

CLINICAL SIGNIFICANCE OF C4D IN SLE



AND ANTIPHOSPHOLIPID SYNDROME



DANIELLE COHEN

CLINICAL SIGNIFICANCE OF C4D IN SLE AND ANTIPHOSPHOLIPID SYNDROME

Dedicated to my parents and grandfather
dr. E.H.Cohen (1916-2011)
who encouraged me to be curious

CLINICAL SIGNIFICANCE OF C4D IN SLE AND ANTIPHOSPHOLIPID SYNDROME

PROEFSCHRIFT
ter verkrijging van
de graad van Doctor aan de Universiteit Leiden
op gezag van Rector Magnificus prof. mr. P.F. van der Heijden
volgens besluit van het College voor Promoties
te verdedigen op 13 juni 2012
klokke 11.15

DOOR
Danielle Cohen
Geboren te Lambeth, Londen in 1983

PROMOTIECOMMISSIE

Promotor:	Prof. Dr. J.A. Bruijn
Co-promotores:	Dr. I.M. Bajema
	Dr. K.W.M. Bloemenkamp
Overige Leden:	Prof. Dr. T.W.J. Huizinga
	Prof. Dr. F.M. Helmerhorst
	Prof. Dr. M. Levi *(Academisch Medisch Centrum, Amsterdam)*

ISBN 9789090268002

The studies described in this thesis were performed at the Department of Pathology (head: Prof. Dr. G.J. Fleuren) of the Leiden University Medical Center, the Netherlands

This thesis was supported by:
* The departments of pathology, obstetrics, rheumatology and radiology of the Leiden University Medical Center
* The MD/PhD fund of the Leiden University Medical Center
* Stichting HELLP-syndroom Nederland
* Nederlandse Vereniging voor Lupus Erythematodes (NVLE)
* Reumafonds Nederland
* BMA BV 'Mosos'

Design: Caroline de Lint, Voorburg (caro@delint.nl)

GENERAL INTRODUCTION

1 Prologue: A case history – *9*
2 General introduction: Complement in auto- and alloimmunity and the introduction of C4d –13
3 Diagnosis and management of the antiphospholipid syndrome – *37*

PART ONE: C4D AND THROMBOTIC COMPLICATIONS

4 Potential for glomerular C4d as an indicator of thrombotic microangiopathy in lupus nephritis – *65*
5 Nationwide autopsy study links complement to thrombo-ischemia in neurolupus – *87*

PART TWO: C4D AND ADVERSE PREGNANCY OUTCOME

6 Classical complement activation as a footprint for murine and human antiphospholipid antibody-induced fetal loss – *115*
7 C4d as a footprint of maternal anti-fetal immunity in recurrent miscarriage. A novel pathogenic mechanism – 141

CONCLUSION AND DISCUSSION

8 General discussion: Pros and cons for C4d as a biomarker – *159*
9 Nederlandse samenvatting – *193*

APPENDICES

Curriculum Vitae – 201
Bibliography – 203
Acknowledgements – 204

GENERAL INTRODUCTION

PART ONE
C4D AND THROMBOTIC COMPLICATIONS

PART TWO
C4D AND ADVERSE PREGNANCY OUTCOME

CONCLUSION AND DISCUSSION

APPENDICES

I

PROLOGUE

A case history

A 27 year old Asian woman presented at the outpatient clinic of the department of rheumatology with a sudden onset of butterfly exanthema, alopecia, fever, and swollen joints of the hands and wrists. Laboratory tests revealed a rapid rise in serum creatinine, leuco- and thrombocytopenia, low levels of complement C3 and C4, positive antinuclear antibodies (ANA) and anti–double-stranded DNA (anti-dsDNA) antibodies. Urinalysis showed an active sediment suggestive of glomerulonephritis. She was diagnosed with systemic lupus erythematosus (SLE). To determine the stage of renal involvement, a renal biopsy was performed, which showed a membranous glomerulopathy and a full house immunofluorescence pattern, compatible with lupus nephritis class V. Prednisone therapy was initiated, after which her renal function stabilized, as did the other clinical symptoms.

Two years later, a second renal biopsy was taken because of declining renal function and pronounced proteinuria. A rise in anti-dsDNA and low levels of C3 and C4 were detected. The renal biopsy confirmed a renal flare, showing a diffuse proliferative glomerulonephritis compatible with lupus nephritis class IV. An abdominal ultrasound obtained for nausea and stomach ache, unexpectedly revealed a renal vein thrombosis in the right kidney. In addition to prednisone, pulse cyclophosphamide was administered, and treatment with vitamin K antagonists as anticoagulant therapy was initiated. The patient's condition and renal function stabilized after 13 cyclophosphamide pulses. The renal vein thrombosis led her clinicians to evaluate antiphospholipid antibody titers. Both anticardiolipin IgG and IgM ELISAs and the lupus anticoagulant test were negative. Therefore, it was decided to terminate the anticoagulant therapy after two years.

During this time, she was referred to a gynecologist for preconceptional counseling, and to assess whether there were signs of premature ovarian failure induced by cyclophosphamide therapy. She had no wish to become pregnant at that moment, but was considering pregnancy in a few years. The gynecologist discussed the potential dangers and difficulties of pregnancy in combination

with renal disease and SLE. The effect of pregnancy on lupus was set out against the effect of lupus on pregnancy and the fetus. Pregnancy in an SLE patient entails a high risk of lupus-flare, a high risk of renal-flare, a higher risk of hypertensive pregnancy disorders such as preeclampsia and HELLP syndrome and a higher risk of thrombosis. Inversely, SLE affects pregnancy and the fetus by inducing a higher risk of miscarriage, intrauterine growth restriction, (iatrogenic) preterm birth and even fetal death. Especially the presence of antiphospholipid antibodies reduces the chance of an uncomplicated pregnancy. Finally, the gynecologist talked about genetics and the teratogenic effect of certain medications.

It appeared that she was using an oral contraceptive pill for heavy menstrual periods, but not for contraception per sé. Due to the renal vein thrombosis, it was advised to stop oral contraception to reduce the risk of further thrombotic complications, even though antiphospholipid antibodies were still undetectable. Furthermore, it was decided that aspirin and a daily prophylactic dose of low molecular weight heparin during pregnancy were indicated in case she would become pregnant.

Unfortunately, within a year after the visit to the outpatient clinic of the department of gynecology she developed menopausal symptoms of flushing and an irregular menstrual cycle. Subsequent analysis showed premature ovarian failure, most likely induced by cyclophophamide pulse therapy.

Despite pulse cyclophosphamide and prednisone therapy, the patient's renal function gradually continued to decline during the following years; her blood pressure slowly increased to a mean of 180/100, and proteinuria rose to a nephrotic range. A third renal biopsy specimen was obtained when she was 36. This time, in addition to lupus nephritis class IV, histological evidence of a thrombotic microangiopathy was found. Anticoagulant treatment was resumed. A thorough diagnostic work-up for underlying causes of thrombotic microangiopathy was performed, in which antiphospholipid antibodies were still negative, but serological complement levels C3 and C4 appeared to be extremely low. Further analysis revealed no evidence for HUS, TTP, or other conditions related to thrombotic microangiopathy apart from active SLE.

Shortly thereafter, she presented with upper body ataxia and progressive aphasia, indicators of cerebral involvement of SLE which is known as neuropsychiatric SLE. She was treated with a high dose of immunosuppression, 10 plasmapheresis sessions and hemodialysis, but despite this treatment, her GFR had now declined to 15 micromoles/liter. Prolonged hemodialysis was started at the age of 39. Her condition stabilized, though permanent neurological injury had developed for which she was admitted to a rehabilitation center.

Two years later, she suffered acute neurologic deterioration and respiratory distress. She was admitted to the intensive care unit, where hemolytic anaemia, deep thrombocytopenia, multiple purpura and an oliguric state pointed at a recurrence of the thrombotic microangiopathy. Despite additional plasmapheresis sessions and high doses of immunsuppression she died, most likely due to diffuse alveolar hemorrhage and bleeding in and around multiple organs caused by severe thrombocytopenia, active lupus nephritis and widespread microthrombotic injury.

At autopsy, the kidneys showed signs of extensive chronic damage, classified as lupus nephritis class IVc. Furthermore, multiple fibrin microthrombi were present in glomeruli. In the brain, diffuse chronic and global ischemia was found, accompanied by several vessels in the white matter with intraluminal thrombi. For research-purposes, her kidney biopsy specimens and available tissue samples from the cerebral cortex were stained for complement factor C4d, which revealed diffuse and intense complement depositions in all affected organs.

This patient appeared as a subject in two out of four research articles described in this thesis (Chapter 4 and 5). The striking observation of widespread thrombo-ischemic injury in combination with extensive complement deposition served as a basis for many ideas and research questions that will be addressed in the following chapters. Most importantly, this case history underlines that all patients tell a story and that all medical science begins and ends with stories like these.

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.

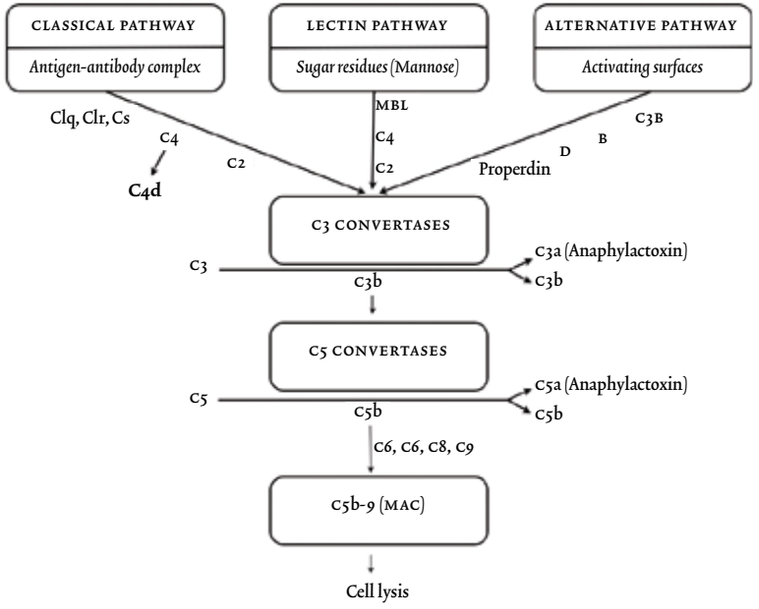
As a biomarker C4d was originally described in solid organ transplantation. Transplantation and the semi-allogeneic fetus in pregnancy provide useful analogous situations for studying the effects of antibody mediated complement activation. In the second part of the introduction, the potential contribution 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.

REFERENCES

1 D'Cruz DP, Khamashta MA, Hughes GR. Systemic lupus erythematosus. *Lancet* 2007; 369(9561):587-596.

2 Pons-Estel GJ, Alarcon GS, Scofield L, Reinlib L, Cooper GS. Understanding the epidemiology and progression of systemic lupus erythematosus. *Semin Arthritis Rheum* 2010; 39(4):257-268.

3 Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF *et al.* The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; 25(11): 1271-1277.

4 Elkon K. Autoantibodies in systemic lupus erythematosus. *Curr Opin Rheumatol* 1995; 7(5):384-388.

5 Arbuckle MR, McClain MT, Rubertone MV, Scofield RH, Dennis GJ, James JA *et al.* Development of autoantibodies before the clinical onset of systemic lupus erythematosus. *N Engl J Med* 2003; 349(16):1526-1533.

6 Cohen D, Berger SP, Steup-Beekman GM, Bloemenkamp KW, Bajema IM. Diagnosis and management of the antiphospholipid syndrome. *BMJ* 2010; 340:c2541.

7 Pierangeli SS, Vega-Ostertag M, Liu X, Girardi G. Complement activation: a novel pathogenic mechanism in the antiphospholipid syndrome. *Ann N Y Acad Sci* 2005; 1051:413-420.

8 Pierangeli SS, Chen PP, Raschi E, Scurati S, Grossi C, Borghi MO *et al.* Antiphospholipid antibodies and the antiphospholipid syndrome: pathogenic mechanisms. *Semin Thromb Hemost* 2008; 34(3):236-250.

9 Allam R, Anders HJ. The role of innate immunity in autoimmune tissue injury. *Curr Opin Rheumatol* 2008; 20(5):538-544.

10 Sarma JV, Ward PA. The complement system. *Cell Tissue Res* 2011; 343(1):227-235.

11 Chen M, Daha MR, Kallenberg CG. The complement system in systemic autoimmune disease. *J Autoimmun* 2010; 34(3):J276-J286.

12 Merrill JT, Neuwelt CM, Wallace DJ, Shanahan JC, Latinis KM, Oates JC *et al.* Efficacy and safety of rituximab in moderately-to-severely active systemic lupus erythematosus: the randomized, double-blind, phase II/III systemic lupus erythematosus evaluation of rituximab trial. *Arthritis Rheum* 2010; 62(1):222-233.

13 Bertsias G, Ioannidis JP, Boletis J, Bombardieri S, Cervera R, Dostal C *et al.* EULAR recommendations for the management of systemic lupus erythematosus. Report of a Task Force of the EULAR Standing Committee for International Clinical Studies Including Therapeutics. *Ann Rheum Dis* 2008; 67(2):195-205.

14 Walport MJ. Complement. First of two parts. *N Engl J Med* 2001; 344(14):1058-1066.

15 Walport MJ. Complement. Second of two parts. *N Engl J Med* 2001; 344(15):1140-1144.

16 Miyano A, Nakayama M, Fujita T, Kitajima H, Imai S, Shimizu A. Complement activation in fetuses: assessment by the levels of complement components and split products in cord blood. *Diagn Clin Immunol* 1987; 5(2):86-90.

17 Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. *Nat Immunol* 2010; 11(9):785-797.

18 Kondos SC, Hatfaludi T, Voskoboinik I, Trapani JA, Law RH, Whisstock JC *et al.* The structure and function of mammalian membrane-attack complex/perforin-like proteins. *Tissue Antigens* 2010; 76(5):341-351.

19 Pipkin ME, Lieberman J. Delivering the kiss of death: progress on understanding how perforin works. *Curr Opin Immunol* 2007; 19(3):301-308.

20 Lachmann PJ. The amplification loop of the complement pathways. *Adv Immunol* 2009; 104:115-149.

21 Zipfel PF, Skerka C. Complement regulators and inhibitory proteins. *Nat Rev Immunol* 2009; 9(10):729-740.

22 Noris M, Remuzzi G. Atypical hemolytic-uremic syndrome. *N Engl J Med* 2009; 361(17):1676-1687.

23 Kavanagh D, Richards A, Atkinson J. Complement regulatory genes and hemolytic uremic syndromes. *Annu Rev Med* 2008; 59:293-309.

24 Salmon JE, Heuser C, Triebwasser M, Liszewski MK, Kavanagh D, Roumenina L *et al.* Mutations in complement regulatory proteins predispose to preeclampsia: a genetic analysis of the PROMISSE cohort. *PLoS Med* 2011; 8(3):e1001013.

25 Ahmed AE, Peter JB. Clinical utility of complement assessment. *Clin Diagn Lab Immunol* 1995; 2(5):509-517.

26 Cameron JS. Lupus nephritis. *J Am Soc Nephrol* 1999; 10(2):413-424.

27 Nickeleit V, Mihatsch MJ. Kidney transplants, antibodies and rejection: is C4d a magic marker? *Nephrol Dial Transplant* 2003; 18(11): 2232-2239.

28 Murata K, Baldwin WM, III. Mechanisms of complement activation, C4d deposition, and their contribution to the pathogenesis of antibody-mediated rejection. *Transplant Rev (Orlando)* 2009; 23(3):139-150.

29 Bohmig GA, Exner M, Habicht A, Schillinger M, Lang U, Kletzmayr J *et al.* Capillary C4d deposition in kidney allografts: a specific marker of alloantibody-dependent graft injury. *J Am Soc Nephrol* 2002; 13(4):1091-1099.

30 Cohen D, Koopmans M, Kremer H, I, Berger SP, Roos van GM, Steup-Beekman GM *et al.* Potential for glomerular C4d as an indicator of thrombotic microangiopathy in lupus nephritis. *Arthritis Rheum* 2008; 58(8):2460-2469.

31 Cohen D, Buurma A, Goemaere NN, Girardi G, le CS, Scherjon S *et al.* Classical complement activation as a footprint for murine and human antiphospholipid antibody-induced fetal loss. *J Pathol* 2011; 225(4):502-511.

32 Berger SP, Roos A, Daha MR. Complement and the kidney: what the nephrologist needs to know in 2006? *Nephrol Dial Transplant* 2005; 20(12):2613-2619.

33 Collins AB, Schneeberger EE, Pascual MA, Saidman SL, Williams WW, Tolkoff-Rubin N *et al.* Complement activation in acute humoral renal allograft rejection: diagnostic significance of C4d deposits in peritubular capillaries. *J Am Soc Nephrol* 1999; 10(10):2208-2214.

34 Feucht HE. Complement C4d in graft capillaries -- the missing link in the recognition of humoral alloreactivity. *Am J Transplant* 2003; 3(6):646-652.

35 Platt JL. C4d and the fate of organ allografts. *J Am Soc Nephrol* 2002; 13(9):2417-2419.

36 Colvin RB. C4d in liver allografts: a sign of antibody-mediated rejection? *Am J Transplant* 2006; 6(3):447-448.

37 Crespo-Leiro MG, Veiga-Barreiro A, Domenech N, Paniagua MJ, Pinon P, Gonzalez-Cuesta M *et al.* Humoral heart rejection (severe allograft dysfunction with no signs of cellular rejection or ischemia): incidence, management, and the value of C4d for diagnosis. *Am J Transplant* 2005; 5(10):2560-2564.

38 Fedrigo M, Gambino A, Tona F, Torregrossa G, Poli F, Benazzi E *et al.* Can C4d immunostaining on endomyocardial biopsies be considered a prognostic biomarker in heart transplant recipients? *Transplantation* 2010; 90(7):791-798.

39 Magro CM, Pope HA, Klinger D, Orosz C, Adams P, Waldman J *et al.* Use of C4d as a diagnostic adjunct in lung allograft biopsies. *Am J Transplant* 2003; 3(9):1143-1154.

40 Abrahamson DR. Structure and development of the glomerular capillary wall and basement membrane. *Am J Physiol* 1987; 253 (5 Pt 2):F783-F794.

41 Savage CO. The biology of the glomerulus: endothelial cells. *Kidney Int* 1994; 45(2):314-319.

42 Hepburn NJ, Ruseva MM, Harris CL, Morgan BP. Complement, roles in renal disease and modulation for therapy. *Clin Nephrol* 2008; 70(5):357-376.

