II

GENERAL INTRODUCTION

Complement in auto- and alloimmunity and the introduction of C4d

Introduction

SLE: A SINGLE DISEASE WITH MANY MANIFESTATIONS. WHAT IS THE COMMON DENOMINATOR? The case presented in the prologue illustrates the potential severity and heterogeneity of symptoms, the difficult treatment choices clinicians are faced with and above all, the unpredictability of the course of the disease of patients with SLE. This puzzling patient history served as a basis for several research questions that have been addressed in this thesis.

SLE is a systemic autoimmune disease occurring mainly in young women of childbearing age.[1] With a prevalence of up to 1-2 cases per 1000 in high risk groups such as Asian, Hispanic and Afro-American women, it is not an uncommon disease.[2] Virtually all organ systems can get involved, but the skin, the kidney and the brain are amongst the most vulnerable.[3] However, not two patients with SLE present alike and the description of the case above thus has limited value as one of a 'typical' SLE patient. This case merely points out that investigating common mechanistic pathways which explain the vast majority of manifestations is valuable, both for diagnostic purposes and the development of guided therapy. The complement system is one of the major contributors to the development of tissue damage in SLE. This thesis will therefore focus on the role of the complement system and on its potential as a diagnostic tool and future therapeutic target.

ANTIBODIES AND COMPLEMENT SLE is caused by an aberrant immune response, and the hallmark of the disease is the presence of autoantibodies.[4] An impressive variety of autoantibodies have been described in SLE and interestingly, the antibodies already present years before actual onset of the disease.[5] Antibodies against nuclear components such as anti-dsDNA antibodies, antinuclear antibodies, anti-SSA, and SSB antibodies are most commonly detected.[1] Antiphospholipid antibodies form a separate group of antibodies that can be detected in about 40% of SLE patients.[6] These antibodies are closely correlated to thrombotic and obstetric complications.

SLE related autoantibodies typically form immune-complexes, which deposit in tissues such as the skin, the kidneys, the brain, and the lungs. Antiphospholipid antibodies on the other hand, can directly bind to endothelial cells and trophoblast cells.[7;8] In both situations complement activation subsequently initiates a damaging sequence of inflammatory events in the tissue where the immune complexes or antibodies are deposited.[9-11]

Herein lies the clue to why this thesis is focused towards complement. When an antibody binds or an immune complex deposits, this will virtually always lead to activation of the complement system. Studying the complement system as a general pathway of injury allows overlooking the variety of different antibodies in order to see the actual effect of this on tissue level. Complement deposition provides an excellent read-out, or tissue-biomarker of previous presence of immune complexes or antibodies, despite their origin or the kinetics of the binding or deposition process.

Furthermore, it seems that the occurrence of immune-complexes or antibodies per se is not by itself sufficient to induce injury.[9] The blockade of antibodies in SLE, for instance with intravenous immunoglobulins or with plasmapheresis, has proven unsuccessful to control disease. The results of the first randomized placebo controlled trials with the anti-B-Cell agent Rituximab, a drug that aims to stop B-cells from producing antibodies, have also failed to show a significant beneficial effect.[12;13] Although some uncontrolled observational studies showed promising results, these were only achieved when Rituximab was used in combination with other immunosuppressants such as prednisone or cyclophosphamide.

A FOCUS ON CLASSICAL COMPLEMENT ACTIVATION This thesis focuses on the complement system as the common denominator, or common mechanism of injury in several manifestations of SLE and antiphospholipid syndrome. In the first part of this introductory chapter, the background and biology of the complement system will be further explained *(Part 1: The human complement system)*. It will become clear that complement activation is an extremely potent mechanism that has the ability to crudely injure microbes and pathogens just as much as self-tissues. Furthermore, the biology and clinical significance of the biomarker C4d will be outlined.

