al., Non-IgE mediated mast cell activation. Eur J Pharmacol, 2015.
31. Ogden, C.A., et al., C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. J Exp Med, 2001. 194(6): p. 781-95.
32. Bossi, F., et al., C1q as a unique player in angiogenesis with therapeutic implication in wound healing. Proc Natl Acad Sci U S A, 2014. 111(11): p. 4209-14.
33. Olszewski, M.B., et al., TNF trafficking to human mast cell granules: mature chain-dependent endocytosis. J Immunol, 2007. 178(9): p. 5701-9.



Chapter 7

Human chondrocytes produce and secrete C1q

Manuscript in preparation

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Abstract

Chondrocytes, the cellular constituents of cartilage, play an important role in the cartilage by producing extracellular matrix molecules. During inflammation both chondrocytes and cartilage can be damaged. Previous studies have shown that chondrocytes express mRNA encoding for C1q. This is surprising as it is currently unclear what the role of such a large molecule is in the cartilage. In this study we demonstrate that C1q is produced and secreted by chondrocytes.
Using western blot and ELISA we analysed the presence of C1q in cultured chondrocytes. By stimulation with pro-inflammatory cytokines we examined the changes in mRNA expression of matrix molecules and of C1q.
Ex-vivo isolated human chondrocytes express mRNA for the three C1q chains. Cultured primary chondrocytes stain positive for C1q and secrete C1q into the culture supernatant. Upon stimulation with pro-inflammatory cytokines, the C1q mRNA expression is upregulated. Staining human cartilage sections for C1q revealed that in-situ chondrocytes are positive for C1q.
In this study we demonstrate that C1q can be produced and secreted by chondrocytes under basal conditions, which can be enhanced by pro-inflammatory stimuli.

Introduction

Chondrocytes are the only cells present in the healthy cartilage matrix. They are responsible for the synthesis and turnover of the extracellular cellular matrix (ECM). Chondrocytes are round cells originating from mesenchymal stem cells that represent 5-10% of the cartilage volume located in matrix cavities called lacunae. The two major ECM macromolecules produced by chondrocytes are collagen type 2 and aggrecan [1].
The complement system is an important part of the innate immune defence, which is able to kill pathogens and remove dying cells and immune complexes. The complement system can be activated via three pathways; the classical pathway, the lectin pathway and the alternative pathway. C1q is the recognition molecule of the classical pathway and together with the proteases C1r and C1s it forms the C1 complex. C1q can activate the classical pathway by binding different ligands such as IgG and IgM antibodies, but also DNA, C-reactive protein (CRP) and lipopolysaccharides [2-4]. C1q producing cells are mainly originating from the haematopoietic stem cells, such as macrophages, immature dendritic cells

and mast cells [4-7]. However, other reports suggest that trophoblasts, decidual endothelial cells, microglia and osteoclasts can also produce C1q [8-10].
Previously studies describe that matrix molecules like aggrecan, fibromodulin and osteoadherin are able to bind C1q and are able to activate the classical pathway of the complement system [11-13]. The cartilage oligomeric matrix protein (COMP) is a strong ligand for C1q, but it is hypothesised that COMP interferes with the binding site of C1r and C1s resulting in inhibition of the classical pathway [14]. This interference is also applies to the cartilage fragments decorin and biglycan [15]. Based on these interesting interactions between C1q and several matrix molecules the question arises if C1q is actually present inside the cartilage.
Proteomic data from synovial fluid from osteoarthritic patients shows the presence of complement components. Wang et al described that complement is important in the pathogenesis of osteoarthritis [16], as the presence of the MAC could be detected on cartilage. Although the authors speculate that complement activation takes place outside the cartilage and then attacks damaged chondrocytes, it cannot be excluded that chondrocytes initiate complement activation leading to MAC deposition takes place in the cartilage [16]. Similarly, In the early 90's it was already described that cartilage is positive for the mRNA for C1q and that deposits of the C1q molecule were present in the cartilage, but the source and function of the production of C1q was not clear [17].
Therefore, we have investigated whether primary human chondrocytes are able to produce and secrete C1q and if the production can be modulated under inflammatory conditions such as present in OA.

Materials and methods

Patients

Cartilage was obtained as left-over tissue from total knee-replacement surgery performed on OA patients in the Alrijne hospital, Leiden, The Netherlands. The local medical ethical committee approved the study.

Isolation of chondrocytes

Chondrocytes were isolated from cartilage obtained from total knee-replacement surgery. Cartilage pieces were incubated with pronase (2 mg/ml, Roche) for 1,5 hours, followed by overnight incubation with collagenase type 2 (225 U/ml, Worthington). After digestion, chondrocytes were directly lysed for western blot analysis or RNA isolation. The purity of the chondrocytes was determined using

Flow Cytometry, whereby CD14 (APC) and CD45 (FITC) were measured using a BD LSRFortessa cell analyser (BD Biosciences, San Jose, CA, USA) and analysed using FlowJo v10.1 software (Tree Star, Ashland, OR, USA).

Stimulation of chondrocytes

Primary chondrocytes were plated in a cell concentration of 5*105 cells/mL in F12 DMEM culture medium (Gibco) supplemented with 10% BSA (Bovine Serum Albumin), L-glutamine, penicillin and streptomycin. Chondrocytes were stimulated for 24 hours with IL-1ß (30 ng/ml, R&D systems), TNF-α (100 ng/ml, R&D systems), TGF-ß (10 ng/ml, peprotech) or LPS (100 ng/ml, Sigma). After stimulation RNA isolation was performed. For western blot analysis primary chondrocytes were lysed and C1qA, C1qB, C1qC was determined as previously described [18]. Culture supernatants of unstimulated chondrocytes were harvested after 72h and analysed by ELISA to determine C1q levels as described before [18].

qPCR

RNA was isolated from cultured chondrocytes using RNeasy mini kit (Qiagen) and analysed with the NanoDrop (NanoDrop Technologies). Subsequently, the RNA was treated with DNase I, Amplification Grade (Invitrogen) and cDNA was synthesized using superscript III (200U/µl, Invitrogen). The cDNA was diluted 1:25 and qPCR was performed using SensiFast Sybr no-ROX (Bioline) and primers with a start concentration of 10 pmol. Primers specific for MMP1, collagen type 1,type 2, type 10, C1r, C1s, C3, C5 and the C1q genes C1qA/B/C, as well as the reference RPL5 were used (see table 1 for primer sequences). The qPCR was performed on the real time PCR system (BioRad CFX-384) with an activation step of 3 min on 95°C (hot start polymerase activation), a melting temperature of 95°C for 5 seconds and an annealing temperature of 64°C for 5 seconds followed by an elongation step for 20 seconds on 72°C for 40 cycles. At the end of the protocol melting curves were performed from 65°C to 95°C to test specific binding of SensiFast Sybr. The qPCR for the C1q genes was performed with annealing temperatures ranging from 58-65°C for 10 seconds.

Immunofluorescence staining of C1q in chondrocytes

Freshly isolated chondrocytes were cultured on poly-d-lysine (Sigma 50 µg/ml) chamber slides (Thermo Scientific) and fixated using 1% paraformaldehyde and acetone. Cells were subsequently incubated with rabbit anti-C1q (DAKO) or isotype control rabbit Ig (DAKO) for 1 hour at RT. After washing, slides were incubated with goat anti-rabbit ALEXA 488 (Invitrogen) and ActinRed 594 (Applied bioprobes) for

1 hour at RT. Finally, the slides were covered with Vectashield containing DAPI (Vector laboratories) to stain nuclei and analysed on a confocal microscope (Leica SP8 confocal).

Gene	5' Forward	3' Reverse
MMP1	CTGGCCACAACTGCCAAATG	ATTCTGTCCCTGAACAGCCC
Collagen type 1	CATGTTCAGCTTTGTGGACCTC	ATGGTACCTGAGGCCGTTCT
Collagen type 2	TCCTAAAGGAGCCCGAGGTGCC	CAGGAGCTTGGAGGCCGGGTT
Collagen type 10	CCCAGCACGCAGAATCCATCT	TTCCAGCCGGTCCAGGGATT
C1qA	CCAGGAAGAACCGTACCAGA	GACGATGGACAGGCAGATTT
C1qB	TCCAGATATGGAGGCCTGAC	TTCACTCAGCAGCATTCACC
C1qC	AAGGARGGGTACGACGGACT	GTAAGCCGGGTTCTCCCTTC
C1r	TCACAGTCCCCACGGGATAC	CCAGTGGAGAACCCAGTTGC
C1s	CTGCAGAGGGAGCGTCAA	TGGTAGGCTCAGCATAAACCC
C3	TGGCCAATGGTGTTGACAGA	GCGTAGACCTTGACTGCTCC
C5	TACCTTGCCTGTTGAAGCCC	CCAGGGAAAGAGCATACGCA
RPL5	TGGAGGTGACTGGTGATG	GCTTCCGATGTACTTCTGC

Table 1. qPCR primers

Immunohistochemical staining of C1q in cartilage

C1q was detected on paraffin embedded cartilage of OA patients (5µm). Slides were deparaffinised and stained with 10 µg/ml rabbit anti-C1q (DAKO) or matching isotype control for 1 hour at RT . Next, slides were incubated with anti-rabbit HRP for 1 hour at RT and were analysed on a Zeiss Axio ScopeA1 microscope.