43 Medawar PB. Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates. Symp.Soc.Exp.Biol. [7], 320-338. 1953. Ref Type: Journal (Full)

44 Hunt JS. Stranger in a strange land. *Immunol Rev* 2006; 213:36-47.

45 Le BP, Mallet V. HLA-G and pregnancy. *Rev Reprod* 1997; 2(1):7-13.
46 Guerin LR, Prins JR, Robertson SA. Regulatory T-cells and immune tolerance in pregnancy: a new target for infertility treatment? *Hum Reprod Update* 2009; 15(5):517-535.
47 Ahrons S. HL-A antibodies: influence on the human foetus. *Tissue Antigens* 1971; 1(3):129-136.
48 Balasch J, Ercilla G, Vanrell JA, Vives J, Gonzalez-Merlo J. Effects of HLA antibodies on pregnancy. *Obstet Gynecol* 1981; 57(4):444-446.
49 Holmes CH, Simpson KL, Okada H, Okada N, Wainwright SD, Purcell DF *et al.* Complement regulatory proteins at the feto-maternal interface during human placental development: distribution of CD59 by comparison with membrane cofactor protein (CD46) and decay accelerating factor (CD55). *Eur J Immunol* 1992; 22(6):1579-1585.
50 Girardi G, Salmon JB. The role of complement in pregnancy and fetal loss. *Autoimmunity* 2003; 36(1):19-26.
51 Girardi G, Yarilin D, Thurman JM, Holers VM, Salmon JE. Complement activation induces dysregulation of angiogenic factors and causes fetal rejection and growth restriction. *J Exp Med* 2006; 203(9):2165-2175.
52 Salmon JE, Girardi G, Holers VM. Activation of complement mediates antiphospholipid antibody-induced pregnancy loss. *Lupus* 2003; 12(7):535-538.
53 Girardi G, Bulla R, Salmon JE, Tedesco F. The complement system in the pathophysiology of pregnancy. *Mol Immunol* 2006; 43(1-2):68-77.
54 Girardi G, Berman J, Redecha P, Spruce L, Thurman JM, Kraus D *et al.* Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. *J Clin Invest* 2003; 112(11):1644-1654.
55 Pierangeli SS, Girardi G, Vega-Ostertag M, Liu X, Espinola RG, Salmon J. Requirement of activation of complement C3 and C5 for antiphospholipid antibody-mediated thrombophilia. *Arthritis Rheum* 2005; 52(7):2120-2124.
56 Ruiz-Irastorza G, Khamashta MA, Castellino G, Hughes GR. Systemic lupus erythematosus. *Lancet* 2001; 357(9261):1027-1032.
57 Di Simone N, Luigi MP, Marco D, Fiorella DN, Silvia D, Clara DM *et al.* Pregnancies complicated with antiphospholipid syndrome: the pathogenic mechanism of antiphospholipid antibodies: a review of the literature. *Ann N Y Acad Sci* 2007; 1108:505-514.
58 Shamonki JM, Salmon JE, Hyjek E, Baergen RN. Excessive complement activation is associated with placental injury in patients with antiphospholipid antibodies. *Am J Obstet Gynecol* 2007; 196(2):167-5.
59 Nielsen HS, Witvliet MD, Steffensen R, Haasnoot GW, Goulmy E, Christiansen OB *et al.* The presence of HLA-antibodies in recurrent miscarriage patients is associated with a reduced chance of a live birth. *J Reprod Immunol* 2010; 87 (1-2):67-73.
60 Girardi G. Complement inhibition keeps mothers calm and avoids fetal rejection. *Immunol Invest* 2008; 37(5):645-659.
61 Pardridge WM. Introduction to the blood-brain barrier. Cambridge University Press; 1998.
62 Abbott NJ, Ronnback L, Hansson E. Astrocyte-endothelial interactions at the blood-brain barrier. *Nat Rev Neurosci* 2006; 7(1):41-53.
63 Barnum SR. Complement in central nervous system inflammation. *Immunol Res* 2002; 26(1-3):7-13.
64 Ward PA. The harmful role of c5a on innate immunity in sepsis. *J Innate Immun* 2010; 2(5): 439-445.
65 Girardi G, Mackman N. Tissue factor in antiphospholipid antibody-induced pregnancy loss: a pro-inflammatory molecule. *Lupus* 2008; 17(10):931-936.
66 Jacob A, Hack B, Chiang E, Garcia JG, Quigg RJ, Alexander JJ. C5a alters blood-brain barrier integrity in experimental lupus. *FASEB J* 2010; 24(6):1682-1688.
67 Ten VS, Sosunov SA, Mazer SP, Stark RI, Caspersen C, Sughrue ME *et al.* C1q-deficiency is neuroprotective against hypoxic-ischemic brain injury in neonatal mice. *Stroke* 2005; 36(10):2244-2250.
68 Joseph FG, Scolding NJ. Neurolupus. *Pract Neurol* 2010; 10(1):4-15.
69 Sibbitt WL, Jr., Brooks WM, Kornfeld M, Hart BL, Bankhurst AD, Roldan CA. Magnetic resonance imaging and brain histopathology in neuropsychiatric systemic lupus erythematosus. *Semin Arthritis Rheum* 2010; 40(1):32-52.
70 Johnson RT, Richardson EP. The neurological manifestations of systemic lupus erythematosus. *Medicine (Baltimore)* 1968; 47(4):337-369.
71 Hanly JG, Walsh NM, Sangalang V. Brain pathology in systemic lupus erythematosus. *J Rheumatol* 1992; 19(5):732-741.
72 Devinsky O, Petito CK, Alonso DR. Clinical and neuropathological findings in systemic lupus erythematosus: the role of vasculitis, heart emboli, and thrombotic thrombocytopenic purpura. *Ann Neurol* 1988; 23(4):380-384.
73 Hess DC. Cerebral lupus vasculopathy. Mechanisms and clinical relevance. *Ann N Y Acad Sci* 1997; 823:154-168.
74 Ruiz-Irastorza G, Crowther M, Branch W, Khamashta MA. Antiphospholipid syndrome. *Lancet* 2010; 376(9751):1498-1509.
75 Pierangeli SS, Colden-Stanfield M, Liu X, Barker JH, Anderson GL, Harris EN. Antiphospholipid antibodies from antiphospholipid syndrome patients activate endothelial cells in vitro and in vivo. *Circulation* 1999; 99(15):1997-2002.

III

DIAGNOSIS AND MANAGEMENT OF THE ANTIPHOSPHOLIPID SYNDROME

DANIELLE COHEN / STEFAN P BERGER / GERDA M STEUP-BEEKMAN
KITTY W M BLOEMENKAMP / INGEBORG M BAJEMA

BRITISH MEDICAL JOURNAL 2010;340:C2541

Introduction

Antiphospholipid syndrome was first described 27 years ago in patients with systemic lupus erythematosus (SLE) and positive anticardiolipin antibodies, who presented with a clotting syndrome that affected arteries and veins.[1] Female patients had a high risk of recurrent miscarriage and late fetal loss. The international classification criteria for this syndrome used today are based on those initial clinical observations.[2]

The syndrome is under-recognised and underdiagnosed and can have devastating consequences if untreated, mainly because of uncontrolled thrombosis. Difficulties in diagnosis are compounded by a lack of standardisation of diagnostic tests. Early recognition is crucial, because treatment can reduce mortality and morbidity in relatively young people who often present with diseases such as stroke, myocardial infarction, and deep vein thrombosis.

Because of its variable clinical presentation, patients with antiphospholipid syndrome present to a variety of medical practitioners. Here, we introduce this complicated and intriguing syndrome, and provide basic guiding principles for the recognition, diagnosis, and management of affected patients.

What is the antiphospholipid syndrome?

Antiphospholipid syndrome is a systemic autoimmune disorder characterized by both arterial and venous thrombosis, adverse outcome in pregnancy (for mother and fetus), and raised titers of antiphospholipid antibodies. It occurs in isolation (primary antiphospholipid syndrome) in more than 50% of patients, but it can be associated with other autoimmune diseases. SLE is the most common–20-35% of patients with SLE develop secondary antiphospholipid syndrome.[2] An acute variant of the syndrome–catastrophic antiphospholipid syndrome–results in widespread thrombotic microangiopathy and multiple organ failure (Box 2).[3] Classification criteria were last updated in 2006 (Box 1). A combination of clinical and laboratory findings is required to confirm the diagnosis.[4]

– BOX 1 –

CLASSIFICATION CRITERIA FOR THE ANTIPHOSPHOLIPID SYNDROME

Antiphospholipid syndrome is present if at least one of the clinical criteria and one of the laboratory criteria that follow are met:

CLINICAL CRITERIA

Vascular thrombosis
One or more clinical episodes of arterial, venous, or small vessel thrombosis, in any tissue or organ. Thrombosis must be confirmed by objective validated criteria (i.e. unequivocal findings of appropriate imaging studies of histopathology). For histopathologic confirmation, thrombosis should be present without significant evidence of inflammation in the vessel wall.

Pregnancy morbidity
One or more unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation, with normal fetal morphology documented by ultrasound or by direct examination of the fetus.
Or one or more premature births of a morphologically normal neonate before 34th week of gestation because of: (I) eclampsia or severe preeclampsia defined according to standard definitions, or (II) recognized features of placental insufficiency.
Or three or more unexplained consecutive spontaneous miscarriages before the 10th week of gestation, with maternal anatomic or hormonal abnormalities excluded and paternal and maternal chromosomal causes excluded.

LABORATORY CRITERIA

Lupus Anticoagulant present in plasma, on two or more occasions at least 12 weeks apart, detected according to the guidelines of the International Society on Thrombosis and Haemostasis (Scientific Subcommittee on lupus anticoagulant/phospholipid – dependent antibodies).[10]

Anticardiolipin antibody of IgG and/or IgM isotype in serum or plasma, present in medium or high titer (i.e. ▸ 40 GPL or MPL, or ▸ the 99th percentile), on two or more occasions, a least 12 weeks apart, measured by standardized ELISA.
Anti-β_2-glycoprotein I antibody of IgG and/or IgM isotype in serum or plasma, present in medium or high titer (i.e. ▸ 40 GPL or MPL, or ▸ the 99th percentile), on two or more occasions, a least 12 weeks apart, measured by standardized ELISA, according to recommended procedures.[59]

Who gets it?

SLE may affect up to 1 in 1000 women (depending on ethnic origin), and around 30% of those develop secondary antiphospholipid syndrome. The population prevalence of primary antiphospholipid syndrome is unknown, although it is estimated that this disease affects up to 0,5% of the general population.

Antiphopholipid syndrome occurs mainly in young women of fertile age, rarely occurs in children and only 12% of patients present over the age of 50. In a large international cohort of patients diagnosed with the syndrome the mean age at diagnosis was 34 + 13 years SD. The male:female ratio was 1:3.5 for primary disease and 1:7 for secondary diagnosis associated with SLE.[2] A recently reported, unique cohort of 121 pediatric cases (primary and secondary) had a mean age of 10.7 at disease onset (range 1.0-17.9), and a male:female ratio of almost 1:1.[5] Patients who present after 50 are more often male and present more often with stroke and coronary heart disease.[2] Less than 1% of patients with primary or secondary antiphospholipid syndrome develop the catastrophic form and in almost half of them, catastrophic antiphospholipid syndrome appears *de novo*, without prior thrombotic events.[2]

What are antiphospholipid antibodies and how are they associated with clinical symptoms?

Antiphospholipid antibodies form a heterogeneous group of autoantibodies directed at plasma proteins that bind to phospholipids.[6] Some antibodies from the antiphospholipid family have a paradoxical effect on coagulation: in vivo they are associated with recurrent thrombosis, but in vitro they increase phospholipid dependent clotting times, a phenomenon known as 'lupus anticoagulant' activity. This peculiar phenomenon was named 'Lupus anticoagulant' and refers to the *ability* of certain antibodies to prolong phospholipid dependent clotting time. The so-called 'lupus anticoagulant assay'

– BOX 2 –

THE CATASTROPHIC ANTIPHOSPHOLIPID SYNDROME

Management of catastrophic antiphospholipid syndrome is an extremely rare life threatening condition, characterized by the rapid development of multiple microthrombi in various organ systems, typically the brain, kidneys, lung and skin.[60] Thrombocytopenia, hemolysis, schistocytes, and activation of the coagulation system are frequent laboratory findings and thus thrombotic thrombocytopenic purpura, hemolytic uremic syndrome and disseminated intravascular coagulation are important differential diagnostic considerations. The mortality of the catastrophic antiphospholipid syndrome approaches 50%.[61] Figure 3 gives examples of schistocutes and microthrombi in the kidneys and the brain.

Data on how to treat the catastrophic antiphospholipid syndrome is limited but current treatment regimes seem to have led to a marked reduction of the mortality when compared to historical case series.[61] Successful treatment regimes include anticoagulation, high dose corticosteroids and plasma exchange with or without intravenous immunoglobulins. Plasma exchange seems to be particularly useful in the setting of thrombotic microangiopathy. Possible precipitating disorders such as infection should be treated promptly.

is a functional assay based on a combination of several clotting tests. Two other antibodies are useful for diagnosing antiphospholipid syndrome: anticardiolipin antibodies and anti-β2 glycoprotein I antibodies (box 1), both of which can be detected by enzyme linked immunosorbent assays (ELISAS).[4]

Antibodies with lupus anticoagulant activity are of major importance clinically as two systematic reviews found them to be strongly correlated with thrombotic and obstetric complications of anti-phospholipid syndrome.[7;8] Table 1 describes assays for lupus anticoagulant, anticardiolipin antibodies, and anti-β2 glyco-protein I antibodies.

Unfortunately, agreement between laboratories for all of these assays is poor. A recent survey that evaluated lupus anticoagulant positive plasma samples found a false positive rate of 24%.[9] This highlights the importance of good communication between the laboratory and the clinician when making a diagnosis and of ensuring that guidelines are followed.[10]

Antiphospholipid antibodies are found in 1-5% of apparently healthy subjects. Prevalence increases with age and may be influenced by chronic disease, infections, malignancies, and the use of certain drugs. Positivity in these conditions usually arises from IgM antibodies at low titres and is not associated with thrombosis or adverse pregnancy outcome.[11]

Persistent positivity is rare. In a cross sectional study of 552 healthy blood donors, 6.5% had anticardiolipin IgG, but fewer than 2% still had increased titres nine months later.[12] A definitive diagnosis of antiphospholipid syndrome requires the presence of clinical criteria and positive results for at least one of the three assays on at least two separate occasions 12 weeks apart because only persistent antiphospholipid antibodies are clinically relevant.[4;13]

The assays currently used for detecting antiphospholipid antibodies show variable correlations with clinical symptoms. Well designed prospective diagnostic studies are scarce. Difficulties in interpreting clinical-serological studies arise from non-standardised assays, variable inclusion criteria, and broad definitions for case selection. Overall, the evidence supports the following:

* Lupus anticoagulant strongly associates with venous thrombosis, both in SLE and in the general population (OR 11).[7] This effect is stronger in younger age groups (◂ 50).[14]
* Lupus anticoagulant strongly associates with stroke, both in SLE and the general population. (OR 8,1 95% CI 2.4–27.5) This effect is stronger in young age groups (◂50).[15;16]
* Lupus anticoagulant is strongly associated with fetal loss ▸ 10 wks of gestation. (OR 7,8 95% CI 2.30–26.45).[8]
* Lupus anticoagulant predicts venous thrombosis and fetal loss more strongly than anticardiolipin antibodies (OR ranging from 1,6-3,5).[7;8]
* The anticardiolipin ELISA is considered to have high sensitivity but low specificity. It has a stronger association with morbidity in pregnancy than with thrombosis.[8;17;18]
* Studies that have investigated the relationship between anti-$\beta_2$glycoprotein I antibodies and clinical symptoms have shown

contradictory findings.[8;13;19] The clinical relevance of isolated a anti-$\beta_2$glycoprotein I antibodies remains uncertain.

* Patients with triple positivity for lupus anticoagulant, anticardiolipin and anti-$\beta_2$glycoprotein I antibodies seem to have a particularly high risk for future pregnancy morbidity or thromboembolism (OR = 34.4, 95% CI 3.5-335).[20;21]
* Established risk factors for thrombosis such as smoking (arterial disease) and oral contraception (venous thrombosis) contribute to a further increased risk of thrombosis in the presence of antiphospholipid antibodies.[15]
* The risk of apparently healthy people with persistently positive antiphospholipid antibodies to eventually develop a clinical event, such as thrombosis or adverse pregnancy outcome remains unknown.

What is known about the aetiology and pathophysiology of antiphospholipid syndrome?

The cause of the production of autoantibodies to phospholipid binding proteins such as anti-β_2-glycoprotein I is largely unknown.[6;22]

EFFECT ON COAGULATION AND INFLAMMATORY PATHWAYS Antiphospholipid antibodies affect the coagulation cascade and inflammation. In a process mediated by β2 glycoprotein I, antiphospholipid antibodies bind to platelets and endothelial cells, activating endothelial cells and inducing a procoagulant state. Antibody binding also activates complement[23], resulting in recruitment of other inflammatory cells, activation of tissue factor, endothelial damage, and finally thrombosis.[24] Although cerebral involvement is thought to be mainly thrombotic in nature, evidence now suggests that antiphospholipid antibodies may have more direct effects, causing neurological impairment unrelated to thrombosis through antibody-cellular interactions, possibly because of complement activation[25] or a disrupted blood-brain barrier.[26;27]

IS THERE AN ADDITIONAL TRIGGER? Most patients develop a discrete thrombotic event at a certain site in the body, suggesting that an additional trigger or risk factor–a 'second hit'–is needed for the development of thrombosis. Infection, local endothelial damage, and pregnancy are possible candidates.

PREGNANCY Thrombosis in the placental vasculature was initially thought to be the main cause of adverse outcomes in pregnancy. However, placental thrombosis and infarction are not specific to antiphospholipid syndrome but occur in other conditions, such as non-antiphospholipid syndrome pre-eclampsia and HELLP syndrome.[28] In vitro and animal studies showing that antiphospholipid antibodies can bind directly to trophoblast cells and cause direct cellular injury, defective invasiveness, and a local inflammatory response as a result of activation of the classical and alternative pathways of complement provided important insights into the pathophysiology of pregnancy loss.[24;29] Moreover, they showed that the protective effect of heparin resulted from its anti-complement activity and not only from its effects on coagulation.[30] Antiphospholipid antibodies seem to cause direct dysfunction of the trophoblast as well as activation of complement at the fetomaternal interface, resulting in an impaired exchange of blood components between mother and fetus, which can lead to early miscarriage, preeclampsia, intrauterine growth restriction or even intrauterine fetal death.

How do patients with antiphospholipid syndrome present?