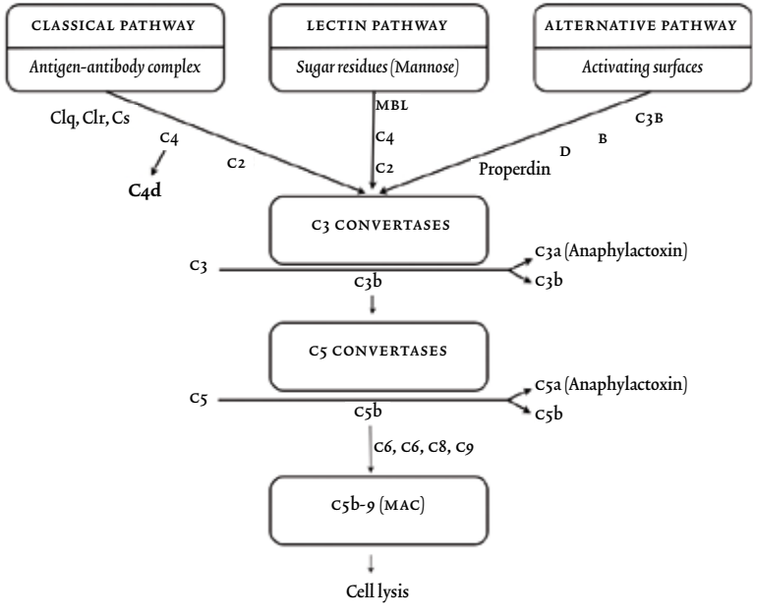
As a biomarker C4d was originally described in solid organ transplantation. Transplantation and the semi-allogeneic fetus in pregnancy provide useful analogous situations for studying the effects of antibody mediated complement activation. In the second part of the introduction, the potential contribution of complement activation to different clinical situations studied in this thesis will be outlined. It will be shown that the kidney, the placenta, and the brain are target organs for classical pathway activation, both in SLE and antiphospholipid syndrome *(Part 2: Clinical settings)*. The third section of this chapter will focus on the research-questions that form the basis of the experiments and the studies performed in this thesis *(Part 3: This thesis)*. Finally, an outline of the chapters will be given.

Part 1 – The human complement system

POWERFUL IMMUNE SURVEILLANCE The complement system is an ancient element of the human immune system.[14;15] Complement components emerge in the blood during fetal development, long before circulating antibodies appear.[16] This probably reflects complement's evolutionary history: Phagocytic cells and complement were major defense mechanisms in vertebrates before they developed the capacity for an adaptive immune response and antibody production.[10]

As a part of the first line of defense, complement can be interpreted as a complex immune surveillance system. Like the coagulation cascade, the complement system needs to be ready for action at all times. More than 30 soluble- and membrane-bound proteins circulate in a 'controlled active state'. Specific triggers, such as for instance an invading pathogen, can set of a cascade of enzymatic reactions and positive feedback loops (known as complement activation pathways, see figure 1), that can lead to the destruction of the potentially dangerous target within seconds.[10]

FIG 1 SCHEMATIC OVERVIEW OF THE COMPLEMENT SYSTEM AND THE DIFFERENT ACTIVATION PATHWAYS



Apart from directly protecting the host from invading pathogens, the proteins of the complement system participate in a variety of other physiological activities, such as the augmentation of adaptive immune responses (by activating upon the binding of antibodies and immune complexes), involvement in tissue regeneration, and participation in apoptotic cell clearance.[10;17]

HOW DOES THE COMPLEMENT CASCADE CAUSE INJURY TO ITS TARGET? The complement system operates in a way that can be summarized as 'Call for help while starting to attack'.[14;17] Firstly, activation of the cascade automatically leads to the generation of the potent anaphylatoxins C3a and C5a, which attract neutrophils and monocytes to the site of inflammation. Furthermore, C3a and C5a cause vasodilatation, smooth muscle contraction, histamine release from mast cells, and oxidative burst from neutrophils. Secondly, the activated complement cascade will generate specific membrane bound fractions and split products that coat the surface of target cells or pathogens, thereby further guiding phagocytosis. Thirdly, the final stage of the complement cascade leads to the formation of the membrane attack complex (MAC). The MAC is an ingeniously formed complex of enzymes that work together to create a pore-like structure.[18;19] This pore inserts itself into cell membranes, thereby causing cell lysis and, subsequently, cell death.