Results

Chondrocytes are able to produce and secrete C1q

Primary human chondrocytes were isolated and the purity of the isolated chondrocyte population was analysed using flow cytometry. As shown in figure 1A, chondrocytes are negative for the hematopoietic marker CD45 and the myeloid marker CD14, indicating no contamination of cells from hematopoietic or myeloid origin (Figure 1A).

Next, we investigated whether chondrocytes can produce and secrete C1q. Using western blot analysis we detected C1q in chondrocyte lysates from 2 OA patients (Figure 1B). We found the molecular size of C1q in the chondrocytes to be similar to the C1q band observed in the lysates of PBMCs and Normal Human Serum (NHS) (positive controls). No C1q was observed in the lysate of T cells (negative control).

Using confocal microscopy we observed C1q inside the cultured chondrocytes (Figure 1C). We confirmed the data by ELISA on lysates of unstimulated chondrocytes cultured for 72 hours (Figure 1D). Moreover, secretion of C1q into the culture supernatant could be shown in one out of 2 OA patients (Figure 1D). To confirm that chondrocytes express C1q in the natural environment of cartilage, we performed an immunohistochemical staining on OA cartilage. Also staining of the human cartilage showed positivity for C1q, which is mainly located around the nucleus of the chondrocytes (Figure 2A-C).

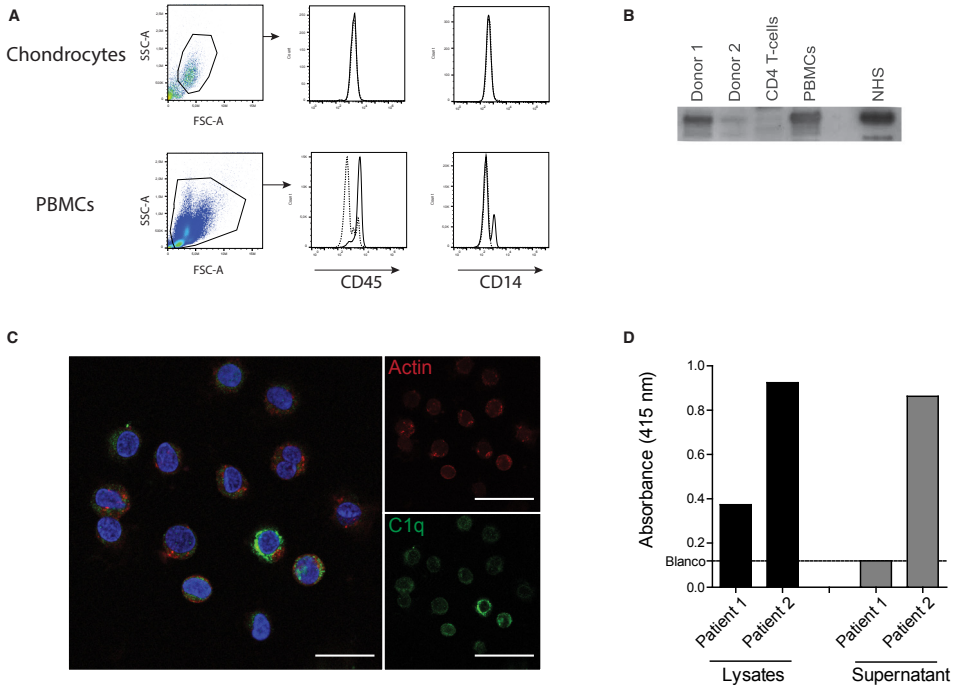

Figure 1. Chondrocytes are positive for C1q.

A. Flow cytometry of chondrocytes isolated from human cartilage compared to PBMCs, **B.** Western blot analysis of lysates of cultured chondrocytes (72 hours, N=2), CD4 T-cells and PBMCs. As positive control NHS is used, **C.** Cultured chondrocytes on chamber slides. C1q is visualized with ALEXA488. Actin is used as counterstaining at 594 nm. The nucleus is stained with DAPI, 630x magnification, the scale bars indicates 6.3 µm, **D.** C1q ELISA on lysates and supernatants of cultured chondrocytes (72h). Read out is the absorbance on 415 nm.

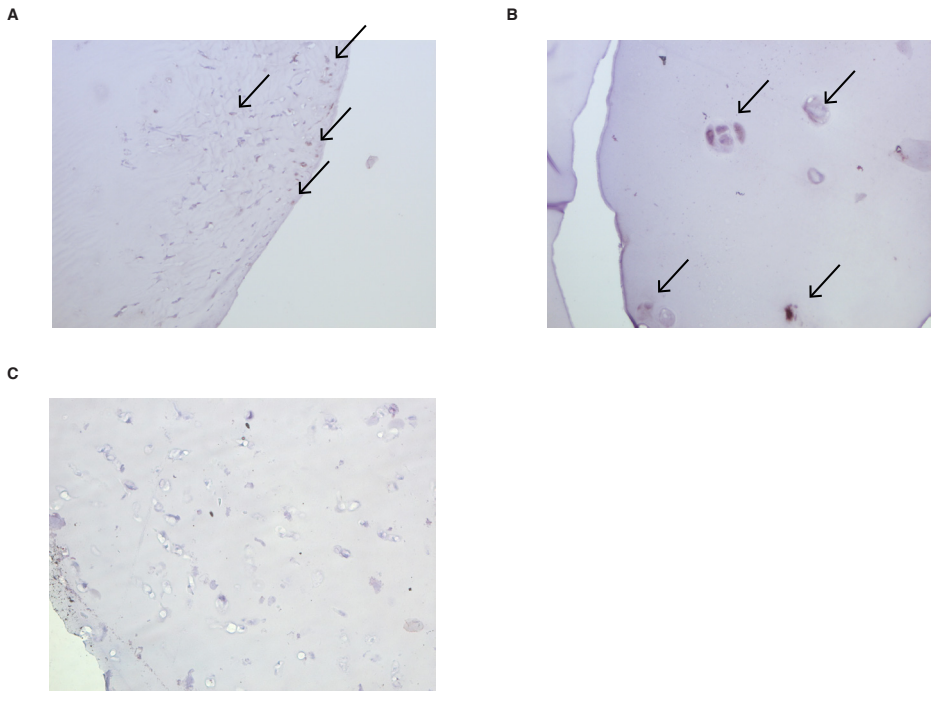

Figure 2. The expression of C1q in human cartilage. A. Expression of C1q (HRP) with a co-staining of haematoxylin with a magnification of 400x, **B.** With a 1000x magnification. **C.** Isotype control on 400x magnification.

mRNA expression of C1q is increased in a pro-inflammatory environment

Next, we wished to determine the mRNA expression pattern of C1qA, C1qB and C1qC by qPCR. We investigated the basal expression pattern as well as the changes that occur after stimulation with cytokines that are relevant for OA [19]. Stimulation with IL-1ß and TNFα led to an increased expression of C1qA, C1qB and C1qC (Figure 3A-C). Furthermore, we investigated whether the expression of other complement components could be modulated by these inflammatory cytokines. Expression levels of C1qA, C1qB and C1qC were upregulated by IL-1ß and TNFα stimulation, while no or limited enhancement was observed for stimulation with TGF-ß and LPS (Figure 3). The expression pattern of the serine protease C1r and C1s and of complement component C3 was largely comparable to the C1q genes, with the exception that LPS did induce a modest increase in their expression after 24 hours of stimulation (Figure 4A, C), while C5 was unaffected by any of the studied cytokines. LPS had little or no effect on the complement genes we studied (Figure 4D).

As a positive control for TGF-ß stimulation we determined the mRNA expression of Collagen type 1, 2 and 10. As expected TGF-ß increases the expression of Collagen

type 1 (figure 5A) [20], while it had little effect on type 2 and type 10 (Figure 5B,C). A small decrease of the expression of Collagen type 1, type 2 and 10 was seen after stimulation with IL-1ß., while TNF or LPS stimulation had little or no effect on the collagen gene expression. MMP1 expression was determined as a positive control for the pro-inflammatory stimulations and shows an increase after stimulation of IL-1ß, TNFα and LPS (figure 5D). The pattern of up/down regulation of the C1q genes is largely similar to C3, C5 and MMP but different from Collagen type 1,2, and 10.

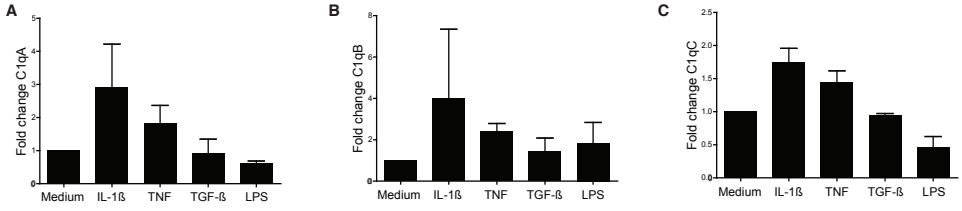

Figure 3. mRNA fold change of C1q genes after 24 hours stimulation (N=3). **A.** Fold change expression after correction for RPL5 of C1qA, **B.** C1qB, **C**. C1qC. Data represents mean and SEM of 3 different OA donors measured in duplicate.