The clinical features of antiphospholipid syndrome are diverse and can affect all organ systems. Figure 1 gives an overview of the most common clinical findings. Venous thrombosis, along with its complications, is more common than arterial thrombosis. In a large cohort of 1000 patients deep vein thrombosis in the leg

was the first symptom in 32%, and pulmonary embolism in 14%.[2] Other vesseIs such as renal veins, hepatic, subclavian, and retinal veins, cerebral sinuses and vena cava are more often affected than in non antiphospholipid syndrome related thrombosis.[2]

The most common arterial thrombotic events are stroke and transient ischaemic attack, which are the initial clinical manifestation in 13% and in 7% of patients, respectively.[2] Recurrent thrombotic events are common. The vascular pattern of recurrent thrombosis is fairly consistent for venous thrombosis (70% venous recurrence) and arterial thrombosis (90% arterial recurrence).[31]

CEREBRAL INVOLVEMENT Cerebral involvement is common in antiphospholipid syndrome[32] and was highlighted in the original description of the syndrome.[1] Cerebral ischaemia, migraine, cognitive dysfunction, seizures, chorea, transverse myelitis, psychosis, depression, and Guillain-Barré syndrome have all been associated with the presence of antiphospholipid antibodies.[27] Despite a strong observed association between chronic headache, including migraine, and antiphospholipid syndrome [33], studies exploring the relationship between headache and antiphospholipid antibodies have shown contradictory results.[34] An association has been reported between valvular heart disease and central nervous system manifestations of the syndrome, which suggests that cerebral emboli from valvular lesions may be a risk.[35]

OTHER ORGAN INVOLVEMENT The most common cardiac abnormality in patients with antiphospholipid syndrome is non-bacterial thrombotic endocarditis characterized by adherent platelet-fibrin thrombi on the endocardial surface of valves, which has been reported in 11.6% of patients during the evolution of disease.[2;36] Myocardial infarction is the presenting symptom of the syndrome in 2.8% of patients.[2] Prospective studies have shown that presence of antiphospholipid antibodies is associated with an increased risk of myocardial infarction.[15;17]

– BOX 3 –

CONDITIONS THAT POINT TO ANTIPHOSPHOLIPID SYNDROME

Red Flags

Unexplained deep vein thrombosis and/or pulmonary embolism under 50
Stroke under 50
Transient ischemic attack under 50
Recurrent thrombosis
Thrombosis in an unusual site
Unexplained fetal loss after 10 weeks gestation
Severe and/or early preeclampsia
Severe intrauterine growth restriction
Preeclampsia with severe thrombocytopenia
Cardiac valve disease *(in combination with other symptoms in this box)*
If a patient is diagnosed with SLE

Yellow Flags

Livedo Reticularis
Raynaud phenomenon
Unexplained persistent thrombocytopenia
Recurrent early pregnancy loss

Thrombosis can occur anywhere in the renal vasculature, ranging from occlusion of the renal veins and arterial trunk to microthrombi in glomerular capillaries. The latter can cause rapid decline of renal function.[2] In secondary antiphospholipid syndrome it is debated whether presence of antiphospholipid antibodies leads to a worsened outcome for traditional lupus nephritis. Although prospective studies have not addressed this matter so far, retrospective analyses provide good evidence for this.[37]

Haematological manifestations such as thrombocytopenia and haemolytic anaemia, and dermal symptoms such as livedo reticularis occur in 10-30% of patients although these features are not included in the classification criteria.[2] Box 3 lists red and yellow flag conditions that indicate when antiphospholipid syndrome should be included in a differential diagnosis.

MATERNAL AND FETAL EFFECTS IN PREGNANCY Obstetric criteria used to define antiphospholipid syndrome are fetal loss after 10 weeks' gestation, three or more unexplained consecutive embryonic losses before the 10th week of gestation, and pre-eclampsia or features of placental insufficiency associated with the premature birth of a morphologically normal neonate before the 34th week of gestation.[4]

Other manifestations that are not stated in the criteria, but are sequelae of the syndrome, are pregnancy-related maternal thrombosis and unexplained intrauterine growth restriction.

Late fetal loss is strongly associated with presence of antiphospholipid antibodies, and particularly lupus anticoagulant. Prospective studies have shown that positive lupus anticoagulant and/or high titers of cardiolipin IgG increase the risk of recurrent adverse outcome in a subsequent pregnancy.[38;39]

Evidence for a causal association between antiphospholipid antibodies and early miscarriage is limited.[8] Early miscarriage is relatively common and has many causes, of which fetal chromosomal abnormalities are the most likely. Observational studies of the association between antiphospholipid syndrome and recurrent early miscarriage are likely to be heavily confounded, especially by inclusion of women with sporadic rather than recurrent miscarriage. Therefore international guidelines (Royal College of Obstetricians and Gynaecologist, European Society of Human Reproduction and Embryology) advise screening for antiphospholipid antibodies in women with more than three early miscarriages.[40;41]

Women with antiphospholipid syndrome have an increased incidence of early or severe pre-eclampsia, which often leads to iatrogenic preterm birth due to termination of pregnancy for maternal or fetal reasons. Pre-eclampsia with severe thrombocytopenia may also point towards the presence of the syndrome, and is a red flag condition (box 3). [42]

Who should be tested for antiphospholipid antibodies?

Box 4 lists the indications for testing for antiphospholipid antibodies.[43]

SLE Testing for antiphospholipid antibodies is recommended in the initial evaluation of patients with SLE and should be reevaluated if new risk factors for thromboembolic events emerge.[44]

– BOX 4 –

SITUATIONS WHEN YOU SHOULD TEST FOR ANTIPHOSPHOLIPID ANTIBODIES

Thrombosis
- Arterial thrombosis before the age of 50
- Unprovoked venous thrombosis before the age of 50
- Recurrent thrombosis
- Thrombosis in an unusual site
- Patients with both arterial and venous thrombotic events
- Any patient admitted with thrombotic microangiopathy of unknown etiology

Obstetric manifestations
- One or more unexplained fetal losses after 10 weeks of gestation
- Unexplained severe intrauterine growth restriction
- Early and/or severe preeclampsia
- Three or more spontaneous miscarriages before 10 weeks of gestation

SLE patients
- At baseline
- Repeat testing prior to pregnancy, surgery, transplant and use of oestrogen-containing treatments, or in the presence of a new neurological, vascular or obstetric event

Lupus anticoagulant and the persistent presence of anticardiolipin antibodies increase the risk of thromboembolic events in patients with SLE. [45;46] Data on antiphospholipid antibodies can help when interpreting new symptoms in these patients and may influence therapeutic decisions in situations with increased thromboembolic risk, such as surgery, pregnancy, puerperium, or the use of oestrogen containing drugs.

PREGNANCY A recent prospective study of pregnant women with only one previous spontaneous abortion before the 10th week of gestation reported that the presence of antiphospholipid antibodies significantly increased the risk of embryonic loss, pre-eclampsia, and intrauterine growth restriction in the next pregnancy.[38] However, after single pregnancy loss, most subsequent pregnancies are uneventful without treatment. Therefore, testing after one early miscarriage, or even testing all women who plan to become pregnant, is not advised. [43]

How to treat antiphospholipid syndrome

Antithrombotic agents aim to reduce the risk of recurrent thromboembolism and are the mainstay of treatment. Recently guidelines on how to treat antiphospholipid syndrome subdivided patients into those with venous thrombosis, those with arterial thrombosis, and obstetric antiphospholipid syndrome.[47] A treatment algorithm containing an overview of these guidelines is presented in figure 2.

FIRST EPISODE For a first episode of unprovoked venous thrombosis or thromboembolism associated with persistent positive antiphospholipid antibodies international guidelines recommend long term anticoagulation with vitamin K antagonists such as warfarin to reduce the risk of recurrence of a thrombotic event.[48] However, if a reversible risk factor for thromboembolism – such as surgery, immobilisation, oestrogen therapy, or pregnancy – is reliably eliminated indefinite anticoagulation may not be justified.[47]

The only prospective study focusing on arterial cerebral events showed similar rates of recurrent thromboembolism and risk of major bleeding in patients treated with warfarin or low dose aspirin.[49] However, inappropriate criteria for defining antiphospholipid antibody positivity limit the generalisability of this study.[50] In patients with antiphospholipid syndrome and stroke, long term anticoagulation with warfarin or low dose aspirin is advised.

Two randomised controlled trials compared high intensity anticoagulation (aimed at an international normalised ratio (INR) of 3.1-4) with moderate intensity anticoagulation (INR 2-3) for the prevention of recurrent venous and arterial thrombotic events in non-pregnant adults with antiphospholipid syndrome. Both trials used oral warfarin and found that high intensity treatment was no better at preventing thrombotic events.[51;52] When results were pooled, the risk of bleeding was slightly increased in patients on high intensity treatment.[53] The limitations of these trials (patients with arterial events were in the minority and many patients randomised to a target INR ▸3 did not achieve this target), and the fact that the results contradict those of observational studies, mean that treatment aims are still a point of ongoing debate.[50] International guidelines and systematic reviews currently recommend aiming for an INR between 2 and 3. [47;48;54;55]

PREVENTING OBSTETRIC COMPLICATIONS Few well designed trials have been carried out and studied populations are heterogeneous, so the level of evidence for all treatment options is low. Table 2 gives suggestions for primary and secondary prevention of thrombosis and adverse pregnancy outcome; these are based on the limited available evidence and our own experience.

PREVENTING MATERNAL THROMBOTIC COMPLICATIONS Warfarin crosses the placenta and is teratogenic in the first trimester of pregnancy so low molecular weight heparins are the agents of choice for antenatal thromboprophylaxis.[41;56] Observational studies have shown that low molecular weight heparin is at least as effective as unfractionated heparin and safer. [41;56] Women who are on long term warfarin because of previous thrombosis should switch to heparin when trying to conceive or on confirmation of conception. The dose of heparin will depend on the woman's clinical history and should be discussed with a haematologist.

PREVENTING ADVERSE PREGNANCY OUTCOME A meta-analysis of intervention trials for recurrent (early) miscarriage have concluded that heparin with low dose aspirin reduces pregnancy loss by 54%.[57]

No randomised controlled trials have investigated prevention in patients with a history of late miscarriage, fetal death, and intrauterine growth restriction. Most clinicians would consider treatment with low dose aspirin and heparin (mostly low molecular weight heparin) in such cases. In patients with antiphospholipid antibodies and a history of severe pre-eclampsia at least low dose aspirin (75-80 mg once daily) is recommended.[47]

Glucocorticoids, cytotoxic agents, and intravenous immunoglobulin have no confirmed benefit and may even be teratogenic.[47]

SLE In patients with SLE and antiphospholipid antibodies, low dose aspirin may be considered for primary prevention of thrombosis and pregnancy loss.[58] In non-pregnant patients with SLE and antiphospholipid syndrome associated thrombosis, long term anticoagulation with vitamin K antagonists is effective for secondary prevention of thrombosis. In pregnant patients with SLE and antiphospholipid syndrome, combined unfractionated heparin or low molecular weight heparin and aspirin reduce pregnancy loss and thrombosis and should be considered.[58]

CATASTROPHIC ANTIPHOSPHOLIPID SYNDROME Box 2 summarises the management and characteristics of this rare manifestation of the syndrome. Figure 3 gives examples of typical histologic findings in patients with catastrophic antiphospholipid syndrome.

Future challenges in for the management of patients with Antiphospholipid syndrome

A reliable diagnostic test is still needed. Antiphospholipid syndrome mimics many other conditions, which leads to misdiagnosis and thwarts efforts to perform studies of sufficient size to give unequivocal support for diagnostic and treatment strategies. However, left untreated the syndrome can have serious sequelae. We advise that any patient with a suspected antiphospholipid syndrome should be seen by a multidisciplinary team of specialists that ideally includes a rheumatologist, haematologist, neurologist, nephrologist, and obstetrician for diagnosis, treatment, and education.

A PATIENT'S PERSPECTIVE

From the age of 16, I had frequent headaches, sometimes with double vision, and I occasionally had pins and needles in one hand. My general practitioner never found an obvious cause. At 21 I was diagnosed with a deep vein thrombosis in my left leg, after a minor car accident. I was treated with heparin and aspirin for a few months.
A year later I had a miscarriage at 9 weeks' gestation. My platelets were low and did not improve. My gynaecologist sent me to a haematologist, who thought of the antiphospholipid syndrome. The blood test was positive. It was a double feeling: on the one hand I felt relieved to have a diagnosis that explained all my medical problems, but I suddenly had a disease that I had never heard of. My friends and family have difficulty understanding when I try to explain what antiphospholipid syndrome is. The most frustrating thing is that even some of the doctors I talk to have never heard of it. Two years ago they found out that two of my heart valves are leaking. I have had surgery for one valve recently, and one more operation is needed for the other. It is scary to think that if my anticoagulation therapy is stopped I will be at risk of developing things like a stroke. It is surreal to have to think about these things in your early 30s. Because of the heart valves I had to postpone further pregnancies. I hope for the best, and hope that with heparin treatment I'll have a fair chance of becoming a mother one day.

A Meijer-Bezema, Stadskanaal, Netherlands

METHODS

In cooperation with a trained librarian, a search strategy was composed. The following databases were searched for evidence from systematic reviews, clinical trials and prospective cohort studies: PubMed (1949 to January 2010), EMBASE (OVID-version, 1980 to January 2010), Web of Science (1945 to January 2010), Cochrane Library (1990 to January 2010), CINAHL (EbscoHost-version, 1982 to January 2010), and Academic Search Premier (EbscoHost-version (1865 to January 2010). All relevant keyword variations were used, not only keyword variations in the controlled vocabularies of the various databases, but the free text word variations of these concepts as well. In general, the search consisted of the combination of the following terms: 'antiphospholipid syndrome', 'Hughes syndrome', 'antiphospholipid antibodies', 'lupus anticoagulant', 'anticardiolipin antibodies', 'anti β_2-glycoprotein I antibodies' and 'catastrophic antiphospholipid syndrome'. The results were limited to articles written in English.

The search was performed on the 27th of January, 2010.

SUMMARY POINTS

Antiphospholipid syndrome is a relatively common autoimmune disorder that mainly affects young adults
If untreated, antiphospholipid syndrome can lead to permanent disability, severe maternal or perinatal morbidity, or even death
Symptoms can occur in virtually all organ systems
Venous thrombosis and stroke are the most common thrombotic manifestations
In pregnancy the syndrome is associated with adverse maternal and fetal outcomes
The lupus anticoagulant test is the most useful because positivity correlates most strongly with clinical manifestations
Cardiac valvular disease is an important clinical manifestation and may contribute to the risk of strokes

CONTINUING MEDICAL EDUCATION RESOURCES

For professionals

How I treat the antiphospholipid syndrome.[47] Excellent review about recent developments in treatment of antiphospholipid syndrome patients.

How we diagnose the antiphospholipid syndrome.[43] Excellent review about recent developments in diagnosing the antiphospholipid syndrome.

Reducing the risk of thrombosis and embolism during pregnancy and the puerperium.[41] Greentop guidelines of the Royal College of Obstetricians and Gynecologists (RCOG). http://www.rcog.org.uk/

Antithrombotic and thrombolytic therapy, and Antithrombotic therapy for venous thromboembolic disease, 8th edition.[48;54] Guidelines of the American College of Chest Physicians (ACCP) http://www.chestnet.org/accp/

Investigation and medical treatment of recurrent miscarriage.[40] Guidelines from the European Society of Human Reproduction and Embryology (ESHRE). http://www.eshre.eu/

Practical management of coagulopathy associated with warfarin.[62] Useful management strategies for patients who are being treated with a vitamin K antagonist and present with an INR outside the therapeutic range.

For patients

Kay Thackray. *Sticky Blood.* ISBN 1-898030-77-4. A personal account of dealing with the condition.

Triona Holden. 'Positive Options for Antiphospholipid Antibody Syndrome' ISBN 0-89793-409-1

UpToDate: Patient information about antiphospholipid syndrome (http://www.uptodate.com/patients/content/topic.do?topicKey=~CQUbGyAA8E5yqH&selectedTitle=1%7E150&source=search_result)

Youtube: Video in which Dr Graham Hughes of the London Lupus Center explains about antiphospholipid syndrome, and in which 2 patients tell about their experiences with recurrent miscarriage and stroke. (http://www.youtube.com/watch?v=V3J8BLkZyhU)

Youtube: Video showing an excellent animation of deep vein thrombosis. (http://www.youtube.com/watch?v=CETfozLocQg)

ONGOING RESEARCH AND FUTURE CHALLENGES

Pathologic mechanism

To clarify the relation between inflammation and thrombosis in antiphospholipid syndrome.

To further unravel the actions of different antiphospholipid antibodies on hemostasis, endothelial activation and placental invasiveness.

Serology

To find more specific tests for antiphospholipid antibodies that correlate better with clinical symptoms. Lupus anticoagulant inducing anti-$\beta_2$glycoprotein I antibodies and anti-$\beta_2$glycoprotein I domain I antibodies are promising new binding targets.[6]

Treatment

To identify the role of newer, preferably oral anticoagulants in antiphospholipid syndrome.

To identify the role of anti-inflammatory drugs in antiphospholipid syndrome (Rituximab, anti-complement agents, statins)

To perform well designed randomized controlled trials in pregnancy related settings.

TIPS FOR NON SPECIALISTS

Early recognition of antiphospholipid syndrome offers a chance for prevention of recurrent thrombosis, and prevention of recurrent maternal and fetal pregnancy morbidity.

A delayed diagnosis can cause permanent disability due to uncontrolled thrombosis formation, or death.

If you consider testing for antiphospholipid syndrome, perform all three laboratory tests mentioned in the classification criteria (BOX 1) or refer the patient to a specialist for testing.

Try to obtain the first test results before starting with anticoagulation therapy, since anticoagulation therapy influences the outcome of the lupus anticoagulant test.

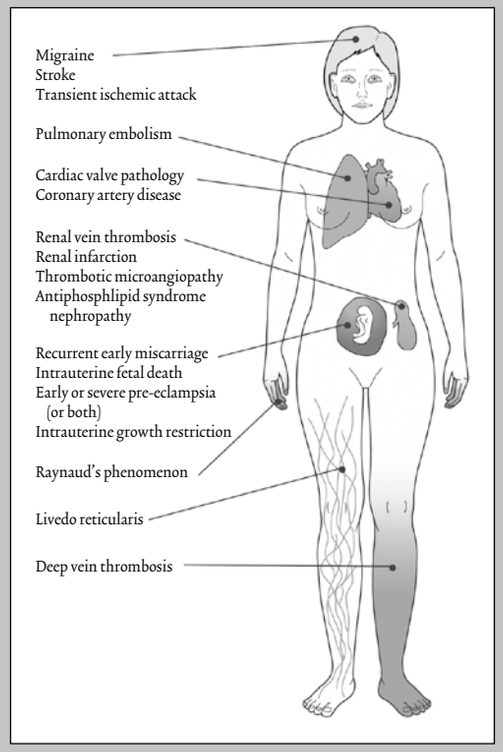
A positive test is an indication to refer the patient to a specialist.

Antiphospholipid syndrome pregnancies are high risk pregnancies, and management at specialized centers is advisable.

Traditional risk factors for cardiovascular disease contribute further to the risk of thrombosis in antiphospholipid syndrome, even at young age.