WHAT SPECIFIC TRIGGERS SET OF THE CASCADE? The three pathways by which the complement system can become activated are the classical, lectin, and alternative pathway (figure 1). All of them converge at the level of C3, and they all have the same final common pathway that leads to the formation of the membrane attack complex (MAC) on complement activating surfaces.[14]

The classical pathway of complement is initiated via binding of its recognition molecule C1q to immune complex deposits, antibody-antigen binding, charged molecules, and apoptotic and necrotic cell debris. This pathway connects innate immunity to adaptive B-cell mediated immunity when an antibody response has developed. However, in the setting of autoimmune disease or transplantation, classical complement is activated due to the deposition of autoantibodies or allo-antibodies and may lead to severe organ damage or graft rejection.[9]

The lectin pathway is activated when mannose-binding-lectin or ficolins of various carbohydrate ligands are recognized. These can be found on the surface of many different pathogens. The lectin pathway does not function via C1, as MBL functions as the recognition molecule instead.[10] Further downstream, this pathway follows the same steps as classical activation: C2, C4, and the final common pathway. Therefore, it can be difficult to distinguish between classical from and MBL pathway activation.

The third and last pathway is known as the alternative pathway. The alternative pathway's main function is to amplify the inflammatory reaction initiated by classical or MBL induced complement

activation.[20] The alternative pathway is, therefore, constantly being activated by the hydrolysis of C3, but at the same time it is tightly regulated to prevent uncontrolled activation. This situation can be compared to a snowball balanced on the top of a hill, on the verge of rolling down. Whenever C3 is activated by the classical or MBL pathway, this immediately leads to instant binding of factor B and D. This complex is subsequently stabilized by properdin forming an enzyme that leads to more C3 activation. The alternative pathway thereby creates a positive feedback loop and amplifies the inflammatory reaction initiated by classical or lectin pathway activation.

MAINTAINING THE BALANCE OF THE CASCADE: COMPLEMENT REGULATION A mechanism with such potent pro-inflammatory and cell-destructing capabilities without any sophisticated strategies to distinguish self- from non-self-tissue needs tight regulation. Nearly half of all complement components have a regulatory function.[21] Their goal is to prevent damage to host tissue by inhibiting the cascade on three vital points: Activation, amplification, and the formation of the membrane attack complex. Complement-regulating proteins can either be membrane-bound or soluble. Membrane-bound proteins [Decay Accelerating Factor (DAF) and Membrane Cofactor Protein (MCP) and CD59] are abundantly present on host-tissues, and they prevent complement from inducing auto-injury. For instance, CD59 binds to components of the membrane-attack complex, preventing it from inserting itself into the cell membrane. DAF works by preventing the formation of an enzyme derived from of C4, which normally leads to C3 activation and the formation of anaphylactoxins C5a and C3a. To prevent 'harmless bystander injury', most self-tissues that are in direct contact with blood, such as for instance endothelium and placental tissues express many different complement regulators.

Powerful soluble regulators are factor I, factor H, and the C4-binding protein. These soluble regulators mainly affect enzymes involved in the alternative route, thereby controlling amplification of the cascade and blocking positive feedback loops.

COMPLEMENT INDUCED TISSUE INJURY: EXCESSIVE ACTIVATION OR INADEQUATE REGULATION? When complement activation is able to injure host tissue, there must either have been so much activation that regulatory mechanisms were overwhelmed, or regulation must have been inadequate. Too much activation is probably the case for most manifestations of SLE and antiphospholipid syndrome. Constant exposure of host tissue to autoantibodies overwhelms the membrane-bound regulatory mechanisms, allowing direct complement mediated injury.

The consequences of inadequate regulation can be observed in patients with genetic mutations and deficiencies in regulatory molecules, such as MCP, factor I, and factor H. These mutations have recently been identified in atypical Hemolytic Uremic Syndrome (aHUS).[22;23] This severe form of thrombotic microangiopathy is a consequence of uncontrolled complement activation, which causes widespread endothelial injury and thrombosis. Recently, the same mutations as those found in aHUS have been detected in patients with severe preeclampsia and HELLP syndrome, providing striking and novel insights into these diseases.[24] It is not unthinkable that many more diseases in which excessive complement deposition is observed have a defect in complement regulation as a basis.

HOW CAN THE ACTIVITY OF THE COMPLEMENT SYSTEM BE MONITORED? There are several different ways to monitor complement activation.[25] In the case report, one method was mentioned, namely, measurement of complement components in serum. Low levels of C3 and C4 indicate that complement is being consumed and deposited in tissues. Although very useful in clinical practice, this method gives little mechanistic insight, because it cannot be determined where the activation takes place and what cells and structures are injured by complement activation.