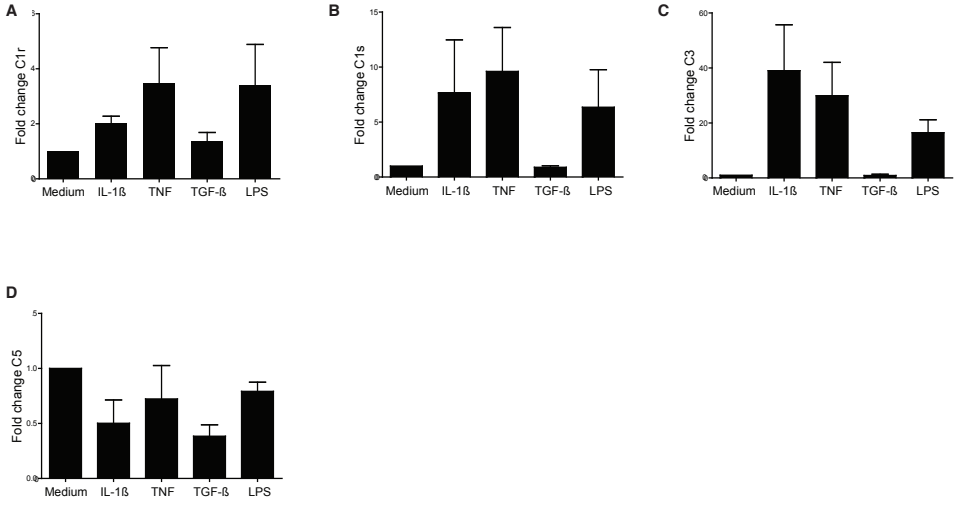

Figure 4. mRNA fold change of complement components after 24 hours stimulation (N=3). A. Fold change expression after correction with RPL5 of C1r, **B.** C1s, **C**. C3. **D.** C5. Data represents mean and SEM of 3 different OA donors measured in duplicate.

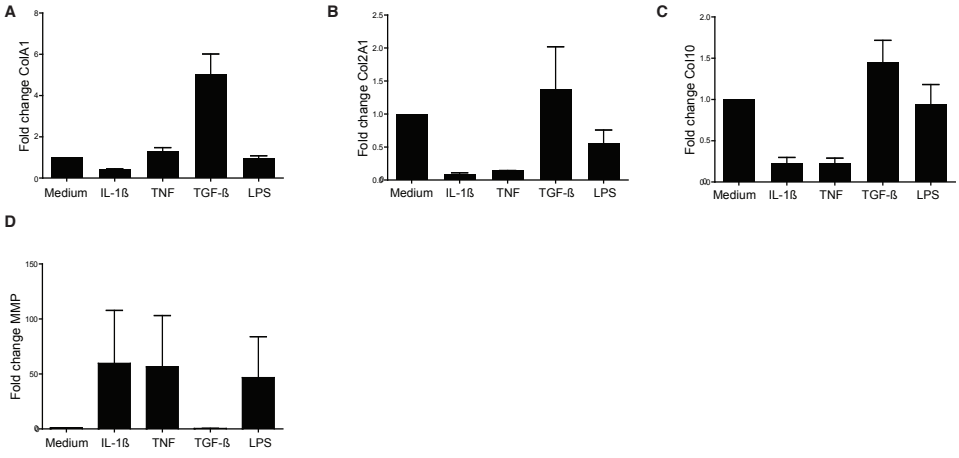

Figure 5. mRNA fold change of chondrocyte components after 24 hours stimulation (N=3). A. Fold change expression after correction with RPL5 of Collagen type 1, **B.** Collagen type 2, **C.** Collagen type 10. **D.** MMP1. Data represents mean and SEM of 3 different OA donors measured in duplicate.

Discussion

The main producers of C1q are originating from haematopoietic stem cells. Chondrocytes are originating from the mesenchymal stem cell and are important in the production of extracellular matrix molecules to build up the cartilage. Wang et al have described that the dysregulation of complement in the synovial joints in has a central role in the pathogenesis of osteoarthritis [16]. In the early 90's it was demonstrated that the cartilage was positive for several complement components like C1q on RNA level, but the presence of C1q could not be shown in vitro. Also the function or relation with the cartilage was not described [17]. Here, we demonstrate that chondrocytes are able to produce and secrete C1q and that the production of C1q is increased by cytokines present in osteoarthritic joints and believed to play a role in disease pathogenesis.

To evaluate which factors could modulate the production of C1q by chondrocytes we stimulated the chondrocytes with cytokines that are known to be present in the joint during inflammation [19]. Stimulation with IL-1ß and TNF-α showed an increase in mRNA expression of C1q compared to unstimulated chondrocytes. Furthermore, the expression patterns of the C1q genes were different compared to the expression of the collagen genes, which are of importance in the maintenance of the cartilage, as seen after TGF-ß stimulation where the gene expression of collagen types 1,2 and 10 were upregulated and the expression of the C1q genes were downregulated. C1q released by chondrocytes could deposit on cartilage or synovium which is damaged by inflammation however, C1q is a large (460kD)

molecule and the size limit of proteins that can move freely in cartilage is estimated to be around 65 kD [17]. Therefore it is likely that C1q produced by chondrocytes must serve a local rather than a systemic function. Conceivably, C1q would function in an autocrine setting in stimulating the chondrocyte or alternatively C1q would be involved in local complement activation, involving C1r and C1s directly outside the chondrocyte. We speculate that C1q and complement activation may be involved in maintenance of a lacuna for the chondrocyte.

Previous studies have demonstrated that the serine protease C1s can degrade collagen type 1 and type 2 when it is activated [21] and that it can play a role in the degenerative cartilage matrix in RA [22]. In our results we also see an increase of RNA expression of C1s and for C1r after stimulation. This could indicate that in pro-inflammatory conditions complement activation will occur in the cartilage or at the surface of the cartilage. Together with the production of C1q and C3 it is highly conceivable that the production of different complement components by chondrocytes plays a role in the degradation of the cartilage.

In our study we only used cartilage and chondrocytes derived from patients who underwent a total knee replacement due to osteoarthritis, indicating that the cartilage is already in inflammatory conditions. In further investigations the role of complement in cartilage it would be interesting to compare these results with healthy donors.

In conclusion, our results indicate that chondrocytes are able to produce and secrete C1q, suggesting a previously unexpected role of this molecule in the cartilage.

Acknowledgement

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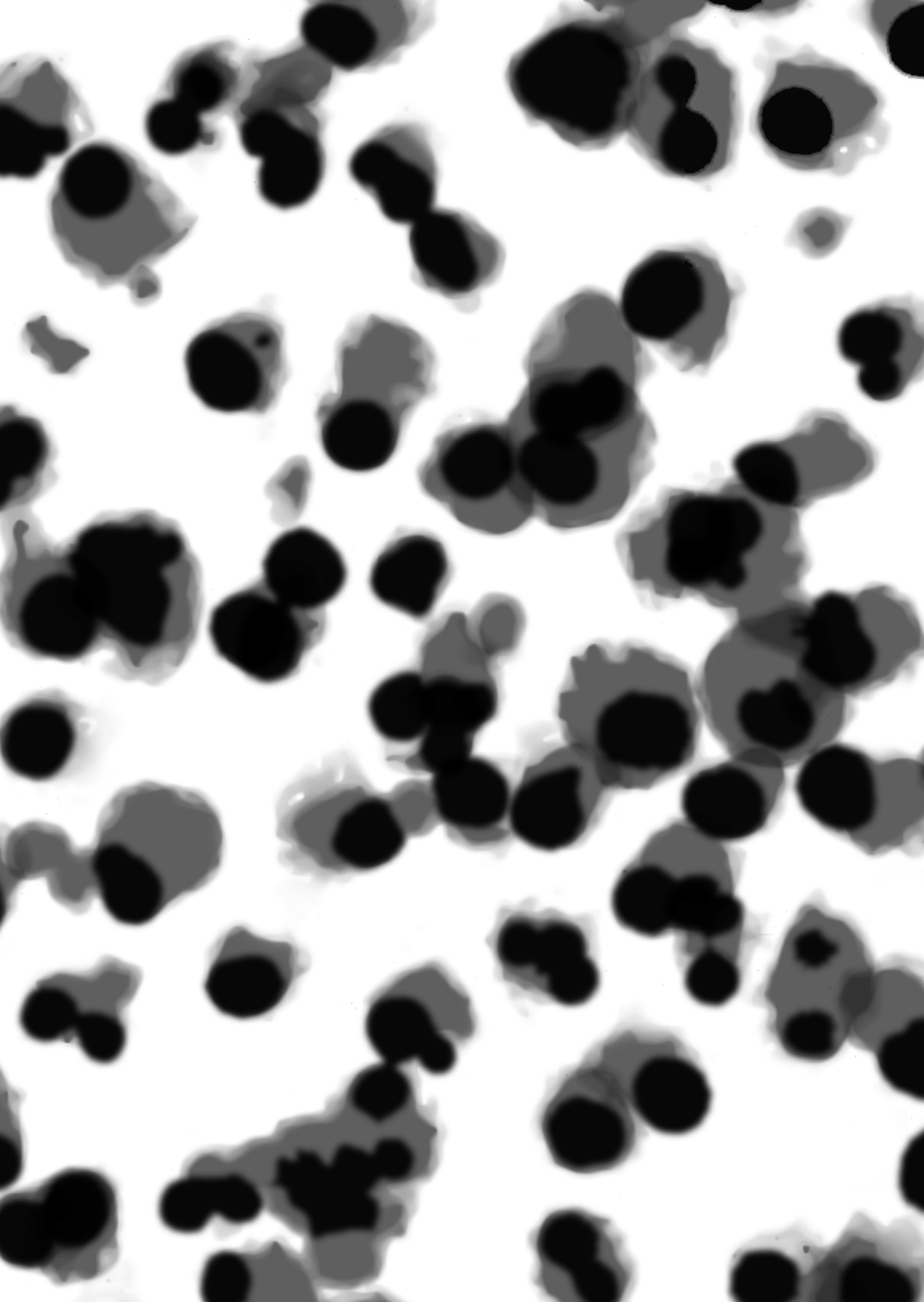
References