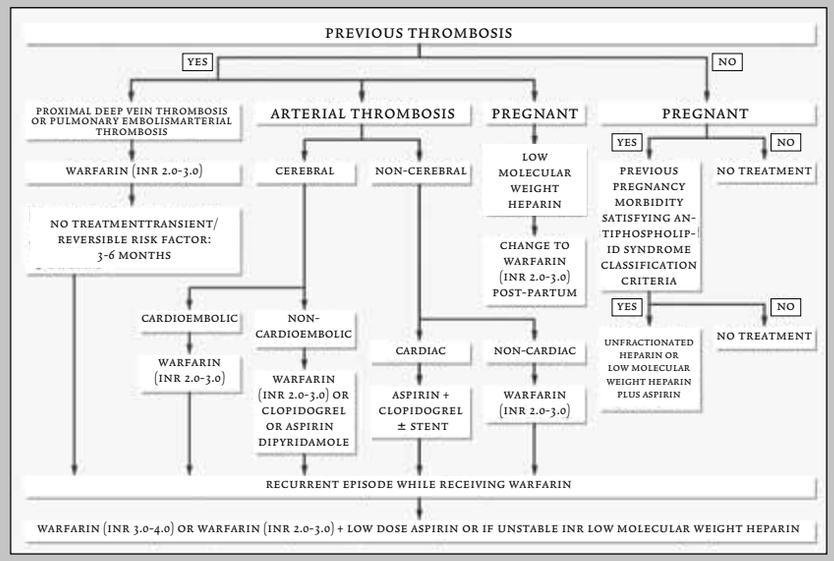
Try to support patients to stop smoking, normalize body weight and try to avoid oral contraception and hormone replacement therapy.

FIG 1 CLINICAL MANIFESTATIONS OF ANTIPHOSPHOLIPID SYNDROME



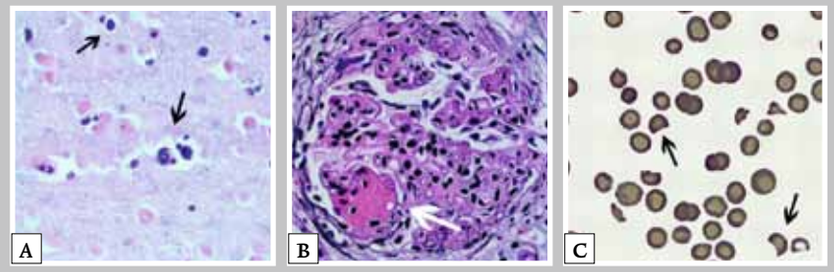
(Illustration by Folkert van Meurs)

FIG 2 TREATMENT ALGORITHM FOR ANTIPHOSPHOLIPID SYNDROME



Adapted, with permission obtained from 'Blood copyright clearance centre', Giannakopoulos *et al*, (2009); How I treat the antiphospholipid syndrome

FIG 3 CLASSIC HISTOLOGICAL FINDINGS IN A PATIENT WITH CATASTROPHIC ANTIPHOSPHOLIPID SYNDROME



(A) Cerebral microthrombi (arrows); the fibrin thrombi are stained blue by phosphotungstic acid haematoxylin. (B) Renal microthrombi, prominently present in a glomerulus (arrow). (C) Blood smear showing schistocytes (fragmented red blood cells), which are formed by fibrin strands that sever red blood cells as they try to move past a (micro)thrombus (arrows), and are indicative of microangiopathic haemolytic anaemia

TABLE 1 ANTIPHOSPHOLIPID ANTIBODIES

	ANTICARDIOLIPIN ANTIBODIES	ANTI-β_2GLYCOPROTEIN I ANTIBODIES	LUPUS ANTICOAGULANT
Test	Anticardiolipin ELISA	anti-$\beta_2$glycoprotein I ELISA	The lupus anticoagulant assay **
Test guidelines	Pierangeli *et al*, 2008 [59]	no guidelines yet	Pengo *et al*, 2009 [10]
Which antibodies are detected?	Antibodies against cardiolipin, and cardiolipin-bound β2-glycoprotein I	Antibodies against beta2-glycoprotein I	Detects immunoglobulins that cause prolonged clotting times in vitro, but are associated with thrombosis in vivo.
Relevant isotypes	IgG +++, IgM +	IgG+++, IgM +	not applicable
What titers are considered positive?	Medium/High: ▸ 99th percentile, or ▸40 GPL or MPL	Medium/High: ▸ 99th percentile, or ▸40 GPL or MPL	not applicable
Is the test influenced by anticoagulation therapy?	no	no	yes: both heparin and warfarin influence the test-results, and testing under coagulation therapy is controversial.
Is there overlap with other tests?	yes, overlap with lupus anticoagulant	yes, overlap with lupus anticoagulant	yes: anti-$\beta_2$glycoprotein I and anticardiolipin antibodies can have an anticoagulant effect, but other antibodies like anti-prothrombin and anti-annexin V antibodies can contribute to this effect too.

** A set of coagulation assays in three steps: Screening (identification of a prolonged clotting time), mixing (confirmation of an inhibitor and exclusion of factor-deficiencies) and confirmation (confirmation of phospholipid dependence of the inhibitor)

TABLE 2 TREATMENT OF PATIENTS WITH PERSISTENT POSITIVE ANTIPHOSPHOLIPID ANTIBODIES IN PREGNANCY

CLINICAL PRESENTATION	TREATMENT REGIMEN IN PREGNANCY	TREATMENT REGIMEN POSTPARTUM	EVIDENCE LEVEL **
Women (including patients with SLE) with prior thrombosis	· Graduated elastic compression stockings · Weight adjusted, full-dose LMWH from ◂ 6 wks gestation	· Graduated elastic compression stockings · 6 weeks LMWH or warfarin*	C
Women with late fetal loss (▸ 10 wks)	Low-dose aspirin and/or LMWH ***	at least 7 days LMWH or warfarin	C
Women with recurrent miscarriage (◂10 wks)	Low-dose Aspirin plus LMWH	at least 7 days LMWH or warfarin	A
Women with history of early and/or severe preeclampsia or intrauterine growth restriction	Low-dose Aspirin Consider additional LMWH ***	at least 7 days LMWH or warfarin	C
Women with persistently positive antiphospholipid antibodies without clinical symptoms	Close surveillance	at least 7 days LMWH or warfarin	C
Women with SLE without previous obstetric or thrombotic complications	Low dose Aspirin ***	at least 7 days LMWH or warfarin	C
Women with SLE with previous obstetric complications	Low-dose Aspirin + LMWH ***	at least 7 days LMWH or warfarin	

* Warfarin crosses the placenta, is teratogenic and must be avoided in pregnancy

** Level A: Consistent randomized controlled trials and/or cohort studies / Level B: Consistent retrospective cohort, exploratory cohort or case control studies or extrapolations from level A studies / Level C: Case-series or extrapolations from level B studies / Level D: Expert opinion without explicit critical appraisal

*** If possible try to include patients in a Rct

REFERENCES

1 Hughes GR. Thrombosis, abortion, cerebral disease, and the lupus anticoagulant. *Br Med J (Clin Res Ed)* 1983; 287(6399):1088-1089.

2 Cervera R, Piette JC, Font J, Khamashta MA, Shoenfeld Y, Camps MT *et al.* Antiphospholipid syndrome: clinical and immunologic manifestations and patterns of disease expression in a cohort of 1,000 patients. *Arthritis Rheum* 2002; 46(4):1019-1027.

3 Asherson RA, Cervera R, de Groot PG, Erkan D, Boffa MC, Piette JC *et al.* Catastrophic antiphospholipid syndrome: international consensus statement on classification criteria and treatment guidelines. *Lupus* 2003; 12(7):530-534.

4 Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R *et al.* International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost* 2006; 4(2):295-306.

5 Avcin T, Cimaz R, Silverman ED, Cervera R, Gattorno M, Garay S *et al.* Pediatric antiphospholipid syndrome: clinical and immunologic features of 121 patients in an international registry. *Pediatrics* 2008; 122(5):e1100-e1107.

6 de Laat B, Mertens K, de Groot PG. Mechanisms of disease: antiphospholipid antibodies-from clinical association to pathological mechanism. *Nat Clin Pract Rheumatol* 2008; 4(4):192-199.

7 Galli M, Luciani D, Bertolini G, Barbui T. Lupus anticoagulants are stronger risk factors for thrombosis than anticardiolipin antibodies in the antiphospholipid syndrome: a systematic review of the literature. *Blood* 2003; 101(5):1827-1832.

8 Opatrny L, David M, Kahn SR, Shrier I, Rey E. Association between antiphospholipid antibodies and recurrent fetal loss in women without autoimmune disease: a metaanalysis. *J Rheumatol* 2006; 33(11):2214-2221.

9 Pengo V, Biasiolo A, Gresele P, Marongiu F, Erba N, Veschi F *et al.* Survey of lupus anticoagulant diagnosis by central evaluation of positive plasma samples. *J Thromb Haemost* 2007; 5(5):925-930.

10 Pengo V, Tripodi A, Reber G, Rand JH, Ortel TL, Galli M *et al.* Update of the guidelines for lupus anticoagulant detection. *J Thromb Haemost* 2009; 7(10):1737-1740.

11 Petri M. Epidemiology of the antiphospholipid antibody syndrome. *J Autoimmun* 2000; 15(2):145-151.

12 Vila P, Hernandez MC, Lopez-Fernandez MF, Batlle J. Prevalence, follow-up and clinical significance of the anticardiolipin antibodies in normal subjects. *Thromb Haemost* 1994; 72(2):209-213.

13 Danowski A, Kickler TS, Petri M. Anti-beta2-glycoprotein I: prevalence, clinical correlations, and importance of persistent positivity in patients with antiphospholipid syndrome and systemic lupus erythematosus. *J Rheumatol* 2006; 33(9):1775-1779.

14 de Groot PG, Lutters B, Derksen RH, Lisman T, Meijers JC, Rosendaal FR. Lupus anticoagulants and the risk of a first episode of deep venous thrombosis. *J Thromb Haemost* 2005; 3(9):1993-1997.

15 Urbanus RT, Siegerink B, Roest M, Rosendaal FR, de Groot PG, Algra A. Antiphospholipid antibodies and risk of myocardial infarction and ischaemic stroke in young women in the RATIO study: a case-control study. *Lancet Neurol* 2009; 8(11):998-1005.

16 Saidi S, Mahjoub T, Almawi WY. Lupus anticoagulants and anti-phospholipid antibodies as risk factors for a first episode of ischemic stroke. *J Thromb Haemost* 2009; 7(7):1075-1080.

17 Levine SR, Salowich-Palm L, Sawaya KL, Perry M, Spencer HJ, Winkler HJ *et al.* IgG anticardiolipin antibody titer ▸ 40 GPL and the risk of subsequent thrombo-occlusive events and death. A prospective cohort study. *Stroke* 1997; 28(9):1660-1665.

18 Naess IA, Christiansen SC, Cannegieter SC, Rosendaal FR, Hammerstroem J. A prospective study of anticardiolipin antibodies as a risk factor for venous thrombosis in a general population (the HUNT study). *J Thromb Haemost* 2006; 4(1):44-49.

19 Galli M, Luciani D, Bertolini G, Barbui T. Anti-beta 2-glycoprotein I, antiprothrombin antibodies, and the risk of thrombosis in the antiphospholipid syndrome. *Blood* 2003; 102(8):2717-2723.

20 Ruffatti A, Tonello M, Del RT, Cavazzana A, Grava C, Noventa F *et al.* Antibody profile and clinical course in primary antiphospholipid syndrome with pregnancy morbidity. *Thromb Haemost* 2006; 96(3):337-341.

21 Ruffatti A, Tonello M, Cavazzana A, Bagatella P, Pengo V. Laboratory classification categories and pregnancy outcome in patients with primary antiphospholipid syndrome prescribed antithrombotic therapy. *Thromb Res* 2009; 123(3):482-487.

22 Pierangeli SS, Chen PP, Raschi E, Scurati S, Grossi C, Borghi MO *et al.* Antiphospholipid antibodies and the antiphospholipid syndrome: pathogenic mechanisms. *Semin Thromb Hemost* 2008; 34(3):236-250.

23 Oku K, Atsumi T, Bohgaki M, Amengual O, Kataoka H, Horita T *et al.* Complement activation in patients with primary antiphospholipid syndrome. *Ann Rheum Dis* 2009; 68(6):1030-1035.

24 Salmon JE, Girardi G, Holers VM. Complement activation as a mediator of antiphospholipid antibody induced pregnancy loss and thrombosis. *Ann Rheum Dis* 2002; 61 Suppl 2:ii46-ii50.

25 Davis WD, Brey RL. Antiphospholipid antibodies and complement activation in patients with cerebral ischemia. *Clin Exp Rheumatol* 1992; 10(5):455-460.

26 Meroni PL, Tincani A, Sepp N, Raschi E, Testoni C, Corsini E *et al.* Endothelium and the brain in CNS lupus. *Lupus* 2003; 12(12):919-928.

27 Katzav A, Chapman J, Shoenfeld Y. CNS dysfunction in the antiphospholipid syndrome. *Lupus* 2003; 12(12):903-907.

28 Sebire NJ, Backos M, El GS, Goldin RD, Regan L. Placental pathology, antiphospholipid antibodies, and pregnancy outcome in recurrent miscarriage patients. *Obstet Gynecol* 2003; 101(2):258-263.

29 Di Simone N, Luigi MP, Marco D, Fiorella DN, Silvia D, Clara DM *et al.* Pregnancies complicated with antiphospholipid syndrome: the pathogenic mechanism of antiphospholipid antibodies: a review of the literature. *Ann N Y Acad Sci* 2007; 1108:505-514.

30 Girardi G, Redecha P, Salmon JE. Heparin prevents antiphospholipid antibody-induced fetal loss by inhibiting complement activation. *Nat Med* 2004; 10(11):1222-1226.

31 Khamashta MA, Cuadrado MJ, Mujic F, Taub NA, Hunt BJ, Hughes GR. The management of thrombosis in the antiphospholipid-antibody syndrome. *N Engl J Med* 1995; 332(15):993-997.

32 Sanna G, D'Cruz D, Cuadrado MJ. Cerebral manifestations in the antiphospholipid (Hughes) syndrome. *Rheum Dis Clin North Am* 2006; 32(3):465-490.

33 Hughes GR. Migraine, memory loss, and 'multiple sclerosis ". Neurological features of the antiphospholipid (Hughes') syndrome. *Postgrad Med J* 2003; 79(928):81-83.

34 Montalban J, Cervera R, Font J, Ordi J, Vianna J, Haga HJ *et al.* Lack of association between anticardiolipin antibodies and migraine in systemic lupus erythematosus. *Neurology* 1992; 42(3 Pt 1):681-682.

35 Krause I, Lev S, Fraser A, Blank M, Lorber M, Stojanovich L *et al.* Close association between valvar heart disease and central nervous system manifestations in the antiphospholipid syndrome. *Ann Rheum Dis* 2005; 64(10):1490-1493.

36 Roldan CA. Valvular and coronary heart disease in systemic inflammatory diseases: Systemic Disorders in heart disease. *Heart* 2008; 94(8):1089-1101.

37 Daugas E, Nochy D, Huong DL, Duhaut P, Beaufils H, Caudwell V *et al.* Antiphospholipid

syndrome nephropathy in systemic lupus erythematosus. *J Am Soc Nephrol* 2002; 13(1):42-52.
38 Chauleur C, Galanaud JP, Alonso S, Cochery-Nouvellon E, Balducchi JP, Mares P *et al.* Observational study of pregnant women with a previous spontaneous abortion before the 10 gestation week with and without antiphospholipid antibodies. *J Thromb Haemost* 2010; 8(4):699-706.
39 Rai RS, Clifford K, Cohen H, Regan L. High prospective fetal loss rate in untreated pregnancies of women with recurrent miscarriage and antiphospholipid antibodies. *Hum Reprod* 1995; 10(12):3301-3304.
40 Jauniaux E, Farquharson RG, Christiansen OB, Exalto N. Evidence-based guidelines for the investigation and medical treatment of recurrent miscarriage. *Hum Reprod* 2006; 21(9):2216-2222.
41 Nelson-Piercy, MacCallum, Mackillop. Reducing the risk of thrombosis and embolism in pregnancy and the puerperium. RCOG *Green-top Guideline No 37* 2009.
42 Duckitt K, Harrington D. Risk factors for pre-eclampsia at antenatal booking: systematic review of controlled studies. *BMJ* 2005; 330(7491):565.
43 Giannakopoulos B, Passam F, Ioannou Y, Krilis SA. How we diagnose the antiphospholipid syndrome. *Blood* 2009; 113(5):985-994.
44 Mosca M, Tani C, Aringer M, Bombardieri S, Boumpas D, Brey R *et al.* European League Against Rheumatism recommendations for monitoring patients with systemic lupus erythematosus in clinical practice and in observational studies. *Ann Rheum Dis* 2010.
45 Martinez-Berriotxoa A, Ruiz-Irastorza G, Egurbide MV, Garmendia M, Gabriel EJ, Villar I *et al.* Transiently positive anticardiolipin antibodies and risk of thrombosis in patients with systemic lupus erythematosus. *Lupus* 2007; 16(10):810-816.
46 Tektonidou MG, Laskari K, Panagiotakos DB, Moutsopoulos HM. Risk factors for thrombosis and primary thrombosis prevention in patients with systemic lupus erythematosus with or without antiphospholipid antibodies. *Arthritis Rheum* 2009; 61(1):29-36.
47 Giannakopoulos B, Krilis SA. How I treat the antiphospholipid syndrome. *Blood* 2009; 114(10):2020-2030.
48 Kearon C, Kahn SR, Agnelli G, Goldhaber S, Raskob GE, Comerota AJ. Antithrombotic therapy for venous thromboembolic disease: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). *Chest* 2008; 133(6 Suppl):454S-545S.
49 Levine SR, Brey RL, Tilley BC, Thompson JL, Sacco RL, Sciacca RR *et al.* Antiphospholipid antibodies and subsequent thrombo-occlusive events in patients with ischemic stroke. *JAMA* 2004; 291(5):576-584.
50 Ruiz-Irastorza G, Hunt BJ, Khamashta MA. A systematic review of secondary thromboprophylaxis in patients with antiphospholipid antibodies. *Arthritis Rheum* 2007; 57(8):1487-1495.
51 Crowther MA, Ginsberg JS, Julian J, Denburg J, Hirsh J, Douketis J *et al.* A comparison of two intensities of warfarin for the prevention of recurrent thrombosis in patients with the antiphospholipid antibody syndrome. *N Engl J Med* 2003; 349(12):1133-1138.
52 Finazzi G, Marchioli R, Brancaccio V, Schinco P, Wisloff F, Musial J *et al.* A randomized clinical trial of high-intensity warfarin vs. conventional antithrombotic therapy for the prevention of recurrent thrombosis in patients with the antiphospholipid syndrome (WAPS). *J Thromb Haemost* 2005; 3(5):848-853.
53 Crowther M, Crowther MA. Intensity of warfarin coagulation in the antiphospholipid syndrome. *Curr Rheumatol Rep* 2010; 12(1):64-69.
54 Hirsh J, Guyatt G, Albers GW, Harrington R, Schunemann HJ, American College of Chest Physician. Antithrombotic and thrombolytic therapy: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). *Chest* 2008; 133(6 Suppl):110S-112S.
55 Lim W, Crowther MA, Eikelboom JW. Management of antiphospholipid antibody syndrome: a systematic review. *JAMA* 2006; 295(9):1050-1057.
56 Bates SM, Greer IA, Pabinger I, Sofaer S, Hirsh J. Venous thromboembolism, thrombophilia, antithrombotic therapy, and pregnancy: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). *Chest* 2008; 133(6 Suppl):844S-886S.
57 Empson M, Lassere M, Craig J, Scott J. Prevention of recurrent miscarriage for women with antiphospholipid antibody or lupus anticoagulant. *Cochrane Database Syst Rev* 2005;(2):CD002859.
58 Bertsias G, Ioannidis JP, Boletis J, Bombardieri S, Cervera R, Dostal C *et al.* EULAR recommendations for the management of systemic lupus erythematosus. Report of a Task Force of the EULAR Standing Committee for International Clinical Studies Including Therapeutics. *Ann Rheum Dis* 2008; 67(2):195-205.
59 Pierangeli SS, Harris EN. A protocol for determination of anticardiolipin antibodies by ELISA. *Nat Protoc* 2008; 3(5):840-848.
60 Asherson RA, Cervera R, Piette JC, Font J, Lie JT, Burcoglu A *et al.* Catastrophic antiphospholipid syndrome. Clinical and laboratory features of 50 patients. *Medicine (Baltimore)* 1998; 77(3):195-207.
61 Bucciarelli S, Erkan D, Espinosa G, Cervera R. Catastrophic antiphospholipid syndrome: treatment, prognosis, and the risk of relapse. *Clin Rev Allergy Immunol* 2009; 36(2-3):80-84.
62 Garcia D, Crowther MA, Ageno W. Practical management of coagulopathy associated with warfarin. *BMJ* 2010; 340:c1813.