To gain this insight, another option is to look at the tissue itself. Renal biopsies are diagnostically stained for complement components in daily clinical practice. This is done by investigating presence of complement components on frozen tissue with immuno-

fluorescence techniques. Both immunoglobulins and complement components can easily be made visible and deposition patterns can be interpreted and used for diagnostic purposes. The kidney of an SLE patient is known for the deposition pattern of IgG, IgM, IgA, C3, and C1q, which is also known as a 'full-house' pattern.[26]

A disadvantage of the use of immunofluorescence techniques on snap-frozen tissue is that the morphology of the tissue cannot be made visible. The interaction of the fluorescent components with specific cells and structures in the tissue is therefore difficult to interpret. In formalin fixed paraffin embedded tissue this problem is solved. Furthermore, using paraffin embedded tissue allows retrospective investigation of stored tissue samples, which is a huge advantage for research into relatively rare diseases such as SLE.

A SPECIAL MARKER: C4D As this thesis mainly addresses antibody-mediated diseases, a special focus is placed on classical complement activation. To visualize classical complement activation, a frequently used marker is C4d.[27] After antigen-antibody complex fixes complement, C4d is generated upon activation of C4. C4d itself has no known biological function. A unique feature of C4d is that it binds covalently to cell-surfaces or collagen basement membranes, thereby avoiding removal and raising the possibility to serve as an immunological footprint of antibody-mediated complement activation. The nickname 'footprint' emphasizes C4d's ability to remain bound at sites of complement activation much better than antibodies, which bind to the tissue by hydrostatic, van der Waals type of interactions. These interactions allow antibodies to dissociate over time, while C4d remains anchored tightly to the tissue.[28]

Presence of C4d first of all tells something about the pathophysiological process going on in tissue where it is detected. However, as a diagnostic tool in daily clinical practice C4d has is mainly used in renal transplantation, where its presence in peritubular capillaries of renal allografts is felt to represent humoral – or antibody-mediated-allograft rejection. Humoral rejection is not diagnosed solely upon C4d positivity in a graft: Only in combination with positivity of allo-

antibody formation in the recipient's serum and histological evidence for tissue injury a diagnosis can be made. This makes it complicated to give reliable sensitivity and specificity data for C4d alone. In a study by Bohmig *et al*, C4d was shown to have a high specificity (93%) but low sensitivity (31%) for the presence of anti-donor antibodies (determined by Flow Cytometric crossmatch testing of anti-HLA panel reactivity), but not for humoral rejection per se. In 58 transplant recipients, 5 immunological graft losses occurred, of which 4 out of 5 had C4d positivity in peritubular capillaries and detectable anti-donor antibodies, and 1 only had detectable antidonor antibodies without C4d. Furthermore C4d positivity was observed to have an independent predictive value for inferior graft function one year after transplantation (P=0,02).[29]

Throughout this thesis, C4d plays a central role as a tissue-biomarker of complement activation. We have studied the clinical significance of C4d in new settings such as auto-immunity and adverse pregnancy outcome. In these studies C4d helps to elucidate so far unknown pathogenic mechanisms. In neuropsychiatric SLE and in lupus nephritis C4d seems to form the link between complement mediated inflammation and the formation of thrombo-ischemic and microthrombotic injury.[30] During pregnancy, placental C4d reveals that complement mediated inflammation at the fetal-maternal interface may contribute to intrauterine fetal death, recurrent miscarriage, and even preeclampsia.[31] The last part of the thesis is dedicated to this marker alone, discussing the pros and cons for C4d as a biomarker in different clinical settings.

Part 2 – Clinical settings

ARE ALL ORGANS EQUALLY VULNERABLE TO COMPLEMENT ACTIVATION? The complement system may act in all organs and tissues. However, it is well known that some organs are more prone to suffer from complement activation than others. The kidney, the brain, and the placenta are predominant localizations for manifestations of SLE and microthrombotic disease.[11] While, at first

sight, it may seem there are few common functional features in these organs that explain this, there may be clues that can be found at the microscopic level. The glomerular filtration barrier, the blood brain barrier, and the fetal-maternal interface all serve as a 'barrier' or have an 'exchange' function that is vital for the health of the organ – and in case of the placenta – foetus. The research in this thesis is directed mainly towards complement activation in these tissues.