1. Sommarin, Y., T. Larsson, and D. Heinegard, Chondrocyte-matrix interactions. Attachment to proteins isolated from cartilage. Exp Cell Res, 1989. 184(1): p. 181-92.
2. Beurskens, F.J., R.A. van Schaarenburg, and L.A. Trouw, C1q, antibodies and anti-C1q autoantibodies. Mol Immunol, 2015.
3. Jiang, H., et al., DNA binds and activates complement via residues 14-26 of the human C1q A chain. J Biol Chem, 1992. 267(35): p. 25597-601.
4. Loos, M., H. Martin, and F. Petry, The biosynthesis of C1q, the collagen-like and Fc-recognizing molecule of the complement system. Behring Inst.Mitt., 1989(84): p. 32-41.
5. Castellano, G., et al., Infiltrating dendritic cells contribute to local synthesis of C1q in murine and human lupus nephritis. Mol Immunol, 2010. 47(11-12): p. 2129-37.
6. Castellano, G., et al., Maturation of dendritic cells abrogates C1q production in vivo and in vitro. Blood, 2004. 103(10): p. 3813-20.
7. Faust, D. and M. Loos, In vitro modulation of C1q mRNA expression and secretion by interleukin-1, interleukin-6, and interferon-gamma in resident and stimulated murine peritoneal macrophages. Immunobiology, 2002. 206(4): p. 368-76.
8. Bulla, R., et al., Decidual endothelial cells express surface-bound C1q as a molecular bridge between endovascular trophoblast and decidual endothelium. Mol Immunol, 2008. 45(9): p. 2629-40.
9. Lynch, N.J., et al., Microglial activation and increased synthesis of complement component C1q precedes blood-brain barrier dysfunction in rats. Mol Immunol, 2004. 40(10): p. 709-16.
10. Teo, B.H., et al., Complement C1q production by osteoclasts and its regulation of osteoclast development. Biochem J, 2012. 447(2): p. 229-37.
11. Melin Furst, C., et al., The C-type lectin of the aggrecan G3 domain activates complement. PLoS One, 2013. 8(4): p. e61407.
12. Sjoberg, A., et al., The extracellular matrix and inflammation: fibromodulin activates the classical pathway of complement by directly binding C1q. J Biol Chem, 2005. 280(37): p. 32301-8.
13. Sjoberg, A.P., et al., Short leucine-rich glycoproteins of the extracellular matrix display diverse patterns of complement interaction and activation. Mol Immunol, 2009. 46(5): p. 830-9.
14. Happonen, K.E., et al., Regulation of complement by cartilage oligomeric matrix protein allows for a novel molecular diagnostic principle in rheumatoid arthritis. Arthritis Rheum, 2010. 62(12): p. 3574-83.
15. Groeneveld, T.W., et al., Interactions of the extracellular matrix proteoglycans decorin and biglycan with C1q and collectins. J Immunol, 2005. 175(7): p. 4715-23.
16. Wang, Q., et al., Identification of a central role for complement in osteoarthritis. Nat Med, 2011. 17(12): p. 1674-9.
17. Bradley, K., et al., Synthesis of classical pathway complement components by chondrocytes. Immunology, 1996. 88(4): p. 648-56.
18. van Schaarenburg, R.A., et al., Identification of a novel non-coding mutation in C1qB in a Dutch child with C1q deficiency associated with recurrent infections. Immunobiology, 2015. 220(3): p. 422-7.
19. Farahat, M.N., et al., Cytokine expression in synovial membranes of patients with rheumatoid arthritis and osteoarthritis. Ann Rheum Dis, 1993. 52(12): p. 870-5.
20. Chin, B.Y., et al., Stimulation of pro-alpha(1)(I) collagen by TGF-beta(1) in mesangial cells: role of the p38 MAPK pathway. Am J Physiol Renal Physiol, 2001. 280(3): p. F495-504.
21. Yamaguchi, K., et al., Degradation of type I and II collagen by human activated C1-s. FEBS Lett,

1990. 268(1): p. 206-8.
22. Nakagawa, K., et al., Complement C1s activation in degenerating articular cartilage of rheumatoid arthritis patients: immunohistochemical studies with an active form specific antibody. Ann Rheum Dis, 1999. 58(3): p. 175-81.



Chapter 8

Summary and discussion

Summary and discussion

In this thesis we report on the production of C1q by immune cells and non-immune cells. Deficiency of C1q as a consequence of a genetic mutation is strongly associated with the development of Systemic Lupus Erythematosus (SLE). In chapter 2 we describe a not previously reported genetic mutation in one of the C1q genes. The patient is currently a teenager and has suffered from infectious problems but did so far not develop SLE. When patients are diagnosed with C1q deficiency due to a genetic mutation the risk to develop SLE is high, but there is high degree of variation between the patients in clinical manifestations. In chapter 3, we used questionnaires to get an overview of C1q deficient patients worldwide, revealing that once the C1q deficient patients reach adulthood that then the chance of fatal infections is reduced. This overview highlighted the importance of a personal approach for therapy, especially in young children.

A mutation in of the genes of C1q can also lead to a non-functional structure of C1q, Low Molecular Weight-C1q (LMW-C1q). In chapter 4 we described a C1q deficient patient who has low levels of LMW-C1q. This patient demonstrates a severe form of SLE and neuropsychiatric SLE (NPSLE). Our data indicate that the classical pathway activity is not required for NP involvement in SLE, but the absence of C1q and the biological consequences may have a role in the pathogenesis of NPSLE.

When we investigated other NPSLE patients we see many different clinical manifestations. To investigate if complement activation and components play a role in NPSLE we performed data analyses and serum analyses. The NPSLE patients have a high degree of complement activation and the levels of anti-C1q and C1q circulating immune complexes are increased compared to healthy controls. The association NPSLE and the levels of anti-C1q, C3/AP50 and C4 are probably due to the disease activity and the presence of anti-phospholipid antibodies (discussed in chapter 5).

In the second part of this thesis we described the production of C1q by different cells. We demonstrate that mast cells, which are originating from the same myeloid progenitor cells as the already known C1q producing cells; macrophages and dendritic cells, are able to produce functional active C1q (chapter 6). We were able to detect C1q secreted from chondrocytes. This was surprising as these cells are originating from mesenchymal stem cells, which is different from haematopoietic stem cells. The main role of chondrocytes is to produce cartilage and maintain the homeostasis of the cartilage (chapter 7).

Overall, all these studies demonstrate the involvement of C1q in disease and the production of C1q by immune cells and non-immune cells.

The role of C1q in disease

In our institute we have identified a C1q deficient patient with a previously unknown mutation in the C1qB chain of C1q resulting in a complete C1q deficiency. Several studies have already shown the association between the absences of C1q in disease like SLE [1-3]. However, this patient did demonstrate several infections but no signs of autoimmunity. Currently, he is receiving prophylactic antibiotics to protect the patient from bacterial infections. If and when patients with a C1q deficiency will develop lupus is unpredictable and it is therefore important to keep him under close control to detect any signs of autoimmunity as early as possible.

Because C1q deficiency is often reported in literature as case reports describing only the initial presentation and no follow-up, we investigated the clinical manifestations of C1q deficient patients around the world. Our data shows that even during follow up the clinical presentation and severity of symptoms in persons that are deficient for C1q is very divers. Even though this case series comprised 45 individuals (comprising the majority of cases known to date) there is no clear algorithm to describe how to manage C1q deficiency clinically. Also differences in clinical presentation are seen within families with the same mutation in one of the C1q genes. Indicating, that other factors like environmental factors or epi-(genetic) changes can influence the different outcome of clinical presentations [4, 5]. From this study we can conclude that the manifestation of the disease in C1q deficient patients is unfortunately not predictable. Especially in young children, where the risk of developing a fatal infectious disease is high. In future studies C1q deficient patients should be monitored regularly from a young age if possible. Together with the familial history and clinical manifestations of the C1q deficient patient, the clinician can decide which treatment will be applied, like FFP or HSCT.

Patients with established SLE have a wide diversity in clinical presentations. Patients can e.g. demonstrate cutaneous lupus or glomerulonephritis, but can also demonstrate symptoms involving the nervous system resulting in neuropsychiatric SLE (NPSLE). In the literature neuropsychiatric involvement in patients with a deficiency in the early components of the classical pathway is only described for patients with a C1q deficiency. We have had the opportunity to investigate in detail a C1q deficient patient demonstrating NPSLE. With this study together with literature research we suggest that the classical pathway activity is not required for NP involvement in SLE but the absence of C1q and subsequently some of its biological functions may have a role in the pathogenesis of NP-SLE.