GENERAL INTRODUCTION

PART ONE
C4D AND THROMBOTIC COMPLICATIONS

PART TWO
C4D AND ADVERSE PREGNANCY OUTCOME

CONCLUSION AND DISCUSSION

APPENDICES

IV

POTENTIAL FOR GLOMERULAR C4D AS AN INDICATOR OF THROMBOTIC MICROANGIOPATHY IN LUPUS NEPHRITIS

DANIELLE COHEN / MARIJE KOOPMANS / IDSKE C. L. KREMER HOVINGA
STEFAN P. BERGER / MARIAN ROOS-VAN GRONINGEN / GERDA M. STEUP-BEEKMAN
EMILE DE HEER / JAN ANTHONIE BRUIJN / INGEBORG M. BAJEMAA

ARTHRITIS AND RHEUMATISM 2008;58:2460–2469

Abstract

OBJECTIVE In patients with systemic lupus erythematosus (SLE) and lupus nephritis, the presence of antiphospholipid antibodies (aPL) is considered to be an indication of increased risk of thrombotic microangiopathy, a serious complication of SLE. Previous studies have demonstrated a critical role for activation of the classical pathway of complement that leads to thrombotic injury in the presence of aPL. This study was undertaken to investigate whether C4d deposition in lupus nephritis is related to circulating aPL and the presence of renal microthrombi.

METHODS Deposition patterns of C4d in 44 renal biopsy samples obtained from 38 patients with biopsyproven lupus nephritis were determined by staining with a polyclonal anti-C4d antibody. A phosphotungstic acid–hematoxylin stain was used to identify fibrin microthrombi. Clinical data (serum creatinine levels and presence or absence of aPL) were obtained and correlated with findings in the renal biopsy specimens. Patients were categorized as having aPL (n = 20) or not having aPL (n = 18).

RESULTS A strong relationship between the intensity of glomerular C4d staining and the presence of microthrombi was found ($P < 0.002$). Intense glomerular C4d deposition was present in 7 of 8 biopsy samples showing renal microthrombi. Neither C4d deposition nor the presence of microthrombi was correlated with aPL status.

CONCLUSION Our findings suggest that activation of the classical pathway of complement plays a pathogenic role in the development of renal tissue injury leading to thrombosis, irrespective of the type of circulating antibodies present. Immunodetection of glomerular C4d deposition in renal biopsy samples could be a convenient method of identifying patients at risk of thrombotic microangiopathy.

Introduction

Lupus nephritis develops in up to 60% of patients with systemic lupus erythematosus (SLE) during the course of the disease.[1] Standard clinical practice is to perform a renal biopsy if clinical parameters suggest renal involvement; the lupus nephritis class[2] can be determined from the biopsy and has an impact on both the choice of treatment and the patient's prognosis. In renal biopsy specimens obtained from SLE patients, evidence of thrombotic microangiopathy is occasionally detected.

Although the occurrence of thrombotic microangiopathy in patients with SLE is rare, about 3% according to various studies[3-6], clinicians are well aware of the serious complications. In its most fulminant form, thrombotic microangiopathy presents with multiple fibrin thrombi in glomeruli and/or arterioles and capillaries and is accompanied by a severe decline in renal function.[7] SLE patients with thrombotic microangiopathy also exhibit extrarenal complications, including thrombocytopenia, microangiopathic hemolytic anemia, neurologic manifestations, and even multiple organ failure.[8]

Thrombotic microangiopathy in SLE patients is typically associated with the presence of antiphospholipid antibodies (aPL), namely anticardiolipin antibodies (aCL) and lupus anticoagulant (LAC).[8-11] However, aPL are detected in up to 40% of SLE patients[12-14], and in many patients with high aPL titers thrombotic microangiopathy never develops[4;10] Conversely, thrombotic microangiopathy has been reported in several SLE patients without aPL.[8] These observations are consistent with suggestions that other factors are necessary to trigger the endothelial damage leading to thrombotic events.

Recently, the role of complement in aPL-induced thrombosis has been thoroughly investigated. Pierangeli *et al* have shown that activation of the complement cascade is necessary for aPL-mediated thrombophilia.[15-16] In addition, Navratil *et al*[17] reported that platelet-bound C4d, a sensitive marker for the classical pathway of complement activation, is highly specific (though not sensitive) for the presence of aPL in SLE patients. Those investigators suggested that platelet-bound C4d may provide clues to the pathogenic mechanisms responsible for the thrombotic and vascular complications of SLE.[17]

C4d is a marker of humoral rejection in renal transplant biopsy samples, and its presence can be interpreted as a footprint of the classical pathway of complement activation and thus of alloantibody-induced damage.[18] In the transplanted kidney, humoral rejection is associated with glomerular and interstitial changes, in which thrombotic lesions may also occur.[19] A mechanism similar to humoral rejection possibly occurs in SLE, in which many different autoantibodies produce an analogous situation. Few reports have described C4d depositions in nontransplanted kidneys.

C4d positivity has been reported in membranous nephropathy and membranoproliferative glomerulonephritis and even in normal kidneys, suggesting that C4d positivity need not always be representative of activation of the classical pathway of complement.[20] C4d was detected by Kim and Jeong [21] in glomeruli in 21 cases of lupus nephritis, and the intensity of C4d staining correlated with the extent of immune complex depositions. In that study, the aPL status of the patients was unknown, and the presence of microthrombi was not investigated.

The recent finding of C4d expression on platelets from SLE patients[17], as well as the results of recent work on complement- and aPL-mediated thrombosis, support the notion of a possible role of C4d in the pathogenesis of thrombosis in SLE. We hypothesized that immunodetection of C4d in renal biopsy samples could facilitate detecting SLE patients at risk of the development of thrombotic complications. In the present study, we investigated the relationship between C4d deposition in renal biopsy samples, the presence of microthrombi in biopsy samples, and aPL status in patients with lupus nephritis.

Patients and methods

PATIENTS We retrospectively studied 38 patients with clinically evident SLE who visited the outpatient clinic at the Department of Nephrology and Internal Medicine of the Leiden University Medical Center between 1983 and 2006. All patients met at least 4 of the American College of Rheumatology criteria for SLE[22], and lupus nephritis was confirmed in all patients by ▸1 renal biopsy. The biopsy specimens were reevaluated and classified according to the most recent modification of the World Health Organization classification from the International Society of Nephrology/Renal Pathology Society (ISN/RPS).[2] Follow-up biopsy samples were available for 4 patients. Three of these patients had 2 biopsies, and 1 patient had 4 biopsies. The medical records of all patients were reviewed independently of the analysis of pathologic features. Most clinical data were available, although some were missing for the older cases. All patients were investigated for the presence of aPL as defined by the Sapporo criteria for antiphospholipid syndrome (APS).[23] In the present study, patients were categorized as having aPL (n = 20) or not having aPL (n = 18). An overview of the clinical data is given in Table 1.

BIOPSY SAMPLES Biopsy samples were fixed in 10% buffered formalin and embedded in paraffin. Paraffin sections (4 micrometer thick) were placed on positively charged slides and kept in a stove at 60°C for 1 hour. Sections were deparaffinized and rehydrated through a series of xylene and graded alcohols. Endogenous peroxidase was blocked in 3% H_2O_2 for 30 minutes. Antigen retrieval was performed by boiling the slides in a microwave at 1,000W for 10 minutes in 10 mM citrate buffer (pH 6.0).

The primary rabbit anti-human C4d polyclonal antibody (BI-RC4d; Biomedica Gruppe, Vienna, Austria) was applied at a dilution of 1:50 in 1% bovine serum albumin/phosphate buffered saline (BSA/PBS), and slides were incubated overnight at room temperature. The slides were then incubated with anti-rabbit EnVision (K5007; DakoCytomation,Glostrup, Denmark) for 30 minutes, and staining was visualized with the Vector Nova Red Substrate Kit (SK-4800; Vector, Peterborough, UK). Sections were washed with PBS (pH 7.4) between each step (3 times for 5 minutes each time). Finally, sections were counterstained with Mayer's hematoxylin, air-dried, cleared in xylene, and coverslipped. A renal specimen from a humoral allograft rejection patient with C4d-positive staining confirmed by immunofluorescence was used as a positive control. Consecutive sections were stained for fibrin with Mallory's phosphotungstic acid–hematoxylin. Fresh frozen tissue sections were incubated with fluorescein isothiocyanate–conjugated polyclonal antibodies directed against human IgA, IgG, IgM, C3, and C1q (Dako).

QUANTIFICATION OF MORPHOLOGY, IMMUNOHISTOCHEMICAL STAINING, HISTOCHEMICAL STAINING, AND IMMUNOFLUORESCENCE The biopsy specimens were scored by a renal pathologist (IMB) who had no prior knowledge of the clinical and laboratory findings in the patients. Peritubular capillary C4d staining was scored as 0 or 1, where 0 represented the absence of C4d in peritubular capillaries, and 1 represented positive peritubular capillary C4d staining in any area of the biopsy sample. C4d positivity in arterioles was scored as 0 or 1, where 0 represented the absence of C4d, and 1 represented the presence of C4d.

Glomerular C4d staining was scored semiquantitatively using the following scoring system: 0 (no glomerular staining), 1 (mild to moderate glomerular staining), and 2 (intense glomerular staining). Typical examples of the different intensities of glomerular C4d staining are shown in Figures 1A–C. Consecutive sections were stained with phosphotungstic acid–hematoxylin and carefully examined for the presence of arterial and/or arteriolar thrombosis and glomerular microthrombi.

A glomerular microthrombus stained with phosphotungstic acid–hematoxylin is shown in Figure 1D. Positive identification of at least 1 microthrombus, either in glomeruli, interstitial capillaries, or small arterioles, was scored as 1. Absence of microthrombi was scored as 0. Intensity of glomerular staining for IgG, IgA, IgM, C3, and C1q was semiquantitatively scored as 0, 1, or 2.

LABORATORY EVALUATION Standard methods were used to determine the levels of antinuclear, anti-DNA, antiextractable nuclear antigen, and anti-C1q antibodies. C3, C4, and C1q levels were also determined by standard protocols. Levels of IgG and IgM aCL were determined using the Varelisa Cardiolipin Antibodies enzyme-linked immunosorbent assay kit (Phadia, Nieuwegein, The Netherlands). Laboratory diagnosis of LAC was performed in citrated plasma prepared by double centrifugation. Two different phospholipid-dependent coagulation assays were used. An activated partial thromboplastin time (APTT) was measured, and, if the APTT was prolonged, patient plasma was mixed with normal plasma (automated APTT reagent; BioMerieux, Marcy l'Etoile, France) at a 1:1 ratio to test for the presence of an inhibitor. Persistence of a prolonged APTT clotting time indicated the presence of an inhibitor. Furthermore, the presence of LAC was tested using a dilute Russell's viper venom time (dRVVT)–based assay (LA screen/LA Confirm; Life Diagnostics, Frenchs Forest, Australia). If the dRVVT obtained using the LA Screen reagent in the patient sample was prolonged ‣20% compared with normal plasma, studies with normal plasma were performed to exclude possible clotting factor deficiencies. If the dRVVT remained prolonged after mixing, then the phospholipid dependency of the possible inhibitor was tested with the LA Confirm reagent, which contains a high phospholipid concentration. Normalization of dRVVT with the LA Confirm reagent confirmed the presence of LAC.

CLINICAL FOLLOW-UP The glomerular filtration rate (GFR) was estimated for all patients at the time of biopsy, as well as during yearly followup visits, using the Modification of Diet in Renal Disease (MDRD) formula.[24] Followup data were used to investigate whether renal biopsy findings at presentation were related to renal outcome. Data regarding the end points of occurrence of end-stage renal failure requiring dialysis and the date and cause of death were obtained from medical records.

STATISTICAL ANALYSIS Categorical variables were compared using Fisher's exact test or the chi-square test. For the statistical analysis of immunofluorescence data, we used the nonparametric Spearman's rank correlation. For the analysis of creatinine level and GFR, we used the Kruskal-Wallis one-way analysis of variance (ANOVA). All analyses were performed using SPSS software, version 12.0.1 (SPSS, Chicago, IL). P values less than 0.05 were considered significant.

Results

Relationships between the presence of aPL, the presence of microthrombi, and C4d staining. Forty-four renal biopsy samples from 38 patients with lupus nephritis were examined. For patients with multiple biopsy samples, only the first biopsy sample in which lupus nephritis was diagnosed was analyzed. The mean (+/-SD) age of patients at the time of biopsy was 31.9 +/- 11.2 years (range 14–66 years). Twenty patients (52.6%) were positive for aPL, according to the Sapporo criteria. IgG aCL was present in 17 of 36 patients (47%), and LAC was present in 10 of 31 patients (32%). A significant relationship was found between the presence of microthrombi and the intensity of glomerular C4d staining. In 15 renal biopsy samples, intense glomerular C4d staining was observed. Of these biopsy samples, 7 had microthrombi, whereas microthrombi were absent in almost all other biopsy samples (P ‹0.002, by Fisher's exact test) (Table 2). Within the renal tissue specimens, microthrombi were found almost exclusively in glomeruli. In some cases, microthrombi were also found in the interstitial arterioles and capillaries. Thrombi were typically not found in areas of active inflammation. Among the 8 patients with biopsy specimens showing microthrombi, 5 were positive for aPL, compared with 15 of 30 patients without microthrombi. This difference was not statistically significant.

Neither the intensity of glomerular C4d staining nor the deposition of C4d in peritubular capillaries was related to aPL status (Table 3). Glomerular C4d staining and C4d in peritubular capillaries were

not related to the presence of aCL or LAC alone, or to the ISN/RPS classification for lupus nephritis. C4d positivity in arterioles occurred in only 3 patients, and none of these arterioles contained thrombi. Relationship between glomerular C4d staining and the presence of antibodies (IgG, IgA, IgM), C3, and C1q. Table 4 shows the relationship between immune complex deposits, glomerular C4d, and aPL. Consistent with results previously reported by Kim and Jeong[21], we found that increased intensity of C4d deposition was significantly correlated with the presence of capillary C3 (r = 0.348, P = 0.023). We also found a nearly significant correlation between the intensity of C4d staining and the presence of capillary IgG (r = 0.316, P = 0.064). There was also a nearly significant correlation between the intensity of C4d staining and the presence of aPL(r = 0.315, P = 0.054).

RESULTS OF CLINICAL FOLLOW-UP The serum creatinine level and the GFR were available for all patients over at least 3 years of followup and for some patients for up to 15 years of followup. Of the 38 patients, 4 died before the study started, and of those, 3 had developed endstage renal failure requiring dialysis. Of the 3 patients who died with end-stage renal failure, 2 had biopsies showing renal microthrombi and intense C4d staining.

Two other patients had developed end-stage renal failure by the time our study started. In these patients, renal biopsy did not show microthrombi, but in 1 patient the biopsy did show intense C4d staining. The mean (+/-SD) GFR after 3 years of followup was 73 +/- 38 ml/minute in the group with no C4d staining (n = 5), 68 +/- 22 ml/minute in the group with mild C4d staining (n = 18), and 62 +/- 23 ml/minute in the group with intense staining (n = 15). There were no statically significant differences between these 3 groups (P = 0.34 by Kruskal-Wallis one-way ANOVA).

FOLLOW-UP BIOPSY FINDINGS Multiple biopsy samples were available for 4 patients. Three patients each had 2 biopsies, and 1 patient had 4 biopsies. Follow-up biopsies showed consistently positive or negative staining in peritubular capillaries (3 of 4 patients had all positive results, while 1 patient had no peritubular capillary staining in either sample). Likewise, followup biopsies showed equally intense glomerular C4d staining at all time points. The patient with 4 biopsy samples showed both intense glomerular C4d staining and positive peritubular capillaries in all biopsy samples (Figure 2). In the diagnostic evaluation for this patient, microthrombi were first detected on the third biopsy. Additional stainings made for the purpose of the present study showed that microthrombi were also present in the first biopsy sample. During the course of the disease, results of tests for aCL and LAC were consistently negative. Because the clinical history of this patient was very illustrative of our results regarding the combination of intense glomerular C4d staining with the presence of microthrombi, we briefly present the case history here.