COMPLEMENT AND THE KIDNEY

VULNERABILITY OF THE GLOMERULAR FILTRATION BARRIER

The kidney is extremely vulnerable to complement-mediated injury, which is shown by the many renal diseases in which complement deposits can be found.[32] The vulnerability of the kidney to antibody-mediated injury and complement becomes clear in diseases with a systemic nature, where the kidney is more often affected than other organs. In SLE, more than 40% of patients develop renal involvement in which excessive complement deposits can be identified in the glomerulus.[2;26] Furthermore, acquired or inherited deficiencies of complement regulation could theoretically cause systemic symptoms, as the complement system is systemically overactive. Clinically, however, this typically leads to kidney-disease in the form of atypical HUS.[22] Finally, in solid organ transplantation, the transplanted kidney is most susceptible to antibody-mediated forms of rejection, where complement is found in peritubular capillaries and is associated with high rates of graft loss.[27;33-35] Liver, pancreas, and cardiac grafts are less often harmed by this form of rejection.[35-39]

Theories about the particular vulnerability of the kidney to complement activation have focused on the anatomic composition of the renal endothelium. The glomerular basement membrane is a unique structure composed of collagen and heparan sulphate molecules in between a layer of fenestrated endothelial cells on the luminal side and an epithelial layer of podocytes on the other side. This fenestrated structure which serves as a filter allows immune-complexes to deposit more easily than in the normal lining of a vessel.[40] On top of this, the glomerular basement membrane lacks endogenous membrane-bound complement regulators; if complement activation occurs there are a limited number of options for preventing damage. Finally, when compared to other organs, the kidney is exposed to extraordinary high levels of shear stress when glomerular endothelium is injured, promoting activation of the alternative pathway on exposed and damaged tissue.[41] This also contributes to the activation of the coagulation cascade, which explains the kidney's vulnerability to microthrombotic injury.[23;42]

The case report in the prologue shows that high levels of glomerular complement, in this particular situation caused by SLE, give rise to severe forms of glomerulonephritis, and subsequent thombotic microangiopathy. In conclusion, the kidney's anatomy provides an exceptional setting for inappropriate complement activation, leading to endothelial injury and, under certain conditions, activation of the coagulation cascade.

COMPLEMENT AND PREGNANCY

IMMUNOLOGICAL TOLERANCE

In 1953 Peter Medawar introduced the concept of 'the fetal allograft'.[43] His work served as a basis for many theories on immunologic maladaption as a cause of pregnancy complications such as recurrent fetal loss, preeclampsia, and HELLP syndrome.

For a successful pregnancy, a fetus should be tolerated by the maternal immune system, even though it is half-allogeneic. The placenta forms the physical barrier between mother and fetus, and it plays a vital role in creating an immune privileged site at the fetal–maternal interface. Trophoblast cells form the outer layer of the placenta, and they are in direct contact with maternal blood and maternal endometrial cells.[44] Specific transport mechanisms on trophoblasts regulate the uptake of nutrients from the maternal blood into the fetal circulation, and they also play a role in the immunological protection of the fetus. Trophoblast cells do not

express the classical HLA-A, HLA-B, HLA-DR, HLA-DQ, and HLA-DP molecules that are the main targets for alloreactive T-cells in transplantation. However, they do express HLA-C, HLA-E, and HLA-G molecules by which they can avoid cell-mediated cytotoxicity. For instance, HLA-G-expressing cells have been shown to induce regulatory T-cell activity and avoid NK-mediated cytotoxicity.[45;46]

Although cellular immune mechanisms have been thoroughly studied, much less is known about humoral immunity and the interplay with the complement system in pregnancy. It is evident that trafficking of maternal immunoglobulins through the placenta is a physiological phenomenon, which is necessary for the protection of the neonate against pathogens. However, as lined out above, trophoblast itself expresses certain fetal antigens to which maternal lymphocytes are exposed, and to which targeted antibodies can be formed by maternal B-cells. The formation of anti-HLA or anti-paternal antibodies does indeed occur in about 30% of pregnant women, but is normally not associated with overt pregnancy morbidity.[47;48] One possible explanation for this puzzling phenomenon is that placental trophoblast is extremely well prepared for maternal anti-fetal antibody mediated attacks. All trophoblast cells that are in contact with maternal blood produce high levels of membrane bound complement regulatory proteins such as Membrane Cofactor Protein, Decay Accelerating Factor, and CD59.[49] By doing this, the placenta can prevent inappropriate activation of the classical complement cascade at the fetal-maternal interface.