Typical phenomena in SLE are B-cell hyper activation, production of autoantibodies and formation of immune complexes [6, 7]. Since immune complexes activate complement a state of secondary complement deficiency can develop. The role

of C1q and other complement components in NPSLE is not exactly known. Due to the complexity of the disease no specific biomarkers are known for NPSLE. As anti-C1q antibodies are very common in SLE patients we measured the presence of anti-C1q antibodies and also the presence of C1q circulating immune complexes in serum of a large cohort of NPSLE patients. As previous studies have reported, the levels of anti-C1q in NPSLE patients is higher compared to healthy controls. These high levels of anti-C1q antibodies correlated with the SLE activity. Unfortunately no association of the presence anti-C1q antibodies and NSPLE patients compared with SLE patients is found, indicating that anti-C1q is not a useful biomarker that can be used to identify NPSLE patients. Furthermore, in NPSLE patients complement activation is taking place, which is seen as decreased levels of C1q and C3 but this is probably the result of the presence of autoantibodies that form immune complexes.

C1q production by immune and non-immune cells

The production and secretion of complement factors by immune cells is of importance in case of local infection or local processes such as clearance of immune complexes or apoptotic cells. In this thesis we demonstrate that mast cells are able to produce and secrete functionally active C1q. This could be of importance in maintaining the homeostasis of tissues were mast cells are abundant, like in the synovial tissue of joints affected by arthritis [8, 9]. In infections mast cells can be of importance due to degranulation and/or the release of IL-8, which is an important chemokine that attracts other immune cells. When mast cells are stimulated with LPS or via FcεRI triggering the mast cells release IL-8 and also C1q. The release of C1q can have a role by activating the complement system resulting in attracting more immune cells due to the cleavage products C3a and C5a [10, 11]. Another role that could be of importance in the release of C1q by mast cells is the clearance of apoptotic cells, macrophage polarization or stimulating cytokine production [12-14].

Previous studies have described a role for C1q in different inflammatory tissues [15, 16]. Like in RA patients the haemolytic activity is up-regulated in synovial fluid as a consequence of complement activation and due to genetic variations in and around the C1q genes, the levels of C1q are increased in RA patients [15, 17]. C1q is predominantly acting in the clearance of apoptotic cells, macrophage polarization or stimulating cytokine production [13, 14, 18]. Several studies demonstrated that C1q is able to bind to cartilage fragments of which could be of importance in the pathogenesis of joint disease. Interference of C1q with the cartilage oligomeric matrix protein (COMP), decorin and biglycan will result in an inhibition of complement activation [19, 20]. On the other site, aggrecan, fibromodulin and osteoadherin are

also able to bind to C1q and enhance the activation of the classical pathway [21-23]. C1q produced by mast cells can play a role in the complement activation in synovial tissue. When mast cells are activated and secrete C1q, the activation of complement can be either dampened or enhanced by binding of certain cartilage fragments. More interestingly is that not only immune cells in the joint are able to produce C1q [24]. In our study we demonstrate that chondrocytes have also the capability to produce C1q. This is quite remarkable, because chondrocytes are originating from mesenchymal stem cells compared to dendritic cells, macrophages and mast cells, which are from haematopoietic origin. The production of C1q by chondrocytes was already described in 1996 [25], but the exact role and the regulation of secretion was not described. We show in our study that non-stimulated chondrocytes are able to produce C1q and that the production is increased after stimulation of pro-inflammatory cytokines including IL-1ß. Also the proteases C1r and C1s had the same mRNA expression pattern as the C1q genes. Compared to the expression of collagen the C1q genes and C1r and C1s expression pattern were opposed. Why chondrocytes produce C1q should be further investigated, due to the high molecular weight of C1q (460 kD) it is unable to diffuse through the cartilage of which the maximum 65 kD is [25].

Perspectives

The involvement of the complement system in inflammation has been studied for many years. Especially the role of complement on cell development, attraction and differentiation is well studied by different research groups [26-28]. Remarkably, the clinical relevance and physiological importance is not yet well understood. Ricklin et al reviewed in 2010 the importance of complement in the maintenance of the homeostasis [29]. For example in normal pregnancy, C1q plays a key role in trophoblast invasion, spiral artery remodelling and the normal placentation [30]. The sources of C1q in the maternal tract are trophoblasts and decidual endothelial cells (DECs) [24]. In mouse studies it is shown that C1q -/- mice were unable to clear apoptotic trophoblasts resulting in an accumulation of apoptotic trophoblasts and an abnormal placentation. These mice showed features of preeclampsia like hypertension, albuminuria, endotheliosis, less placenta vascular endothelial growth (VEGF), an increase of soluble VEGF receptor 1 (sFlt-1) and oxidative stress. Furthermore, decreased blood flow, increased fetal death, diminished litter size, abnormal invasion of trophoblasts and increased levels of STAT-8 (inhibitor trophoblast migration) [31]. Unfortunately, this is all based on mouse studies and

no literature is known about pregnancy in women with C1q deficiency. In our questionnaires no problems with pregnancy were described. However, since half the cohort was male and different age groups were present, such an effect could easily have been missed. Apparently C1q is of major importance in pregnancy and therefor it would be of importance and interesting to know if there are problems with pregnancy in C1q deficient women.

Another important role for C1q is the role in synaptic pruning [32]. During developmental stage the neural network is growing continuously leading to excessive synaptic formation, which needs to be under control to maintain a proper functioning of the central nervous system (CNS). The role of C1q in this stage is to contribute to the elimination of synapses during developmental stages of the CNS. This process is also well studied in mice. C1q -/- mice have aberrant synaptic connectivity and show forms of epilepsy, which is probably a result of excessive excitatory synapses [33, 34]. Also this phenomenon is not observed in our questionnaires. The clinicians treating C1q deficient patients describe no epilepsy or other CNS abnormalities. However, in the literature NPSLE symptoms are described in patients with a C1q deficiency in previous studies[3, 35-38]. For future research it would be interesting to find out if there is an association with C1q deficiency and the development of NPSLE. Due to absence of C1q in the brain during the developmental stage, the synaptic connectivity could also be aberrant as in C1q -/- mice leading to NPSLE events during aging.

A standard treatment for C1q deficiency is not available. Most of the patients receive immunosuppressive drugs or are receiving fresh frozen plasma (FFP) on a regularly basis [39, 40]. Another therapy, which is more radical, is hematopoietic stem cell transplantation (HSCT). Already 2 patients are treated successfully after HSCT. Following HSCT, the levels of C1q in serum are in the normal range and they are cured from SLE [41, 42]. Because there are people with C1q deficiency that live a normal life without any infections or symptoms of autoimmunity the question is raised if those people should also undergo a HSCT, because the chance to develop SLE is almost 100%. If the patient is feeling healthy and does not have signs of autoimmunity it may not be ethical to perform HSCT, because it will be 'cutting in a healthy body'. Also one patient, who underwent HSCT to normalize the C1q levels in the serum, died of multiorgan failure and an intracerebral hemorrage. For the clinicians treating C1q deficiency patients it should be important to check the patient on a regular basis and to determine if the patient is vulnerable to infections in his surroundings. An infection will trigger the immune system, which can also be a trigger the development of SLE. An extra consideration the clinicians should make is to vaccinate the patient with additional vaccinations like, Pneumococcus,

Meningococcus, Hepatitis B and the seasonal influenza vaccinations next to the regular childhood vaccines such as measles, diphtheria, tetanus and poliomyelitis. As described before the role of complement in the human body is diverse. The complement system and especially C1q can be involved in many processes. C1q can be involved in the induction of angiogenesis and tissue repair. In mouse studies C1q is believed to be a unique player in the angiogenesis and thereby be a potential therapeutic target in wound healing of the skin [43]. In this study they demonstrate that C1q will bind to the endothelial cells by their globular head and without activating the complement system will induce an angiogenic phenotype in endothelial cells. The suggested source of C1q is probably plasma, but by analysing our data we can consider that the mast cells in the skin are a source of C1q production as the mast cells in the skin are the most abundant C1q positive cells. Vascular endothelial growth factor (VEGF) plays a major role angiogenesis and mast cells are an important source of VEGF. This together will suggest that C1q producing mast cells have a potential role in inducing an angiogenic phenotype in endothelial cells and thereby promote vascularization.

Angiogenesis is a phenomenon that also takes plays in the bone repair [44-46]. For example, chondrocytes are able to produce VEGF and mice lacking the VEGF gene in chondrocytes have an impaired embryonic bone development, reducing angiogenesis and reduced removal of terminally differentiated hypertrophic chondrocytes [47-49]. In the mouse studies they suggested gC1qR on the endothelial cells as potential receptor of C1q inducing the angiogenic phenotype of the endothelial cells. In a study in 2012 it is also demonstrated that chondrocytes are positive for gC1qR [50]. This could indicate that C1q produced by chondrocytes can regulate a feedback loop to chondrocytes by binding to gC1qR and subsequently induce VEGF resulting in the survival of the chondrocytes. When C1q is produced by chondrocytes C1r and C1s are also produced. These proteases are able to degrade collagen, which is an important matrix molecule [51, 52]. This is also an indication that C1q and other complement components could be of importance in the survival of chondrocytes. C1q, C1r and C1s can bind matrix molecules surrounding the chondrocytes and subsequently degrade the collagen to maintain the lacunae.