CASE HISTORY The patient, a 27-year-old woman, presented with butterfly exanthema, alopecia, and renal involvement with proteinuria and active urinary sediment, and SLE was diagnosed. Positive antinuclear factor and anti–double-stranded DNA antibodies were present, and no aPL were found. The first renal biopsy specimen obtained indicated lupus nephritis class V, and prednisone therapy and azathioprine were initiated. At age 28 years, a second renal biopsy specimen obtained, showing a diffuse proliferative glomerulonephritis compatible with lupus nephritis class IV (A/G). Additionally, renal vein thrombosis developed, but the patient was still negative for aPL. She was treated with vitamin K antagonists as anticoagulant therapy. Pulse cyclophosphamide (CYC) was administered, and the patient's condition stabilized. Because aPL did not seem to play a role, anticoagulant therapy was terminated after 2 years. Because the patient's renal function continued to decline during the following 2 years, and proteinuria continued to increase during pulse CYC and prednisone therapy, a third renal biopsy specimen was obtained. Intra- and extracapillary proliferative lupus nephritis class IV (A/C) and evidence of a thrombotic microangiopathy were found. Shortly thereafter,

the patient presented with upper body ataxia and aphasia. These neurologic complications were most likely associated with the thrombotic microangiopathy in combination with active SLE. The patient remained negative for aPL, but complement values were very low. She was treated with 10 plasmapheresis sessions and hemodialysis, and her GFR declined to 15 mmoles/liter. At age 39 years, prolonged hemodialysis was started. Two years later, acute neurologic deterioration and respiratory distress occurred, and the patient was admitted to the intensive care unit, where she died, likely due to diffuse alveolar hemorrhage in the lungs and bleeding in and around multiple organs during active lupus nephritis. At autopsy, the kidneys showed extensive chronic damage, classified as lupus nephritis class IV-C (G). In the brain, diffuse chronic and global ischemia was detected, accompanied by a few vessels in the white matter with intraluminal thrombi and extensive cerebral gliosis. For the present study, all of the kidney biopsy specimens obtained from the patient were stained for C4d, and all of them were positive for similar high-intensity glomerular C4d staining (Figure 2).

Discussion

We observed a striking relationship between the intensity of glomerular C4d deposition and the presence of renal microthrombi in lupus nephritis. This report describes the novel finding of the presence of C4d in lesions indicative of thrombotic microangiopathy in nontransplant biopsy specimens. It is likely that activation of the classical pathway of complement is a crucial pathogenic intermediate in the development of thrombosis in lupus nephritis. In the group of patients included in the present study, there was no definitive relationship between C4d deposition and the presence of aPL. The presence of aPL was not correlated with the presence of microthrombi. This suggests that additional factors, such as other autoantibodies that have not yet been identified and assessed, may trigger activation of the classical pathway of complement in these patients. Although its function is closely related to the classical pathway, C4d is also involved in the lectin pathway. Therefore, we cannot exclude the possibility that C4d deposition partly reflects activation of the lectin pathway due to, for example, IgM autoantibodies.[25]

The case report presented in this study illustrates that C4d deposition can be found in the kidney years prior to the time when actual thrombotic microangiopathy is revealed. Regarding the clinical outcome in the patients in the present study, there appeared to be a trend toward a less favorable renal outcome in the group of patients whose biopsy samples showed more intense C4d staining. However, this trend did not reach statistical significance. Of the 5 patients in whom end-stage renal failure developed, 2 had renal microthrombi and 3 had intense C4d staining, suggesting that the presence of intense C4d staining may be related to a less favorable clinical outcome. Based on the findings of our study, positive C4d staining in a renal biopsy sample from a patient with lupus nephritis should raise suspicion of thrombotic complications, even in the absence of aPL. However, further studies are needed to define the clinical implications of C4d deposition as a biopsy finding, because in this retrospective study, our results were obviously biased by several factors that were uncontrolled, such as ongoing disease activity at the time of measurement and co-medication. Our findings therefore indicate the need for future prospective and controlled studies in larger groups of patients, in order to investigate the potential of C4d staining in the renal biopsy specimen to predict the serious clinical complication of thrombotic microangiopathy in SLE and APS.

Our study was initiated by the results of the recent study by Navratil *et al*[17], which showed a specific relationship between platelet C4d and the presence of aPL in SLE. Platelet C4d was detected in 18% of 105 patients with SLE, and was 100% specific for a diagnosis of SLE compared with healthy controls, and 98% specific for SLE compared with patients with other diseases. The authors suggested that, apart from indicating that platelet-bound C4d may be a biomarker for SLE, their findings provided a clue to the pathogenic mechanisms responsible for the myriad thrombotic and vascular complications

of lupus associated with aPL. Thus far, the exact role of aPL in the development of lupus-related thrombotic microangiopathy has not been elucidated. The ability of aPL to activate endothelial cells in vitro and in vivo, induce platelet activation, and interact with elements of the coagulation cascade has been well established.[26] These findings indicate that aPL seem to play an important role in the initial phase of a cascade leading to thrombotic events. In addition to this, many studies have shown complement activation to be essential. The detrimental role of complement is underlined in experimental studies, such as that by Nangaku *et al*[27], demonstrating that thrombotic microangiopathy is prevented if C5b–9 (the membrane attack complex) is temporarily inhibited. Pierangeli *et al*[15] demonstrated that C3- and C5-deficient mice were resistant to aPL-induced thrombosis. How would complement activation result in a thrombotic event? A novel finding is the role of tissue factor activation as an inducer of thrombosis downstream of complement. It was recently demonstrated that aPL-induced complement activation and downstream signaling via C5a receptors in neutrophils lead to the induction of tissue factor, a key initiating component of the blood coagulation cascade[28]. Another study identified tissue factor as an important mediator of the C5a-induced oxidative burst in neutrophils in the setting of aPL-induced fetal injury[29]. These findings are interesting in light of our results, since they may explain why such a strong relationship was found between the presence of C4d and microthrombi in this study. In patients with SLE, APS may become clinically apparent only after a major thrombotic event. In many hospitals, patients with SLE are not routinely tested for aPL but are tested only 'on clinical indication.' In some instances, the detection of microthrombi in the renal biopsy specimen is the first clue that thrombotic microangiopathy is present. However, the detection of microthrombi in renal biopsy specimens is not very sensitive, which is likely due to sampling error.

We have illustrated the difficulty of identifying and managing thrombotic microangiopathy in an SLE patient in the case history presented in this study. In this patient, the presence of thrombotic microangiopathy became evident fairly late in the clinical course, namely when a microthrombus was detected on the third renal biopsy specimen, which was obtained 7 years after disease presentation. Earlier tests for aPL were negative, which contributed largely to the decision to stop oral anticoagulant therapy after renal vein thrombosis. The patient experienced severe renal and neurologic complications, which were confirmed at autopsy to have been caused by microthrombi. In this patient, C4d staining was notably intense in all biopsy samples, as shown in Figure 2, and could have indicated the risk of a thrombotic microangiopathy much earlier in the time course of the disease. Our results further suggest the potential for C4d staining in guiding the therapeutic strategy, such as the earlier and prolonged use of immunosuppressive therapy or anticoagulant therapy. Recently, immunoreactivity to C4d protein was reported to be significantly stronger in the placentas of patients with aPL than in the placentas of healthy controls.[30] These findings are consistent with the results of murine studies by Girardi *et al*[31; 32], indicating that low molecular weight heparin, even at doses that do not interfere with coagulation, protects pregnancies against aPL-induced damage mor thrombosis, because it blocks the activation of complement. Interestingly, anticoagulant treatment without any anticomplement effect was not protective, suggesting that anticoagulation in and of itself is not sufficient for patients with APS-related miscarriage. If complement is indeed a critical factor in the development of endothelial damage and microthrombi in kidneys of patients with SLE and/or APS, the possibility that treatment with heparin at subcoagulant doses could have beneficial effects in these patients should be investigated. Notably, the patient described in the case history presented in this study was treated with vitamin K antagonists as anticoagulant therapy after renal vein thrombosis and received several plasmapheresis sessions when thrombotic microangiopathy was discovered, but this therapeutic intervention was not successful. She never received heparin during the course of her disease.

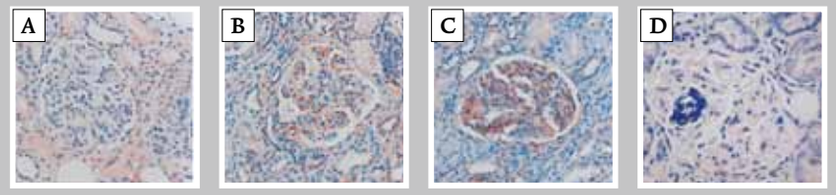
A shortcoming of the present study regards the detection of microthrombi in renal biopsy specimens. Given their relatively sparse

presence, it is likely that microthrombi are easily missed in the small tissue sample obtained at renal biopsy. Therefore, we cannot exclude the possibility that renal microthrombi were missed due to sampling error in the 8 patients whose biopsy samples showed intense glomerular C4d staining but no thrombotic lesions when stained with phosphotungstic acid–hematoxylin. It is also possible that some focal microthrombi were missed in patients whose biopsy samples showed only mild or no staining for C4d.

In this study, only 1 patient had renal microthrombi in the absence of C4d deposition. In future studies, more patients should be included, and thrombotic microangiopathy should be investigated more closely, by examining more biopsy slides or by taking into account other clinical parameters indicative of thrombotic microangiopathy.

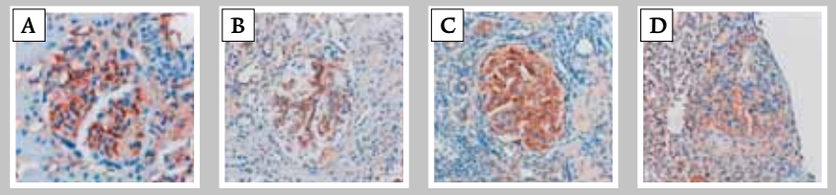
Importantly, not all patients with SLE and aPL develop thrombotic microangiopathy. According to Harris and Pierangeli[33], a 'second hit,' e.g., infection or trauma, is necessary to trigger thrombosis at the vessel site where aPL have deposited. Conversely, not all patients with SLE and thrombotic microangiopathy have aPL. This suggests that the deposition of antibodies other than aPL may lead to a thrombotic event, or that aPL are only transiently present in some SLE patients. Although the etiology of neither situation is completely understood, it seems wise to look at complement deposition, and deposition of C4d in particular, in all patients with lupus nephritis. Because activation of the classical pathway of complement is likely to be a marker of endothelial damage leading to thrombotic microangiopathy in lupus nephritis, C4d immunodetection may be a useful tool in determining whether patients are at risk of this complication. In our experience, the serologic detection of aPL is only marginally related to evidence of microthrombi in the kidney. Of 8 patients with microthrombi, aPL were found in 5, and of 30 patients without microthrombi, aPL were present in 15. This difference was not statistically significant. The results of the present study strongly support the notion that activation of the classical pathway of complement is a crucial factor in the development of thrombosis in lupus nephritis. Furthermore, we propose that C4d may be an important additional tool in the evaluation of renal biopsy specimens obtained from patients with SLE. Future prospective studies are needed to investigate this possibility. Our findings also have therapeutic implications, in that staining renal biopsy specimens for C4d could be an easy and elegant method of identifying patients with lupus nephritis who are at risk of thrombotic microangiopathy.

FIG 1 DIFFERENT INTENSITIES OF GLOMERULAR C4D STAINING IN LUPUS NEPHRITIS



Renal biopsy samples obtained from patients with lupus nephritis, showing different intensities of glomerular C4d staining and a glomerular microthrombus. A, No glomerular C4d staining. B, Mild to moderate glomerular C4d staining. C, Intense glomerular C4d staining. D, A glomerular microthrombus stained bright blue with a phosphotungstic acid–hematoxylin stain for fibrin. (Original magnification X400.)

FIG 2 FOUR BIOPSY SAMPLES OF ONE PATIENT



Four renal biopsy samples obtained from a patient with lupus nephritis complicated by a thrombotic microangiopathy during the disease course. The patient was negative for antiphospholipid antibodies. Glomerular C4d staining intensity was uniformly strong in all biopsy samples. (A) First biopsy sample in which lupus nephritis was diagnosed. Microthrombi were not detected. (B) Biopsy sample obtained after development of renal vein thrombosis and a decline in renal function. (C) Biopsy sample showing an intensely stained glomerulus. Thrombotic microangiopathy was diagnosed, and neurologic complications developed. (D) Biopsy sample obtained at autopsy, showing extensive chronic damage. C4d deposition was still detectable. (Original magnification X400.)

TABLE 1 CLINICAL CHARACTERISTICS AND LABORATORY FINDINGS IN THE 38 PATIENTS WITH LUPUS NEPHRITIS*

Age at biopsy, mean ± SD (range) years	31.9 ± 11.2 (14–66)
Sex, no. female	35
ISN/RPS class for lupus nephritis, no. of samples†	
I	0
II	1
III	9
IV	24
V	2
VI	0
Unclassified	2
Immunologic disorders, no. (%) positive‡	
Antiphospholipid antibody disorders§	20 (53)
Anticardiolipin antibodies, IgG	17 (47)
Anticardiolipin antibodies, IgM	11 (32)
Lupus anticoagulant	10 (32)
Antinuclear antibodies	34 (90)
Anti-EMA antibodies	22 (59)
Anti-dsDNA antibodies	28 (74)
Anti-C1q antibodies	8 (33)
Serum creatinine level, median (range) μmoles/liter	95.0 (56–485)
GFR, median (range) μmoles/liter	70.1 (9.6–119.4)
Serum C3 level, median (range) μmoles/liter	45.5 (1–115)
Serum C4 level, median (range) μmoles/liter	17.0 (2–46)
Serum C1q level, median (range) μmoles/liter	11.5 (1–21)

* ISN/RPS = International Society of Nephrology/Renal Pathology Society; anti-dsDNA = anti–double-stranded DNA; GFR = glomerular filtration rate.

† Subclasses are not shown.

‡ IgG anticardiolipin antibodies were tested in 36 patients; IgM anticardiolipin antibodies were tested in 34 patients; lupus anticoagulant was tested in 31 patients; anti–extractable nuclear antigen (anti-ENA) antibodies were tested in 37 patients; and anti-C1q antibodies were tested in 24 patients.

§ Defined by the Sapporo criteria, specifically by the presence of IgG anticardiolipin antibody or lupus anticoagulant

TABLE 2 RELATIONSHIP OF GLOMERULAR C4D STAINING TO THE PRESENCE OF MICROTHROMBI IN THE 38 PATIENTS WITH LUPUS NEPHRITIS*

NO DETECTABLE MICROTHROMBI (N = 30)	MICROTHROMBI PRESENT (N = 8)	TOTAL (N = 38)
4	1	5
18	0	18
8	7†	15

* Values are the number of patients. P ◂ 0.002 for the presence of microthrombi in samples with intense staining versus samples with no staining or mild to moderate staining.

TABLE 3 RELATIONSHIP OF C4D STAINING AND PRESENCE OF MICROTHROMBI TO APL STATUS IN THE 38 PATIENTS WITH LUPUS NEPHRITIS*

	PATIENTS WITH APL (N = 20)	PATIENTS WITHOUT APL (N = 18)
C4d peritubular capillary staining		
Positive	10	12
Negative	10	6
C4d glomerular staining		
No staining	1	4
Mild to moderate staining	9	9
Intense staining	10	5
Presence of microthrombi		
Microthrombi present	5	3
No detectable microthrombi	15	15

* Values are the number of patients. APL = antiphospholipid antibodies

TABLE 4 RELATIONSHIP BETWEEN IMMUNE COMPLEX DEPOSITS, GLOMERULAR C4D STAINING, AND THE PRESENCE OF ANTIPHOSPHOLIPID ANTIBODIES IN THE 38 PATIENTS WITH LUPUS NEPHRITIS*

PATIENT	GLOMERULAR C4D STAINING	APL	ARTERIOLAR C4D	MICRO-THROMBI	IGG	IGM	IGA	C3	C1Q
1	0	0	0	0					
2	0	0	0	0					
3	0	0	0	1	2	2	2	2	2
4	0	0	0	0	2	1	1	2	1
5	0	1	0	0	1	1	2	1	2
6	1	0	0	0	1	1	0	2	1
7	1	0	0	0	1	0	1	0	0
8	1	0	0	0	1	2	2	2	2
9	1	0	0	0	1	1	1	0	1
10	1	0	0	0	1	2	1	1	2
11	1	1	0	0	1	1	1	1	1
12	1	1	0	0	2	2	1	1	1
13	1	1	0	0	1	1	1	1	1
14	1	0	0	0	1	1	0	1	0
15	1	0	0	0	0	1	0	1	1
16	1	0	0	0	2	2	1	2	2
17	1	0	0	0	2	1	1	2	1
18	1	1	0	0	0	1	1	1	1
19	1	1	0	0	2	2	1	2	2
20	1	1	0	0	1	1	2	2	2
21	1	1	0	0					
22	1	1	1	0	1	1	1	1	1
23	1	1	0	0	1	1	1	1	1
24	2	0	0	1	2	2	1	2	0
25	2	0	0	0	1	1	1	1	1
26	2	0	0	0	2	2	2	2	2
27	2	0	0	1	2	2	1	2	2
28	2	0	1	0	2	1	0	2	1
29	2	1	0	1	2	2	2	2	2
30	2	1	0	0	0	1	1	1	1
31	2	1	0	0	1	0	2	2	1
32	2	1	0	1	2	2	2	2	2
33	2	1	0	0	2	2	1	2	2
34	2	1	0	0	2	2	1	2	2
35	2	1	0	1	2	2	2	2	2
36	2	1	1	1	2	0	1	2	2
37	2	1	0	1	2	2	1	2	2
38	2	1	0	0	1	2	2	1	2

* Values are the score. Glomerular C4d staining of each sample was scored on a scale of 0–2, where 0 = no glomerul ar staining, 1 = mild to moderate glomerular staining, and 2 = intense glomerular staining. Samples were given a score of 1 or 0 for the presence or absence, respectively, of antiphospholipid antibodies (aPL), arteriolar C4d, and microthrombi. The intensity of immunofluorescence for IgG, IgM, IgA, C3, and C1q was scored on a scale of 0–2. Data on IgG IgM, IgA, C3, and C1q were not available for patients 1, 2, and 21.

REFERENCES

1 Cameron JS. Lupus nephritis. J Am Soc Nephrol 1999;10:413–24.

2 Weening JJ, D'Agati VD, Schwartz MM, Seshan SV, Alpers CE, Appel GB, *et al.* The classification of glomerulonephritis in systemic lupus erythematosus revisited [published erratum appears in J Am Soc Nephrol 2004;15:835–6]. J Am Soc Nephrol 2004;15:241–50.

3 Descombes E, Droz D, Drouet L, Grunfeld JP, Lesavre P. Renal vascular lesions in lupus nephritis. Medicine (Baltimore) 1997;76:355–68.

4 Appel GB, Pirani CL, D'Agati V. Renal vascular complications of systemic lupus erythematosus. J Am Soc Nephrol 1994;4:1 499–515.