WHAT HAPPENS IN ABNORMAL PREGNANCIES AND WHAT ROLE DOES COMPLEMENT PLAY? Experimental models produced evidence that excessive complement activation at the fetal-maternal interface plays a crucial role in inducing pregnancy morbidity. Studies in murine models of immunologically-mediated pregnancy loss and preeclampsia indicate that complement activation targeting the placenta drives placental inflammation and leads to recurrent miscarriage, hypertension, proteinuria, and glomerular endotheliosis.[50-52]

The most elegant work in this field comes from experiments with complement deficient animal models. In mouse models of antibody-mediated pregnancy loss in which pregnant mice are injected with antiphospholipid antibodies, this leads to extensive fetal loss. Complement can be detected at the fetal maternal interface. When the same procedure is carried out in mice with a deficiency of complement components C3 or C5, fetal loss can be prevented.[53-55] These studies underline the vital importance of adequate complement regulation in murine pregnancy.

When focusing on complement activation as a cause for adverse outcome in human pregnancy, SLE and antiphospholipid syndrome are rational diseases to start with given the high prevalence of pregnancy morbidity in these women and the role of complement in other manifestations of those diseases. As was shown in the case-report, pregnancy and SLE can be a problematic combination.[56] When accompanied by antiphospholipid antibodies, pregnancies are complicated by preeclampsia, fetal death, or recurrent miscarriage 20 times more often than in the normal population.[6] It has been shown in vitro that antiphospholipid antibodies can bind to and affect trophoblast cells, leading to an impaired production of progesterone and placental growth factor.[57] Furthermore, an association between antiphospholipid antibodies and placental complement deposits was reported, showing that these antibodies can indeed activate the complement system at the fetal maternal interface.[58] Whether this leads to an adverse maternal and fetal outcome will be studied further in this thesis.

Another question that arises is whether the complement system could also play a role in recurrent miscarriage in women without underlying autoimmune diseases. In recurrent miscarriage, antibodies against HLA have recently been associated with a worse outcome in subsequent pregnancies.[59] Furthermore, low complement levels in mothers with recurrent miscarriages also predict a worse outcome. In preeclampsia, placental complement components have been identified and were reported to be present in abundance when compared to placentas of uneventful pregnancies.

Interestingly, a recent cohort study revealed that many patients with severe preeclampsia have mutations in genes coding for complement regulatory mechanisms, similar to those found in atypical HUS.[24]

All in all, excessive complement deposition at the fetal maternal interface seems to be a pathological finding, which is associated with adverse pregnancy outcome.[24;50;52;53;60] The placenta is normally very well prepared for complement mediated injury. Whenever complement deposits manage to injure the placenta, there is either a lack of regulation (such as in some patients with severe preeclampsia) or too much activation (such as in antiphospholipid syndrome and SLE, where there is constant exposure of antibodies that may overwhelm regulatory mechanisms). The presence of complement in placental tissue of a fetal loss might provide clues for an antibody mediated pathogenesis. This idea will be further explored in this thesis.

COMPLEMENT AND THE BRAIN

CEREBRAL IMMUNOLOGY AND THE CONSEQUENCES OF CEREBRAL COMPLEMENT ACTIVATION Through which mechanisms could complement cause injury in the brain? To find answers to this question it is necessary to focus on the immunology of the brain, which is essentially different from other organs. The cerebral microenvironment needs to be precisely regulated and strongly protected from invasion of microorganisms for optimal functioning. Since the brain has limited capacity for repair and regeneration of neurons, the immunological barriers in the brain help to minimize damage.[61] This is done mainly through mechanisms at the interface where the blood meets the nervous tissue, better known as the blood-brain barrier. The blood-brain barrier is composed of polarized brain endothelial cells connected by tight intercellular junctions. The endothelial layer is further supported by the footprocesses of astrocytic glial cells.[62] This construction limits the cellular permeability of plasma constituents (including immunoglobulins), whilst carefully regulating the uptake of nutrients and efflux of toxins and metabolites into, and out of the brain. Moreover, the blood-brain barrier allows for a reduced immune surveillance from cellular immune mechanisms. Consequently, under normal circumstances, there is little T-cell trafficking to the central nervous system and a negligible production of antibodies by B-cells within the brain.[61] The current understanding is that this attenuated cellular immune response limits 'harmless bystander injury' of neurological tissue that would occur during regular immune responses.