In diseased joints as in rheumatoid arthritis (RA) and in osteoarthritis (OA) higher levels of complement components are detected in the synovial fluid. As described by Wang et al more deposition of MAC is found on the cartilage of osteoarthritic joints [16]. Together with our findings and with the findings of Bradley et al, who described that chondrocytes are positive for C1q [25], we can suggest that C1q produced by chondrocytes can play a role in the pathogenesis of OA and maybe

also in RA. However, the major source of C1q is possibly C1q from the circulation that deposits on the cartilage due to damage by pro-inflammatory cytokines. So the additional value of C1q producing chondrocytes should be investigated.

Conclusion

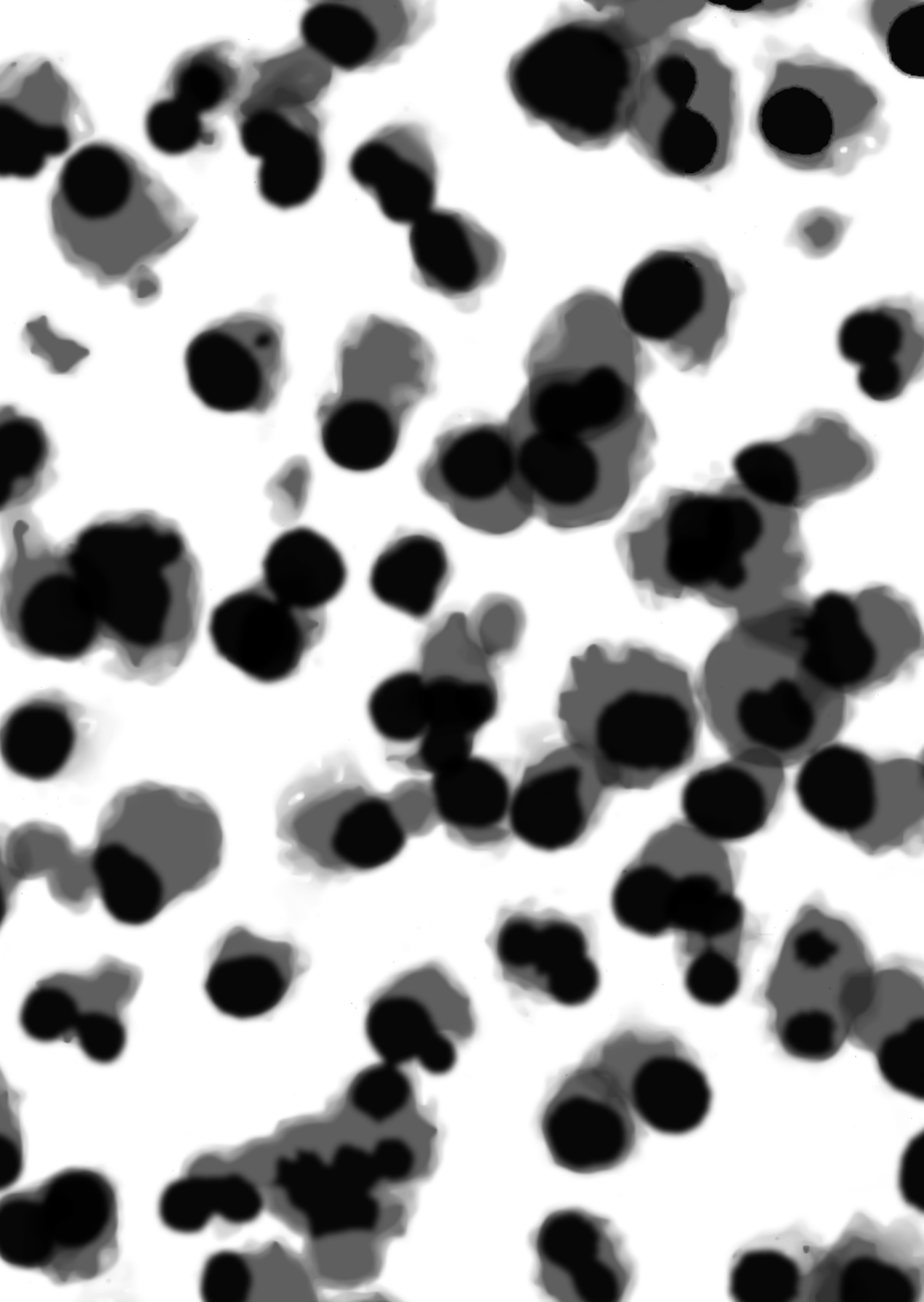
In this thesis the combination of epidemiological research of C1q deficient patients and NPSLE patients in combination with cell biology of C1q producing cells is described. This combination provides more insight into how C1q producing cells can play a role in autoimmune diseases like SLE, NPSLE, RA and OA.

References

1. Walport, M.J., K.A. Davies, and M. Botto, C1q and systemic lupus erythematosus. Immunobiology, 1998. 199(2): p. 265-85.
2. Walport, M.J., et al., Complement deficiency and autoimmunity. Ann N Y Acad Sci, 1997. 815: p. 267-81.
3. Schejbel, L., et al., Molecular basis of hereditary C1q deficiency--revisited: identification of several novel disease-causing mutations. Genes Immun, 2011. 12(8): p. 626-34.
4. Ballestar, E., Epigenetic alterations in autoimmune rheumatic diseases. Nat Rev Rheumatol, 2011. 7(5): p. 263-71.
5. Salvetti, M., et al., Twins: mirrors of the immune system. Immunol Today, 2000. 21(7): p. 342-7.
6. Mok, C.C. and C.S. Lau, Pathogenesis of systemic lupus erythematosus. J Clin Pathol, 2003. 56(7): p. 481-90.
7. Lipsky, P.E., Systemic lupus erythematosus: an autoimmune disease of B cell hyperactivity. Nat Immunol, 2001. 2(9): p. 764-6.
8. de Lange-Brokaar, B.J., et al., Characterization of synovial mast cells in knee osteoarthritis: association with clinical parameters. Osteoarthritis Cartilage, 2016. 24(4): p. 664-71.
9. Suurmond, J., et al., Mast cells in rheumatic disease. Eur J Pharmacol, 2016. 778: p. 116-24.
10. Halova, I., L. Draberova, and P. Draber, Mast cell chemotaxis - chemoattractants and signaling pathways. Front Immunol, 2012. 3: p. 119.
11. Hartmann, K., et al., C3a and C5a stimulate chemotaxis of human mast cells. Blood, 1997. 89(8): p. 2863-70.
12. Korb, L.C. and J.M. Ahearn, C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited. J Immunol, 1997. 158(10): p. 4525-8.
13. Nauta, A.J., et al., Direct binding of C1q to apoptotic cells and cell blebs induces complement activation. Eur J Immunol, 2002. 32(6): p. 1726-36.
14. Spivia, W., et al., Complement protein C1q promotes macrophage anti-inflammatory M2-like polarization during the clearance of atherogenic lipoproteins. Inflamm Res, 2014. 63(10): p. 885-93.
15. Trouw, L.A., et al., Genetic variants in the region of the C1q genes are associated with Rheumatoid Arthritis. Clin.Exp.Immunol., 2013.
16. Wang, Q., et al., Identification of a central role for complement in osteoarthritis. Nat Med, 2011. 17(12): p. 1674-9.
17. Pekin, T.J., Jr. and N.J. Zvaifler, Hemolytic Complement in Synovial Fluid. J Clin Invest, 1964. 43: p. 1372-82.
18. Bohlson, S.S., et al., Complement, c1q, and c1q-related molecules regulate macrophage polarization. Front Immunol, 2014. 5: p. 402.
19. Groeneveld, T.W., et al., Interactions of the extracellular matrix proteoglycans decorin and biglycan with C1q and collectins. J Immunol, 2005. 175(7): p. 4715-23.
20. Happonen, K.E., et al., Regulation of complement by cartilage oligomeric matrix protein allows for a novel molecular diagnostic principle in rheumatoid arthritis. Arthritis Rheum, 2010. 62(12): p. 3574-83.
21. Melin Furst, C., et al., The C-type lectin of the aggrecan G3 domain activates complement. PLoS One, 2013. 8(4): p. e61407.
22. Sjoberg, A.P., et al., Short leucine-rich glycoproteins of the extracellular matrix display diverse patterns of complement interaction and activation. Mol Immunol, 2009. 46(5): p. 830-9.
23. Sjoberg, A., et al., The extracellular matrix and inflammation: fibromodulin activates the classical