5 Grishman E, Venkataseshan VS. Vascular lesions in lupus nephritis. Mod Pathol 1988;1:235–41.

6 Magil AB, McFadden D, Rae A. Lupus glomerulonephritis with thrombotic microangiopathy. Hum Pathol 1986;17:192–4.

7 Bridoux F, Vrtovsnik F, Noel C, Saunier P, Mougenot B, Lemaitre V, *et al.* Renal thrombotic microangiopathy in systemic lupus erythematosus: clinical correlations and long-term renal survival [published erratum appears in Nephrol Dial Transplant 1998;13: 1328]. Nephrol Dial Transplant 1998;13:298–304.

8 Jain R, Chartash E, Susin M, Furie R. Systemic lupus erythematosus complicated by thrombotic microangiopathy. Semin Arthritis Rheum 1994;24:173–82.

9 Hughson MD, Nadasdy T, McCarty GA, Sholer C, Min KW, Silva F. Renal thrombotic microangiopathy in patients with systemic lupus erythematosus and the antiphospholipid syndrome. Am J Kidney Dis 1992;20:150–8.

10 Tektonidou MG, Sotsiou F, Nakopoulou L, Vlachoyiannopoulos PG, Moutsopoulos HM. Antiphospholipid syndrome nephropathy in patients with systemic lupus erythematosus and antiphospholipid antibodies: prevalence, clinical associations, and long-term outcome. Arthritis Rheum 2004;50:2569–79.

11 Nochy D, Daugas E, Huong DL, Piette JC, Hill G. Kidney involvement in the antiphospholipid syndrome. J Autoimmun 2000;15:127–32.

12 Cervera R, Piette JC, Font J, Khamashta MA, Shoenfeld Y, Camps MT, *et al*, for the Euro-Phospholipid Project Group. Antiphospholipid syndrome: clinical and immunologic manifestations and patterns of disease expression in a cohort of 1,000patients. Arthritis Rheum 2002;46:1019–27.

13 Hughes GR. The antiphospholipid syndrome: ten years on. Lancet 1993;342:341–4.

14 Alarcon-Segovia D, Deleze M, Oria CV, Sanchez-Guerrero J, Gomez-Pacheco L, Cabiedes J, *et al.* Antiphospholipid antibodies and the antiphospholipid syndrome in systemic lupus erythematosus: a prospective analysis of 500 consecutive patients. Medicine (Baltimore) 1989;68:353–65.

15 Pierangeli SS, Girardi G, Vega-Ostertag M, Liu X, Espinola RG, Salmon J. Requirement of activation of complement C3 and C5 for antiphospholipid antibody–mediated thrombophilia. Arthritis Rheum 2005;52:2120–4.

16 Pierangeli SS, Chen PP, Gonzalez EB. Antiphospholipid antibodies and the antiphospholipid syndrome: an update on treatment and pathogenic mechanisms. Curr Opin Hematol 2006;13:366–75.

17 Navratil JS, Manzi S, Kao AH, Krishnaswami S, Liu CC, Ruffing MJ, *et al.* Platelet C4d is highly specific for systemic lupus erythematosus. Arthritis Rheum 2006;54:670–4.

18 Collins AB, Schneeberger EE, Pascual MA, Saidman SL, Williams WW, Tolkoff-Rubin N, *et al.* Complement activation in acute humoral renal allograft rejection: diagnostic significance of C4d deposits in peritubular capillaries. J Am Soc Nephrol 1999;10:2208–14.

19 Crespo M, Pascual M, Tolkoff-Rubin N, Mauiyyedi S, Collins AB, Fitzpatrick D, *et al.* Acute humoral rejection in renal allograft recipients. I. Incidence, serology and clinical characteristics. Transplantation 2001;71:652–8.

20 Zwirner J, Felber E, Herzog V, Riethmuller G, Feucht HE. Classical pathway of complement activation in normal and diseased human glomeruli. Kidney Int 1989;36:1069–77.

21 Kim SH, Jeong HJ. Glomerular C4d deposition indicates in situ classic complement pathway activation, but is not a marker for lupus nephritis activity. Yonsei Med J 2003;44:75–80.

22 Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, *et al.* The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271–7.

23 Wilson WA, Gharavi AE, Koike T, Lockshin MD, Branch DW, Piette JC, *et al.* International consensus statement on preliminary classification criteria for definite antiphospholipid syndrome: report of an international workshop [published erratum appears in Arthritis Rheum 1999;42:1997]. Arthritis Rheum 1999;42:1309–11.

24 Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D, for the Modification of Diet in Renal Disease Study Group. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Ann Intern Med 1999;130: 461–70.

25 McMullen ME, Hart ML, Walsh MC, Buras J, Takahashi K, Stahl GL. Mannose-binding lectin binds IgM to activate the lectin complement pathway in vitro and in vivo. Immunobiology 2006; 211:759–66.

26 Pierangeli SS, Colden-Stanfield M, Liu X, Barker JH, Anderson GL, Harris EN. Antiphospholipid antibodies from antiphospholipid syndrome patients activate endothelial cells in vitro and in vivo. Circulation 1999;99:1997–2002.

27 Nangaku M, Alpers CE, Pippin J, Shankland SJ, Kurokawa K, Adler S, *et al.* CD59 protects glomerular endothelial cells from immune mediated thrombotic microangiopathy (TMA). J Am Soc Nephrol 1997;8:590–7.

28 Ritis K, Doumas M, Mastellos D, Micheli A, Giaglis S, Magotti P, *et al.* A novel C5a receptor-tissue factor cross-talk in neutrophils links innate immunity to coagulation pathways. J Immunol 2006; 177:4794–802.

29 Redecha P, Tilley R, Tencati M, Salmon JE, Kirchhofer D, Mackman N, *et al.* Tissue factor: a link between C5a and neutrophil activation in antiphospholipid antibody induced fetal injury. Blood 2007;110:2423–31.

30 Shamonki JM, Salmon JE, Hyjek E, Baergen RN. Excessive complement activation is associated with placental injury in patients with antiphospholipid antibodies. Am J Obstet Gynecol 2007;196:167–5.

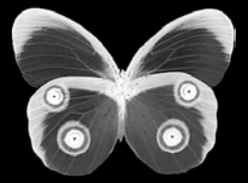
31 Girardi G, Redecha P, Salmon JE. Heparin prevents antiphospholipid antibody-induced fetal loss by inhibiting complement activation. Nat Med 2004;10:1222–6.

32 Salmon JE, Girardi G. Antiphospholipid antibodies and pregnancy loss: a disorder of inflammation. J Reprod Immunol 2008;77:51–6.

33 Harris EN, Pierangeli SS. Primary, secondary, catastrophic antiphospholipid syndrome: is there a difference? Thromb Res 2004;114:357–61.

V

NATIONWIDE AUTOPSY STUDY LINKS COMPLEMENT TO THROMBO-ISCHEMIA IN NEUROLUPUS



DANIELLE COHEN / ROBERT JA NABUURS / GERDA M STEUP-BEEKMAN
MAARTEN J VERSLUIS / BART J EMMER / MALU ZANDBERGEN / JASPER LUYENDIJK
MARK A VAN BUCHEM / CORNELIA F ALLAART / RON WOLTERBEEK / JAN A BRUIJN
SJOERD G VAN DUINEN / TOM WJ HUIZINGA / INGEBORG M BAJEMA

SUBMITTED

Abstract

OBJECTIVE Neuropsychiatric (NP) involvement is a poorly understood manifestation of systemic lupus erythematosus (SLE). Studies in humans have failed to provide clues for interactions between autoantibody-mediated inflammation and thrombo-ischemic lesions observed in brains of NP-SLE patients. We hypothesized that cerebral complement activation could induce microthrombotic injury in NP-SLE. Furthermore, we aimed to correlate post-mortem histopathology to ex-vivo7 Tesla MRI imaging in three brains of SLE patients.

METHODS A nationwide search for autopsy material from SLE patients resulted in brain tissue from 16 patients with NP-SLE and 18 patients with SLE. Brains from 24 previously asymptomatic patients who died from acute cardiac events served as controls. Paraffin embedded tissue of the cerebral cortex was stained for complement components of the classical- and lectin pathway. Of three NP-SLE patients, whole formalin fixed brains were available for MRI analysis.

RESULTS Cerebral complement deposition was strongly associated with both SLE and NP-SLE compared to controls (P‹0,001) and C1q and C4d deposits were typically seen in small vessels affected by vasculopathy. Microthrombi were exclusively found in NP-SLE and were associated with C4d deposition (P=0,029). MRI analysis revealed that the majority of small vessel injury was found in the vicinity of white matter hyperintensities.

INTERPRETATION Cerebrovascular complement activation is common in SLE patients but not in controls, and deposits of C4d are closely associated to microthrombotic injury. These findings provide a novel explanation for the interaction between circulating autoantibodies and the development of thrombo-ischemic lesions observed in NP-SLE. Complement activation has potential as a novel therapeutic target in NP-SLE.

Introduction

Systemic lupus erythematosus (SLE) is a severe systemic autoimmune disease that primarily affects women of childbearing age.[1] The presence of circulating auto-antibodies and immune complexes is the hallmark of SLE, and disease manifestations can occur in virtually all organ systems.[2] The devastating consequences of cerebral involvement of SLE are commonly referred to as neuropsychiatric SLE (NP-SLE).[3] NP-SLE develops in about 50-70% of SLE patients who may show symptoms varying from stroke to psychosis.[4;5] Due to the heterogeneity of the condition and the lack of both etiological insight and evidence based therapeutic interventions, the clinical management of these patients is complex.

In the extensive work-up of patients with suspected NP-SLE, magnetic resonance imaging (MRI) of the brain plays a prominent role[4] although the interpretation is often hampered by a so-called 'clinicoradiological paradox': Some patients with SLE and severe neurological symptoms only show minor abnormalities on MRI, whereas the reverse also occurs. In a recent systematic analysis of MRI findings in the setting of NP-SLE, absence of MRI abnormalities despite signs and symptoms of active disease was found in 42% of all patients. Overall, the most common MRI findings were white matter hyperintensities, suggestive of cerebral hypoperfusion and infarction.[6]

The few studies on histopathological findings in brains obtained from SLE patients with neuropsychiatric symptoms showed that the most prominent pathological lesions were related to ischemic injury in the vicinity of small vessels.[3;7-10] Microthrombosis, microinfarction, and microhemorrhage were present in the majority of cases. These findings suggest a thrombo-ischemic pathofysiology, whereas SLE is known as a disease with an autoantibody-mediated inflammatory nature. So far, studies in humans have failed to provide clues for interactions between autoantibody- mediated inflammation and the thrombo-ischemic lesions observed in the brain.

We recently demonstrated that the intensity of classical complement activation in glomeruli corresponds to the presence of glomerular microthrombi in lupus nephritis.[11] This is in line with findings in antiphospholipid syndrome (APS)[12-14] and atypical hemotylic uremic syndrome (HUS)[15] where there is good evidence that complement activation can induce endothelial activation, endothelial injury and (micro)thrombosis.[13;16;17] We hypothesize that this mechanism could also be responsible for the vascular pathology observed in NP-SLE.

To test this hypothesis we performed a nationwide search for cerebral autopsy material of SLE patients with and without NP symptoms. In this unique cohort we investigated the presence of complement depositions and their correlation to thrombo-ischemic lesions. As a secondary aim, we questioned whether our post-mortem findings can be potentially detected clinically by correlating ex- vivo imaging with 7 Tesla MRI with post-mortem histology of three brains of SLE patients.

Methods

PALGA search for cerebral tissue of SLE patients

To study the role of complement in NP-SLE we conducted a nationwide search for cerebral autopsy-tissue from SLE patients with and without clinical signs of neuropsychiatric involvement. For this purpose we used the Dutch PALGA system (www.palga.nl), a unique histopathology data network, encompassing data from the archives of all sixty-four pathology laboratories in The Netherlands.[18] Our search parameters included 'SLE', 'Systemic Lupus Erythematosus', 'Lupus' and 'Cerebral autopsy', which led to 296 hits from the PALGA database. We subsequently excluded patients who only had cutaneous or discoid lupus erythematosus, and included all patients with *systemic* LE of which cerebral autopsy tissue was available. This resulted in 48 appropriate cases. Of 14 cases paraffin blocks were no longer available or tissue quality was too poor for analysis. Finally, 34 formalin-fixed tissue samples from autopsied SLE cases were retrieved from 12 Dutch pathology laboratories for analysis.

PATIENTS We studied brain tissue of 34 patients (27 females, 7 males) with SLE. All patients fulfilled the 1982 American College of Rheumatology (ACR) revised criteria for SLE.[19] Cases were divided in patients with neuropsychiatric symptoms that could be attributed to SLE (NP-SLE group, n=16) and patients without neuropsychiatric symptoms or with neuropsychiatric symptoms that were evidently caused by other factors than SLE such as medication toxicity or infection (SLE group, n=18). Classification of patients in different groups was performed by two rheumatologists with extensive experience in diagnosing NP-SLE (MSB and TH) using the available patient data from clinical records and autopsy reports.

The following neuropathological symptoms (ACR case definitions) were present in our NP-SLE group: Cerebrovascular disease (n=11), movement disorder (chorea) (n=2), seizures and seizure disorders (n=2), acute confusional state (n=2), cognitive disorder (n=2), plexopathy (1) and psychosis (n=1). There were 11 patients with one symptom, 4 patients with 2 symptoms and 1 patient with 3 symptoms.

In our SLE group, 3 patients had neuropsychiatric symptoms that could be evidently attributed to other factors than SLE. One patient suffered large a intracerebral hemorrhage associated with anticoagulant treatment, one patient had a severe cerebral mycotic infection in association with high dose immunosuppressive therapy for class IV lupus nephritis and a third patient died from a uremic coma following acute renal failure caused by fulminant lupus nephritis.

Control cases were identified from the archives of the Leiden University Medical Centre (LUMC) and the Reinier de Graaff Hospital (Delft, the Netherlands), which included previously healthy patients who died from acute cardiac events confirmed by autopsy (control group, n=24). An overview of the clinical characteristics of all patients, derived from the autopsy-reports and from clinical data when available, is given in table 1.

BRAIN TISSUE For post-mortem preservation, the brain and spinal cord were fixed in 10% neutral buffered formalin for 14 days. The cerebral and cerebellar hemispheres were sectioned in the coronal plane. Regions of interest were dissected into tissue blocks, processed using standard tissue processing methods and embedded in paraffin.

HISTOLOGY AND IMMUNOHISTOCHEMISTRY Sections of all tissue blocks were stained with haematoxylin and eosin using standard methodologies. To study cerebral complement activation, we investigated the presence of C1q (representing activation of the classical pathway), Mannose Binding Lectin (MBL) (representing activation of the MBL pathway) and C4d (a split product of C4 which binds covalently to the target tissue, and is widely used as a tissue biomarker for antibody mediated endothelial injury. In transplant pathology C4d-staining is incorporated in the standard work-up for diagnosing antibody mediated rejection of a renal allografts.[20])

IMMUNOHISTOCHEMISTRY Immunohistochemistry was performed after deparaffinized sections were subjected to antigen retrieval by EDTA-TRIS (pH 9.0) or 10 mM citrate buffer (pH 6.0) (waarom twee methods). Sections were subsequently stained with antibodies to C1q (Dako Cytomation, Denmark, 1:800), C4d (Biomedica Gruppe, Austria, 1:50) and MBL (Sigma-Aldrich Biotechnology, 1:500). Staining was visualized with appropriate secondary antibodies and diaminobenzidine as the chromagen. Finally, sections were counterstained with haematoxylin. Optimal antibody dilutions and incubation times for the different antibodies were pre-determined by means of titration on positive control sections.

QUANTIFICATION OF HISTOPATHOLOGIC MORPHOLOGY AND IMMUNOHISTOCHEMISTRY All sections were evaluated by an experienced neuropathologist who was blinded to the patients' clinical data. Each case was scored separately for the presence (1) or absence (0) of microinfarction, macroinfarction, large hemorrhage, microbleeds, cerebral infection and vasculitis. Vasculopathy was defined as endothelial cell proliferation, thickening of the vessel wall and narrowing

of the capillary lumen, and was scored semiquantitatively as 'no vasculopathy' (total absence of vasculopathy in all low powerfields), 'mild vasculopathy' (1-2 vessels showing vasculopathy per low powerfield) or 'diffuse vasculopathy' (several vessels showing vasculopathy in all low powerfields).

Positivity for immunohistochemical stainings was scored by two independent observers blinded to the patients' clinical data. A similar semi-quantitative scoring system was used for C1q and C4d, which both mainly stained positive on endothelial cells of small vessels in the white and grey matter. C1q and C4d depositions were scored as 'no staining' (total absence of C1q or C4d staining in all low powerfields), 'mild staining' (1-2 vessels showing C1q or C4d positivity per low powerfield) or 'diffuse staining' (several vessels showing C1q or C4d positivity in all low powerfields). MBL was never positive in a vascular pattern, but deposited on single cells throughout the grey and white matter. Therefore, MBL-positive cells were scored as present (1) or absent (0).

WHOLE FORMALIN FIXED BRAINS: CLINICAL CASE HISTORIES In three subjects, whole formalin fixed brains were available that permitted direct comparison of postmortem MRI with cerebral histopathology. Detailed case histories of these patients are given below.

* PATIENT 1. NP-SLE, ANTIPHOSPHOLIPID SYNDROME AND CEREBROVASCULAR DISEASE This 57-year-old female patient with a 28-year history of SLE complicated by arthritis, endocarditis, epilepsy, cerebral infarctions and antiphospholipid syndrome, suffered an epileptic attack at home and was admitted in a confusional state. MRI imaging (1,5T) of 2 weeks before her death revealed diffuse cortical atrophy, multiple old cortical infarcts and diffuse white matter hyperintensities. She developed a myocardial infarction, severe pulmonary embolism, acute renal failure and multiple cerebral infarctions. Antinuclear, anti-dsDNA and antiphospholipid antibodies were repeatedly positive. There was no sign of CNS infection. She died in a coma from multiorgan failure due to active SLE and diffuse thrombotic complications. Cerebral autopsy revealed atrophy of the cerebral cortex, laminar cortical necrosis, old and recent micro- and macroinfarctions and diffuse vasculopathy.

* PATIENT 2. NP-SLE, ACUTE NEUROLOGICAL DETERIORATION AND VASCULITIS This 38-year-old man with a 10-year history of SLE was admitted in a subcomatose condition, a state which had developed the night before. His SLE was associated with skin lesions, pleuritis, pericarditis, arthritis and hypocomplementemia. Antinuclear and anti-dsDNA were positive, antiphospholipid antibodies were negative. All cerebrospinal fluid cultures at admission were negative. Premortem CT and MRI (1,5T) imaging did not show any abnormalities. Upon clinical diagnosis of NP-SLE the patient was treated with high dose immunosuppression (cyclophosphamide and prednisone). However, the clinical course was complicated by the development of an opportunistic pulmonary infection with Klebsiella pneumonia and the patient died from respiratory distress at the intensive care unit. Autopsy revealed venous abnormalities compatible with venous vasculitis (invasion of lymphocytes within the vascular wall and fibrinoid necrosis), and diffuse vasculopathy.