Complement deposition may occur in cerebral tissue. As complement components circulate in the blood, activation of the cascade in the brain is equal to activation elsewhere in the body. This is reflected by the relatively large amount of complement regulatory mechanisms expressed on cerebral endothelial cells.[63] Theoretically, the direct binding of autoantibodies or deposition of C1q-binding immunecomplexes may trigger classical complement activation at the blood brain barrier. There are many potential pathways through which complement may induce damage. For instance, C5a can induce heparan-sulphate release from endothelial cell membranes, promoting endothelial proliferation and the upregulation of e-selectin and VCAM.[64] Also, the membrane attack complex might cause lysis and it can mediate von Willebrand secretion and Tissue Factor expression in response to endothelial injury, contributing to a procoagulant state.[8;55;65] In experimental lupus models, Alexander *et al* showed that the inhibition of complement attenuates disease symptoms in the brain. This group further demonstrated that C5a production can alter the integrity of the blood-brain barrier.[66] Furthermore, the complement system seems to be able to amplify thrombo-ischemic damage. In an elegant study performed in neonatal mice, it was shown that the infarcted area that developed after clipping of a cerebral artery was more than 3 times smaller in C1q deficient animals compared to wild type mice.[67]

Taken together, experimental models show that complement activation at the blood-brain barrier can cause endothelial damage and mediate thrombo-ischemic injury, and that it also has the ability to induce a breach in the blood-brain barrier.

NEUROPSYCHIATRIC SLE Between 20-70% of patients with SLE develop nervous system involvement, and even in those without overt neurological symptoms, cognitive deficits can often be detected by careful testing.[68] The culprit (or culprits) causing neuropsychiatric symptoms in SLE remains an enigma with few therapeutic options. Both clinically and histologically, neuroinvolvement of SLE seems to be caused by a vascular, thrombo-ischaemic pathomechanism. Microvascular occlusions with hyaline or platelet microthrombi, microinfarctions and small vessel vasculopathy are the most common findings in all neuropathological studies that have been performed so far.[69-72] Affected vessels are virtually always arterioles and capillaries, with endothelial proliferation, hyalinization, and thickening of the vessel wall also known as 'vasculopathy'.[73] Interestingly, evidence of immunecomplex deposition in the small cerebral vessels has never been demonstrated.[71] In contrast to the renal endothelium, the blood-brain barrier is better able to prevent trapping of immune-complexes, which can probably be attributed to the tight junctions between adjacent endothelial cells.

As complement is a key event in many of the other organs that are involved in SLE, this mechanism could play a similar role in cerebral lupus. Although immunecomplexes are not detected in cerebral vessels, any explanation for the mechanism of NP-SLE must take into account the role of antibodies and in particular that of anti-phospholipid antibodies.[6;74] The latter can directly bind to endothelial cells, and they can activate the classical complement cascade.[6;55;75] The small vessel injury observed in NP-SLE could be caused by complement activation at the blood-brain barrier. If complement activation is mutilating enough to damage the blood-brain barrier, it may even lead to entering of neurotoxic autoantibodies into the parenchyma, causing direct neuroinflammation. These mechanisms will be investigated further in this thesis.