pathway of complement by directly binding C1q. J Biol Chem, 2005. 280(37): p. 32301-8.
24. Bulla, R., et al., Decidual endothelial cells express surface-bound C1q as a molecular bridge between endovascular trophoblast and decidual endothelium. Mol Immunol, 2008. 45(9): p. 2629-40.
25. Bradley, K., et al., Synthesis of classical pathway complement components by chondrocytes. Immunology, 1996. 88(4): p. 648-56.
26. Son, M., et al., C1q limits dendritic cell differentiation and activation by engaging LAIR-1. Proc Natl Acad Sci U S A, 2012. 109(46): p. E3160-7.
27. van Kooten, C., et al., Complement production and regulation by dendritic cells: molecular switches between tolerance and immunity. Mol Immunol, 2008. 45(16): p. 4064-72.
28. Trouw, L.A. and M.R. Daha, Role of complement in innate immunity and host defense. Immunol Lett, 2011. 138(1): p. 35-7.
29. Ricklin, D., et al., Complement: a key system for immune surveillance and homeostasis. Nat Immunol, 2010. 11(9): p. 785-97.
30. Agostinis, C., et al., An alternative role of C1q in cell migration and tissue remodeling: contribution to trophoblast invasion and placental development. J Immunol, 2010. 185(7): p. 4420-9.
31. Singh, J., A. Ahmed, and G. Girardi, Role of complement component C1q in the onset of preeclampsia in mice. Hypertension, 2011. 58(4): p. 716-24.
32. Stevens, B., et al., The classical complement cascade mediates CNS synapse elimination. Cell, 2007. 131(6): p. 1164-78.
33. Chu, Y., et al., Enhanced synaptic connectivity and epilepsy in C1q knockout mice. Proc Natl Acad Sci U S A, 2010. 107(17): p. 7975-80.
34. Ma, Y., et al., Remodeling of dendrites and spines in the C1q knockout model of genetic epilepsy. Epilepsia, 2013. 54(7): p. 1232-9.
35. Jlajla, H., et al., New C1q mutation in a Tunisian family. Immunobiology, 2014. 219(3): p. 241-6.
36. Kirschfink, M., et al., Complete functional C1q deficiency associated with systemic lupus erythematosus (SLE). Clin Exp Immunol, 1993. 94(2): p. 267-72.
37. Macedo, A.C. and L. Isaac, Systemic Lupus Erythematosus and Deficiencies of Early Components of the Complement Classical Pathway. Front Immunol, 2016. 7: p. 55.
38. Petry, F., et al., Non-sense and missense mutations in the structural genes of complement component C1q A and C chains are linked with two different types of complete selective C1q deficiencies. J Immunol, 1995. 155(10): p. 4734-8.
39. Mehta, P., et al., SLE with C1q deficiency treated with fresh frozen plasma: a 10-year experience. Rheumatology (Oxford), 2010. 49(4): p. 823-4.
40. Topaloglu, R., et al., C1q deficiency: identification of a novel missense mutation and treatment with fresh frozen plasma. Clin Rheumatol, 2012. 31(7): p. 1123-6.
41. Arkwright, P.D., et al., Successful cure of C1q deficiency in human subjects treated with hematopoietic stem cell transplantation. J Allergy Clin Immunol, 2014. 133(1): p. 265-7.
42. Olsson, R.F., et al., Allogeneic Hematopoietic Stem Cell Transplantation in the Treatment of Human C1q Deficiency: The Karolinska Experience. Transplantation, 2016. 100(6): p. 1356-62.
43. Bossi, F., et al., C1q as a unique player in angiogenesis with therapeutic implication in wound healing. Proc Natl Acad Sci U S A, 2014. 111(11): p. 4209-14.
44. Gerber, H.P., et al., VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med, 1999. 5(6): p. 623-8.
45. Chim, S.M., et al., Angiogenic factors in bone local environment. Cytokine Growth Factor Rev, 2013. 24(3): p. 297-310.
46. Hausman, M.R., M.B. Schaffler, and R.J. Majeska, Prevention of fracture healing in rats by an

inhibitor of angiogenesis. Bone, 2001. 29(6): p. 560-4.
47. Carlevaro, M.F., et al., Vascular endothelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation. J Cell Sci, 2000. 113 (Pt 1): p. 59-69.
48. Maes, C., et al., Impaired angiogenesis and endochondral bone formation in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. Mech Dev, 2002. 111(1-2): p. 61-73.
49. Zelzer, E., et al., VEGFA is necessary for chondrocyte survival during bone development. Development, 2004. 131(9): p. 2161-71.
50. Dembitzer, F.R., et al., gC1qR expression in normal and pathologic human tissues: differential expression in tissues of epithelial and mesenchymal origin. J Histochem Cytochem, 2012. 60(6): p. 467-74.
51. Nakagawa, K., et al., Complement C1s activation in degenerating articular cartilage of rheumatoid arthritis patients: immunohistochemical studies with an active form specific antibody. Ann Rheum Dis, 1999. 58(3): p. 175-81.
52. Yamaguchi, K., et al., Degradation of type I and II collagen by human activated C1-s. FEBS Lett, 1990. 268(1): p. 206-8.



Chapter 9

Nederlandse samenvatting

Nederlandse samenvatting

Het molecuul C1q speelt een belangrijke rol in de homeostase van het menselijk lichaam. Het wordt voornamelijk geproduceerd door immature dendritische cellen en macrofagen.
Zonder C1q hebben individuen een verschuiving binnen hun immuunsysteem, waardoor ze een verhoogd risico op infecties hebben maar ook daardoor een auto-immuunziekte kunnen ontwikkelen. Tot nu toe zijn dergelijke C1q-deficiënte patiënten vooral per individu beschreven betreffende de mutaties van het C1q gen en de klinische presentatie.
In de studies beschreven in dit proefschrift hebben we in detail gekeken naar twee individuele C1q deficiënte patiënten maar ook naar de variabele klinische presentatie van een groot aantal C1q deficiënte patiënten. Tevens worden studies beschreven naar de rol van C1q en anti-C1q antistoffen bij Neuro-Psychiatrische Systemische Lupus Erythematosus (NP-SLE). Daarnaast hebben we de productie van C1q door mestcellen en chondrocyten onderzocht.

C1q en anti-C1q antistoffen in relatie tot klinische presentatie
C1q is het herkenningsmolecuul van de klassieke route van het complementsysteem. Een belangrijke taak van C1q is het opruimen van immuuncomplexen en dode cellen. De 'waste disposal' theorie beschrijft hoe SLE zich ontwikkelt doordat immuuncomplexen en dode cellen niet kunnen worden opgeruimd vanwege de afwezigheid van C1q.
Patiënten met een deficiëntie voor C1q zijn vatbaarder voor bacteriële infecties. Ongeveer 90% van de patiënten met een deficiëntie voor C1q ontwikkelen SLE.
In **hoofdstuk 2** wordt een Nederlands kind beschreven dat gediagnosticeerd is met een C1q-deficiëntie. Door middel van het uitlezen van het genomische DNA hebben we kunnen vaststellen dat deze patiënt een mutatie heeft in een splitsingsplaats bij het gen C1qB. Opvallend is dat deze patiënt alleen te maken heeft gehad met bacteriële infecties, maar geen SLE heeft ontwikkeld. Omdat deze patiënt (nog) geen SLE heeft ontwikkeld waren wij benieuwd naar de klinische presentatie van andere C1qdeficiënte patiënten.
In **hoofdstuk 3** hebben we door middel van enquêtes 45 C1q-deficiënte patiënten in beeld gebracht. Hieruit bleek dat de klinische presentatie van C1q-deficiënte patiënten erg variabel is. Ongeveer 80% van deze patiënten heeft SLE ontwikkeld. De overige 20% van de patiënten heeft alleen bacteriële infecties of helemaal geen infecties en is volledig vrij van symptomen. Vanwege het variërende ziektebeeld is het bijzonder lastig om uit te zoeken welke therapie voor deze patiënten het

meest geschikt is. Daarom is het van belang dat de arts patiënten met een C1q-deficiëntie regelmatig ziet en op deze manier een therapie kan toepassen die persoonsgebonden is. Uit de enquêtes bleek ook dat de kans op sterfte op jonge leeftijd hoog is maar, als de patiënt een volwassen leeftijd heeft bereikt, de kans op sterfte weer afneemt. Deze informatie geeft duidelijk aan dat het van belang is juist jonge kinderen met een C1q-deficiëntie goed te volgen.

C1q-deficiëntie kan veroorzaakt worden door een mutatie in het DNA van één van de C1q genen. Dit kan tot gevolg hebben dat er helemaal geen C1q wordt geproduceerd of dat er een niet-functionele variant van C1q wordt geproduceerd.

In **hoofdstuk 4** beschrijven wij een Nederlandse patiënt die door een mutatie in het C1qC gen een heel lage concentratie van een andere vorm van C1q maakt, low molecular weight C1q (LMW-C1q). Door middel van serum analyse hebben wij dit LMW-C1q in beeld gebracht. Deze patiënt heeft een ernstige vorm van SLE wat zich uit in NP-SLE. In dit project hebben we gekeken naar de mogelijke relatie van C1q-deficiëntie en de ontwikkeling van NP-SLE. Daarnaast hebben we de biochemische structuur van LMW-C1q onderzocht. Uit literatuuronderzoek blijkt dat C1r/s C4 en C2 deficiëntie ook een verhoogde kans geeft op het ontwikkelen van SLE, maar dat NP-SLE symptomen in deze groepen nooit zijn beschreven. Doordat de patiënt geen activatie heeft van de klassieke route, suggereren wij dat de klassieke route niet noodzakelijk is voor het ontwikkelen van NP-SLE, maar dat de afwezigheid van C1q zeker een aandeel heeft in de pathogenese van NP-SLE.

NP-SLE is een vorm van SLE, waarbij SLE patiënten neurologische en psychiatrische symptomen ontwikkelen. Door de aanwezigheid van ontstekingen en immuuncomplex deposities in de hersenen kunnen deze symptomen worden ontwikkeld, die ernstige gevolgen kunnen hebben. Patiënten hebben zeer variabele symptomen. Dit kan de diagnose van NP-SLE bemoeilijken. Immuuncomplexen die neerslaan in weefsels zoals in het brein zullen C1q binden en complement activeren. De aanwezigheid van anti-C1q antilichamen zal dit proces verder versterken. Dit zal zorgen voor meer complement activatie wat kan leiden tot meer schade van de organen.