Part 3 – This thesis

In SLE and antiphospholipid syndrome, but also in pregnancy-associated morbidity, vital organs can be injured by means of antibody-mediated complement activation. The vast majority of these patients are young women of childbearing age. This impels efforts to further unravel these disease mechanisms and search for diagnostic tools and therapeutic targets. The complement system is a double-edged sword. When excessively activated, its beneficial effects can become detrimental and cause serious organ damage. In this thesis we focus on the role of classical complement activation, and especially on the clinical significance of C4d in the following clinical settings:

* Complement induced endothelial injury in SLE and antiphospholipid syndrome
* Complement induced injury at the fetal-maternal interface

AIMS OF THIS THESIS

* To investigate the relation between C4d and microthrombotic injury in lupus nephritis
* To explore the presence of classical complement deposition in cerebral tissue of patients with SLE, and investigate the association of C4d with thrombo-ischemic brain damage
* To determine the role of placental C4d in SLE and Antiphospholipid syndrome related adverse pregnancy outcome
* To explore the possibility of antibody mediated fetal loss in patients with recurrent miscarriage of unknown etiology
* To set out the historical role of C4d as a biomarker in solid organ transplantation against novel insights, and explore whether C4d has potential as a biomarker for other clinical settings (such as autoimmunity and pregnancy)

THESIS OUTLINE This thesis is divided into two parts. The first part will uncover the role of classical complement activation in the development of endothelial injury and microthrombotic complications of SLE and antiphospholipid syndrome. The second part will

focus on classical complement activation in adverse pregnancy outcome related to SLE and antiphospholipid syndrome, and in patients with recurrent miscarriage of unknown etiology.

The role of complement in thrombosis and complicated pregnancy cannot be investigated without attention being given to the antiphospholipid syndrome. Therefore, after this introductory chapter, the diagnosis and management of this relatively new disease is reviewed in **chapter 3.** The antiphospholipid syndrome overlaps with SLE, and is a good example of an autoimmune disease in which complement activation is the consequence of antibody binding, leading to thrombosis and complications in pregnancy. This chapter highlights the difficulty of treating APS patients with a wide variety of distressing clinical symptoms and sets the clinical scene for the rest of the thesis.

Interest in the subject was originally triggered when an unexpected association was observed between signs of earlier complement activation (complement factor C4d) and the presence of glomerular microthrombi in patients with lupus nephritis. This confirmed our suspicion that complement activation can induce endothelial injury to such an extent that thrombotic microangiopathy can develop. **Chapter 4** describes these first experiments.[30]

Neuropsychiatric involvement is a severe but poorly understood manifestation of SLE in which microthrombotic injury is often observed. So far, studies in humans have failed to provide clues for interactions between autoantibody-mediated inflammation and thrombo-ischemic lesions observed in brains of SLE patients. In line with our previous study in the kidney, we hypothesized that cerebral complement activation could play a role in inducing microthrombotic injury in NP-SLE. A nationwide search for brain tissue of SLE patients provided a unique cohort to study deposition patterns of C4d and several other complement components. Furthermore, in this study we aimed to correlate post-mortem histopathology to ex-vivo 7Tesla MRI imaging in three brains of SLE patients. The results of this study can be found in **chapter 5**.

The second part of the thesis is devoted to adverse pregnancy outcome in auto- and alloimmune settings. In **chapters *6* and *7*** the role of placental complement is investigated in two clinical settings. First, **chapter *6*** evaluates pregnancies of patients with SLE and antiphospholipid syndrome. This study describes the role of classical complement in inducing antiphospholipid antibody-mediated fetal loss, showing that C4d has potential as a diagnostic marker to detect antibody-mediated morbidity in pregnancy. This prompted us to perform the studies described in **chapter *7***, in order to investigate whether the same mechanism could play a role in recurrent miscarriage of unknown etiology. Here, anti-HLA or anti-paternal antibodies are likely candidates to cause placental insufficiency via a humoral immune-attack at the fetomaternal interface.

Throughout the whole thesis C4d plays an important role as a disease marker and as an indicator of classical complement activation. For decades, C4d has been used as a marker for antibody mediated rejection in solid organ transplantation. Will C4d remain as diagnostic tool in solid organ transplantation, or will newer molecular techniques replace it? Are findings in this thesis and in other work in this field interesting enough to start using C4d as a marker in pregnancy or in native renal disease? And finally: Is complement a target for treatment and could C4d be used as a marker to identify patients who would benefit from these treatments? These questions will be addressed in **chapter 8**, the general discussion**,** where the findings of this thesis will be summarized and placed in a more general perspective. This chapter is accompanied by several audio-interviews with experts in the field who comment on controversies surrounding the use of C4d as a biomarker. The general discussion will be followed by a summary in Dutch.

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