In **hoofdstuk 5** hebben wij onderzocht of de aanwezigheid van anti-C1q autoantistoffen en C1q-bindende circulerende immuuncomplexen (C1q-CIC) associëren met NP-SLE. Door middel van serum analyses konden we aantonen dat patiënten met NP-SLE een hogere concentratie anti-C1q en C1q-CIC hebben dan gezonde individuen. Verder zagen we een associatie tussen diffuus NP-SLE met anti-C1q antistoffen, een verlaagde concentratie van C3 en een verlaagde activatie van de alternatieve route van het complementsysteem. Binnen de groep van patiënten die gediagnosticeerd zijn met focaal NP-SLE was een associatie

te zien met een lage concentratie van C4. Het verschil van de verschillende subgroepen van NP-SLE en de associaties met complementcomponenten kan worden verklaard door de mate van ontsteking die de patiënten hebben met diffuus NP-SLE en door de aanwezigheid van anti-fosfolipiden antistoffen in de patiënten met focaal NP-SLE. Daarnaast zal verder onderzoek moeten worden gedaan naar de mogelijke rol van andere complementcomponenten, zoals van de alternatieve route. De alternatieve route zou een rol kunnen spelen bij de ontwikkeling van NP-SLE, omdat de concentratie van C3 een associatie heeft met cognitieve dysfunctie en psychoses in lupus patiënten.

De productie van C1q door verschillende cellen

De voornaamste C1q producerende cellen zijn immature dendritische cellen en macrofagen. Deze cellen hebben eenzelfde voorloper cel namelijk de myeloïde voorlopercel. Mestcellen hebben ook deze voorloper cel en daarom waren wij nieuwsgierig of mestcellen ook C1q kunnen produceren.

De studies beschreven in **hoofdstuk 6** laten zien dat mestcellen inderdaad C1q kunnen produceren en uitscheiden. De productie van C1q is niet afhankelijk van de gelijktijdige immunologische activatie van de mestcel. Dit is bevestigd door middel van het analyseren van de uitscheiding van IL-8. Mestcellen staan bekend om de granules die binnen de cel aanwezig zijn. Het degranuleren van mestcellen is een bekend fenomeen in de pathogenese van allergieën. Bij deze degranulatie komen enzymen vrij zoals tryptase, chymase en β-hexosaminidase. C1q komt ook vrij bij degranuleren en kan dus mogelijk een rol spelen bij allergieën en ontstekingen waarbij mestcellen betrokken zijn. Met immunofluorescentie tonen wij C1q-positieve mestcellen aan in verschillende humane weefsels zoals synovium, huid van psoriasis patiënten en in de normale huid.

Complement speelt een belangrijke rol in de pathogenese van reumatische aandoeningen zoals reumatoïde artritis en osteoartritis. Deposities van verschillende complementcomponenten en complement-activatie producten zijn te vinden in het synovium, synoviaal vocht en in het kraakbeen van aangetaste gewrichten. In de jaren 90 is al beschreven dat de cellen die verantwoordelijk zijn voor de aanmaak van kraakbeen, chondrocyten, positief zijn voor C1q.

In **hoofdstuk 7** hebben wij onderzocht of chondrocyten inderdaad C1q kunnen produceren en ook uitscheiden. Wij hebben de chondrocyten gestimuleerd met verschillende cytokines zoals IL-1β en TNF-α. Deze cytokines zijn aanwezig in de gewrichten van patiënten met reumatoïde artritis en osteoartritis. Hieruit bleek dat IL-1β en TNF-α de RNA expressie van C1q verhoogt. Dit kan betekenen dat het C1q dat aanwezig is in ontstoken gewrichten, ook deels door chondrocyten

geproduceerd kan zijn als er sprake is van afbraak van het kraakbeen. Daarnaast hebben we geanalyseerd of de expressie van C1q associeert met de expressie van genen die coderen voor matrix-eiwitten zoals collageen types 1, 2 en 10. Door de chondrocyten te stimuleren met TGF-β zagen we dat de relatieve RNA expressie van alle types collageen was verhoogd. Deze verhoging was niet aanwezig bij de RNA expressie van C1q. Deze data suggereren dat de expressie van C1q en de expressie van matrix-opbouwende eiwitten niet aan elkaar gerelateerd zijn.

De precieze rol van C1q dat geproduceerd wordt door chondrocyten moet nog verder worden onderzocht.
Samenvattend: de studies beschreven in dit proefschrift laten zien dat C1q een belangrijke bijdrage levert bij auto-immuunziektes. Het feit dat C1q geproduceerd kan worden zowel door afweercellen als door niet-afweercellen suggereert dat C1q bij mogelijk meer lichamelijke processen mogelijk betrokken is dan eerder gedacht. Het begrijpen van de precieze rol van C1q in verschillende weefsels zal nieuwe belangrijke inzichten verschaffen op het gebied van het behandelen van patiënten met een auto-immuun ziekte waarbij C1q betrokken is.

Curriculum Vitae

Rosanne van Schaarenburg werd op 27 augustus 1988 geboren in Zoeterwoude. In 2006 slaagde zij voor haar HAVO examen op het Bonaventura College te Leiden. Hierna is zij begonnen aan de opleiding Biologie en Medisch Laboratoriumonderzoek aan de Hogeschool Utrecht. Tijdens haar bachelor heeft Rosanne stage gelopen op de afdeling Immunologie in het Wilhelmina Kinderziekenhuis te Utrecht en bij het biofarmaceutisch bedrijf Arthrogen BV. in Amsterdam. Zij behaalde haar bachelor diploma in juli 2010 met een specialisatie op dierkunde.
In september 2010 begon zij aan de master Medische Biologie aan de Universiteit van Amsterdam. Zij heeft haar master stages gelopen bij de afdeling pathologie in het Amsterdam Medisch Centrum en de afdeling auto-immuun ziektes in het Erasmus Medisch Centrum. Zij behaalde haar masterdiploma in juli 2012, waarna zij in september 2012 als promovendus begon op de afdeling Reumatologie aan het onderzoek beschreven in dit proefschrift, onder leiding van prof. Dr. René Toes, prof. Dr. Tom Huizinga en Dr. Leendert Trouw.
Momenteel is Rosanne werkzaam als scientist bij Charles River Laboratories te Leiden.

List of publications

Rosanne A. van Schaarenburg*, C. Magro-Checa*, I. Bajema, T.W.J. Huizinga, G.M. Steup-Beekman1, L.A. Trouw. C1q deficiency and neuropsychiatric systemic lupus erythematosus. Frontiers Immunology 2016
*Both authors contributed equally to this manuscript

Rosanne A. van Schaarenburg, J. Suurmond, K.L. Habets, M.C. Brouwer, D. Wouters, F.A. Kurreeman, T.W.J. Huizinga, R.E.M. Toes, L.A. Trouw. The production and secretion of complement component C1q by human mast cells. Molecular Immunology 2016

Rosanne A. van Schaarenburg*, C. Magro-Checa*, H.J. Beaart, T.W.J. Huizinga, G.M. Steup-Beekman, Leendert A. Trouw. Complement levels and anti-C1q autoantibodies in patients with neuropsychiatric systemic lupus erythematosus. Lupus 2016
*Both authors contributed equally to this manuscript

Rosanne A. van Schaarenburg, L. Schejbel, L.Truedsson, R. Topaloglu, S.M. Al-Mayouf, A. Riordan, A. Simon, M. Kallel-Sellami, P.D. Arkwright,A. Åhlin, S. Hagelberg, S. Nielsen, A. Shayesteh, A. Morales, S. Tam, F. Genel, S. Berg, A.G. Ketel, M.J. van den Berg, T.W. Kuijpers, R.F. Olsson, T.W.J. Huizinga, A.C. Lankester, L.A. Trouw. Marked variability in clinical presentation and outcome of patients with C1q immunodeficiency. Autoimmunity 2015

F.J. Beurskens, **Rosanne A. van Schaarenburg,** L.A. Trouw. C1q, antibodies and anti-C1q autoantibodies. Molecular Immunology 2015

Rosanne A. van Schaarenburg, N.A. Daha, J.J.M. Schonkeren, E.W.N. Levarht, D.J. van Gijlswijk-Janssen, F.A. Kurreeman, A. Roos, C. van Kooten, C.A. Koelman, M.R. Ernst-Kruis, R.E.M. Toes, T.W.J. Huizinga, A.C. Lankester, L.A. Trouw. Identification of a novel non-coding mutation in C1qB in a Dutch child with C1q deficiency associated with recurrent infections. Immunobiology. 2015

M.A. Dragon-Durey, C. Blanc, M.C. Marinozzi, **Rosanne A. van Schaarenburg,** Leendert A. Trouw. Autoantibodies against complement components and functional consequences. Molecular Immunology 2013

M. Mahler, **Rosanne A. van Schaarenburg,** L.A. Trouw. Anti-C1q autoantibodies, novel tests, and clinical consequences. Frontiers Immunology 2013

T.P. Rygiel, G. Karnam, G. Goverse G, A.P. van der Marel, M.J. Greuter, **Rosanne A. van Schaarenburg**, W. Visser, A.B. Brenkman, R. Molenaar, R.M Hoek, R.E. Mebius, L. Meyaard. CD200-CD200R signaling suppresses anti-tumor responses independently of CD200 expression on the tumor. Oncogene 2012

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