* PATIENT 3. SLE, ACUTE MYOCARDIAL INFARCTION AND NO NEUROPSYCHIATRIC SYMPTOMS This 63-year-old female patient with a 30-year history of SLE complicated by arthritis, glomerulonephritis, pleuritis and skin lesions died from a myocardial infarction. During the course of her disease she never had neuropsychiatric symptoms. She was positive for antinuclear antibodies and anti-dsDNA antibodies, and negative for antiphospholipid antibodies.

POST-MORTEM NEUROIMAGING AND EVALUATION OF ACQUIRED IMAGES Directly after autopsy formalin fixed brains were sectioned into approximately 1 centimeter thick coronal sections and stored

accordingly. Prior to imaging, remnants of the dura and vasculature were removed from the pial surface, and residual formalin was washed out by immersion in phosphate buffered saline (PBS) for at least one day to partially restore transverse relaxation parameters.[21] The brain specimens were placed in between two adjustable polymethyl methacrylate plates (170 mm long, 80 mm wide) and immersed in a proton free fluid (Fomblin LC55, Solvay). Postmortem MRI was acquired at a whole body 7 T system (Philips Healthcare, Best, The Netherlands) using a Nova Medical transmit coil with 16 channel receive array.

A modified protocol described before was used with echo times (TE) ranging from 20 – 40 ms.[22] After visual inspection the protocol with a TE of 35 ms was chosen for displaying the best image quality and contrast, and was subsequently used for imaging of all other brain specimens. Scan parameters were: Voxel resolution 0.3 x 0.3 x 0.3 mm^3 for a 3D T_2*-weighted gradient echo sequence with repetition time / TE / flip angle = 60 ms / 35 ms / 10°. The number of slices was adjusted to match the size of the brain specimen and varied between 60 and 80 slices, resulting in an approximate scan duration of 2h30min, 7 signal averages were acquired to obtain sufficient image quality.

Images were concurrently reviewed by two neuroradiologists with extensive experience in NP-SLE (MvB and BE) who identified areas of interest.

HISTOLOGICAL ANALYSIS OF POSTMORTEM MRI SCANNED BRAINS After radiological analysis, tissue blocks of those areas that were selected on postmortem MR images were sampled, embedded in paraffin and stained with haematoxylin and eosin. The neuropathologist then independently prepared a report detailing the histopathological changes in each sampled area, and pathological lesions were correlated to MRI findings.

ETHICAL CONSIDERATIONS Tissues and patient data were used according to the guidelines of the ethics committee of the LUMC. Patient anonymity was strictly maintained. All tissue samples were handled in a coded fashion, according to Dutch national ethical guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of Medical Scientific Societies).

STATISTICAL ANALYSIS Categorical variables were compared using the Chi-square test and its trend version (linear-by-linear analysis). Differences in quantitative parameters between groups were assessed using one-way ANOVA (for data normally distributed) or the non-parametric Kruskall Wallis-test (for non-normally distributed data). All analyses were performed using SPSS statistical software package (version 16.0; Chicago, IL). A *p*-value equal to or less than 0,05 was considered statistically significant.

Results

LIGHT MICROSCOPY AND NEUROPATHOLOGY The preponderance of pathology was found in the cerebral cortex, equally distributed over white and grey matter. Cases (NP-SLE and SLE) differed significantly on all histological parameters from controls, in which neuropathological abnormalities were rarely present. When comparing cases with and without neuropsychiatric symptoms, microinfarction (P=0,016), macroinfarction (P=0,002) and vasculitis (P=0,048) were more often present in NP-SLE than in SLE. Microthrombi were exclusively found in patients with NP-SLE, in 7 out of 16 patients (44%) (P=0,002) (figure 1).

The presence of vasculopathy and its distribution in either a focal or diffuse pattern differed significantly among the three groups (i.e. patients with NP-SLE, SLE and controls) in a chi-square linear by linear association analysis ($p<0.00001$). Whereas focal vasculopathy was present in all groups in circa 40-60% of subjects, diffuse vasculopathy was almost uniquely present in patients with NP-SLE, and significantly more often than in SLE patients without neuropsychiatric symptoms (P=0,001) (figure 2). Diffuse vasculopathy occurred in 9/16 patients with NP-SLE (56%) and in only 1/18 SLE patients (6%). Diffuse vasculopathy was not observed in controls.

In total, vasculitis was present in 6 patients, of which 5 in the NP-SLE-group and 1 in the SLE group (P=0,048). Every patient with NP-SLE and vasculitis also had vasculopathy (4 in a diffuse pattern, 1 in a focal pattern). Vasculitis was associated with cerebrovascular disease in 4 patients, and acute confusional state in one case. The patient with vasculitis with SLE (without NP-SLE) had a severe mycotic infection in association with immunosupression.

Typical histological examples of vasculopathy and cerebral microthrombi are shown in figure 3A and B respectively.

IMMUNOHISTOCHEMISTRY Staining patterns for C1q, C4d and MBL were investigated in all tissue samples. In figure 3C-H typical examples of immunohistochemical staining in cases and controls are shown.

C1Q C1q deposition was observed on endothelial cells of small vessels, as shown in figure 3D. Vessels in tissue of NP-SLE and SLE patients both had significantly more C1q depositions than controls (Table 2, P<0,001). A diffuse staining pattern for C1q was present in 12 cases, of which 5 (31%) with NP-SLE and 7 with SLE (38%). In controls diffuse C1q depositions never occurred. Focal deposition was present in 11 NP-SLE cases (69%) and 10 SLE cases (56%). All cases of NP-SLE showed diffuse or focal C1q deposition, whereas total absence of C1q was seen only once in SLE. Between SLE and NP-SLE there was no significant difference (P=0,531) with respect to C1q deposits. Nine out of 24 controls (38%) had detectable C1q depositions in a focal deposition pattern. In all other controls C1q deposits were undetectable. An overview of the different staining intensities in patients and controls is given in figure 4a.

C4D C4d depositions were also observed on endothelial cells, as shown in figure 3F. Similar to C1q, small vessels in tissue of NP-SLE and SLE patients both showed significantly more C4d depositions than controls (Table 2, P<0,001). A diffuse staining pattern was present in 5 patients, of which 2 patients with NP-SLE (13%) and 3 with SLE (17%). Focal deposition of C4d was present in 11 patients with NP-SLE (69%) and 14 patients with SLE (78%). Between SLE and NP-SLE there was no significant difference (P=0,800). C4d deposition in controls occurred in 6 out of 24 controls in a focal pattern. In all other controls, C4d deposits were negative. The different staining intensities in patients and controls are given in figure 4b.

MBL MBL did not deposit in a vascular pattern and as a consequence co-localization between MBL and C1q/C4d never occurred. However, MBL positivity was observed on star-shaped cells, most likely astrocytes. These MBL-positive cells were detected both in patients and controls (no statistically significant difference, data not shown) Examples of MBL-positive cells are given in figure 3G and 3H.

COMPLEMENT DEPOSITION, MICROTHROMBI AND MICRO-INFARCTION To find out whether complement deposition was associated with histological lesions that were associated to NP-SLE, the relation between C1q and C4d and presence of microthrombi, micro- and macro-infarction, vasculitis and vasculopathy was investigated. Of those, only the presence of microthrombi was significantly related to the presence of C4d staining (p=0,029, table 3) All cases with cerebral microthrombi showed focal or diffuse C4d staining. Three had a diffuse C4d staining pattern whereas the other four had a focal C4d staining pattern. Because microthrombi uniquely occurred in the NP-SLE patient group, the association between C4d and microthrombi within this group was analysed separately, and was significant in a linearbylinear association chi square analysis (p=0.029).

RELATION BETWEEN C4D AND C1Q IN SLE AND NP-SLE
To gain more insight in the cascading events of classical complement activation, staining-patterns of C1q and C4d were correlated to each other, within patient groups with SLE and NP-SLE. In general, diffuse C1q staining was present more often than diffuse C4d staining (35% vs 15%). Patients with positive C4d staining (focal or diffuse) had co-localized C1q deposits in 29 out of 30 positive cases

(97% overlap). Conversely, patients with positive C1q (focal or diffuse) had co-localized C4d in 28 out of 33 cases (85% overlap) In table 4 it can be appreciated that for both the SLE and the NP-SLE group the distribution of C1q and C4d are similar.

POST MORTEM MRI SCANS AND CORRELATIONS WITH HISTOPATHOLOGY AND IMMUNOHISTOCHEMISTRY Results of post mortem MR Imaging of two NP-SLE patient and one SLE patient are shown in figure 5.

* PATIENT 1: POST MORTEM MR IMAGING Imaging studies revealed extensive confluent periventricular and deep white matter lesions with notable sparing of U-fibers (figure 5, patient 1A and B). Furthermore, central lacunes were identified in the deep white matter suggesting tissue loss as can be seen in lacunar infarction. The white matter lesions peripheral to the confluent white matter lesions and adjacent to the deep white matter lesions had a perivascular distribution.

* PATIENT 1: HISTOPATHOLOGY Sections taken from both deep and periventricular white matter lesion showed areas of recent and older micro- and macroinfarction. In one deep white matter lesion multiple microthrombi (figure 5, patient 1C and D) were identified. In all sections prominent vasculopathy in both grey and white matter was found (figure 5, patient 1 E). Vasculopathy was seen within white matter hyperintensities and infarcted areas, but also in normal appearing white and grey matter. Furthermore, vast areas of the atrophied cortex, especially in proximity of infarctions, showed laminar necrosis (figure 5, patient 1F)

* PATIENT 2: POST MORTEM MR IMAGING Imaging studies revealed normal gray and white matter differentiation (figure 5, patient 2A and B) Several linear hyperintensities were characteristic of normal Virchow Robin spaces (figure 5, patient 2A). The white matter in patient 2 was homogenous compared to the white matter in patient 1 and there was a normal cortical thickness.

* PATIENT 2: HISTOPATHOLOGY As no abnormalities were identified on MRI, pathological sections were taken from various areas in the cortex. All sections revealed severe vasculopathy (figure 5, patient 2C and D). Furthermore, as described in the original autopsy-report, several venes and venules showed invasion of lymphocytes within the vascular wall, associated with fragmented nuclei and fibrinoid material (figure 5, patient 2E) No intracascular microthrombi, infarctions or gliosis lesions were discovered.

* PATIENT 3: POST MORTEM MR IMAGING Imaging studies revealed a prominent virchow robin space (figure 5, patient 3A) Furthermore, a linear perivascular white matter hyperintensity was discovered in the internal capsule. Another white matter lesion was identified in the frontal white matter (figure 5, patient 3B).

* PATIENT 3: HISTOPATHOLOGY Sections taken from the frontal white matter lesion showed a mild vasculopathy, but no other abnormalities. Vasculopathy was seen throughout all other sections as well in a similar focal distribution pattern (figure 5, patient 3C and D).

Discussion

The understanding of mechanisms involved in NP-SLE is poor. This lack of insight has major consequences for both patients and clinicians, mainly because there is no gold standard for diagnosis and no targeted treatment options. Our aim was to test the hypothesis that complement activation is involved in the pathofysiology of NP-SLE. With this study we are the first to show that vascular depositions of C1q and C4d, both components of the classical pathway of complement, were detected in cerebral vessels of patients with SLE and were present significantly more often in SLE and NP-SLE than in controls. Thrombo-ischemic injury was closely associated to NP-SLE. Interestingly, cerebral microthrombi were associated with the presence of C4d, and were found exclusively in

patients with NP symptoms. Our data support a pathogenic mechanism where SLE-related autoantibodies bind to endothelial cells of small vessels in the brain leading to local activation of the classical complement cascade, widespread vasculopathy, impaired cerebral perfusion, and finally, development of cerebral microthrombi and microinfarction.

The role of complement activation in the development of thrombosis and ischemia has been extensively studied outside the field of SLE. In the setting of antiphospholipid syndrome, both animal studies and studies in humans have shown that complement activation is essential for the development of antiphospholipid antibody-mediated thrombosis[13] and antiphospholipid antibody mediated fetal loss[23], the latter being characterized by placental infarction and fetal growth restriction.[14] By using complement deficient mice, or blocking complement activation pharmacologically, it was possible to reduce or even prevent the development of thrombosis.[24;25] In a non auto-immune model of hypoxic-ischemic brain injury in neonatal mice, Ten *et al* showed that significantly greater neurologic damage developed after an induced hypoxic-ischemic insult in wild type mice compared with C1q deficient mice.[26] The infarct volume that developed in the wild type mice was more than twice as large as in C1q deficient mice. This study provides strong evidence that hypoxic-ischemic brain injury can be mediated by C1q deposition. Given the fact that we observed C1q deposits in the majority of SLE patients, this mechanism might also contribute to cerebrovascular injury seen in SLE and NP-SLE.

Studies in animal models are less explicit about the histopathology of experimental NP-SLE, which in these studies is defined by altered animal behavior. The group of Diamond *et al* provided a body of evidence focusing on the role of autoantibodies in the development of neuropsychiatric symptoms. They demonstrated that anti-dsDNA antibodies derived from human SLE patients can enter the murine brain through a lipopolysaccharide induced breach in the blood-brain barrier and can cross-react with NMDA receptors on neurons.[27-29] In mice, this led to impaired cognition and emotional disturbance. Thus far it has not been possible to link the work of Diamond *et al* to the thrombo-ischemic injury observed in human NP-SLE cases.

With our study a new pathogenic factor in the form of complement activation enters the field. Our findings suggest that the constant exposure of autoantibodies to the cerebral endothelium of lupus patients causes continuous complement activation and endothelial injury in all SLE patients. Complement may activate endothelial cells leading to upregulation of adhesion molecules such as ICAM-1, e-selectin, p-selectin and VCAM promoting coagulation and proliferation. This mechanism, which has been also described in conditions like HUS[30], post-transplant ischemia-reperfusion damage[31] and severe malaria[32], provides a model for the development of small vessel injury and vasculopathy seen in SLE.[33] Furthermore, the effect of complement on the cerebral endothelium may damage the blood-brain barrier. In experimental lupus it was shown by Alexander *et al* that C5a indeed is able to alter blood-brain barrier integrity[34] and that inhibition of the C5a receptor alleviates neurological symptoms.[35] If complement fulfills this role also in the human brain, this may explain the passage of neurotoxic autoantibodies in SLE, causing a neuroinflammatory state.

The fact that we observed complement activation in SLE patients with and without neuropsychiatric symptoms may explain the clinically well-known 'lupus fog' of the brain of which many patients complain, but which is not incorporated in the ACR *'99* criteria as an official form of neuro-involvement of SLE. Apparently, a second hit is necessary for overt clinical disease as defined in the ACR *'99* criteria.[36] Infection, pregnancy, medication toxicity[15], or defects in complement regulatory mechanisms[15;37] have been described as triggering factors. Whether these also play a role in NP-SLE could be subject for further studies.

Three 'whole' human brains of patients with SLE were subjected to 7Tesla MRI imaging to investigate whether microvascular and thrombo-ischemic injury could be detected. High field MRI examinations can provide images at a higher spatial resolution, resulting

in more detailed information of microvascular injuries. Interestingly, in our patients (2 with NP-SLE and 1 with SLE, figure 5) even high resolution MR imaging could not detect the majority of small vessel injury observed histologically. Microvascular injury (vasculopathy, microinfarctions, microbleeds) were most prominently found in the vicinity of white matter hyperintensities. In our opinion, white matter hyperintensities in SLE patients should therefore not be considered innocent, but instead might mark the initial phase of vascular damage that could eventually lead to NP-SLE. Prospective studies on the role of 7Tesla MRI in the detection of these lesions in SLE and NP-SLE should be performed to further unravel this issue.

Currently, neurological involvement of SLE is a poorly understood manifestation of the disease causing major decline in quality of life of young patients. NP-SLE is often treated with aggressive immunosuppression, which is beneficial in some but certainly not all patients.[4;38] Our findings demonstrate that complement activation is present in patients with SLE and NP-SLE and suggest that this mechanism may contribute to the development of thrombo-ischemic injury and potentially disruption of the blood brain barrier, thus facilitating antibody-mediated neuroinflammation. Eculizumab is the first complement inhibitor that is already used extensively in other settings of complement-mediated disease with fewer side effects than high dose corticosteroids or cyclosporine.[39] We therefore suggest complement as a promising target for treatment in NP-SLE.

FIG 1 HISTOLOGICAL PARAMETERS IN NP-SLE, SLE AND CONTROLS

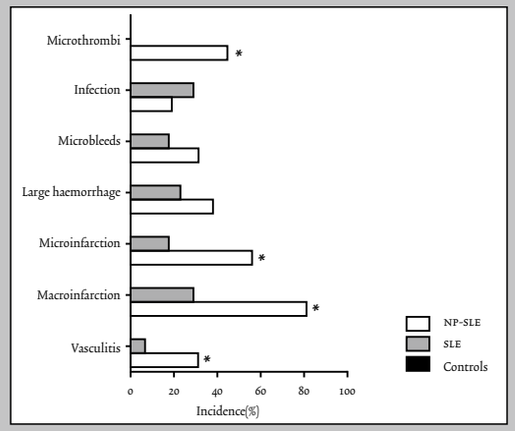


Figure 1 shows the presence of different histological parameters in brain tissue of patients with SLE, neuropsychiatric SLE (NP-SLE) and controls. Microthrombi, microinfarction, macroinfarction and vasculitis were present statistically significantly more often in NP-SLE compared to SLE. None of these parameters were present in brains of controls.

FIG 2 VASCULOPATHY IN NP-SLE, SLE AND CONTROLS

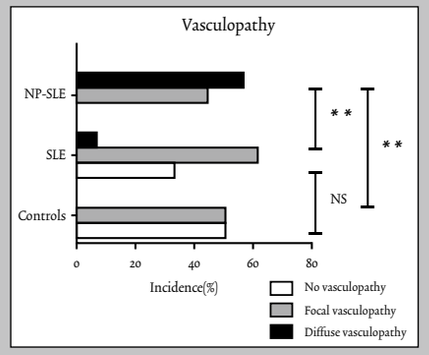


Figure 2 shows the results of semi-quantitative scoring of vasculopathy in brain tissue of patients with SLE, neuropsychiatric SLE (NP-SLE) and controls. Vasculopathy was defined as endothelial cell proliferation, thickening of the vessel wall and narrowing of the capillary lumen, and was scored semiquantitatively as 'no vasculopathy' (total absence of vasculopathy in all low powerfields), 'mild vasculopathy' (1-2 vessels showing vasculopathy per low powerfield) or 'diffuse vasculopathy' (several vessels showing vasculopathy in all low powerfields). Diffuse vasculopathy was present significantly more often in NP-SLE than in SLE and controls.

FIG 3 HISTOPATHOLOGICAL FINDINGS AND IMMUNOHISTOCHEMICAL STAINING PATTERNS

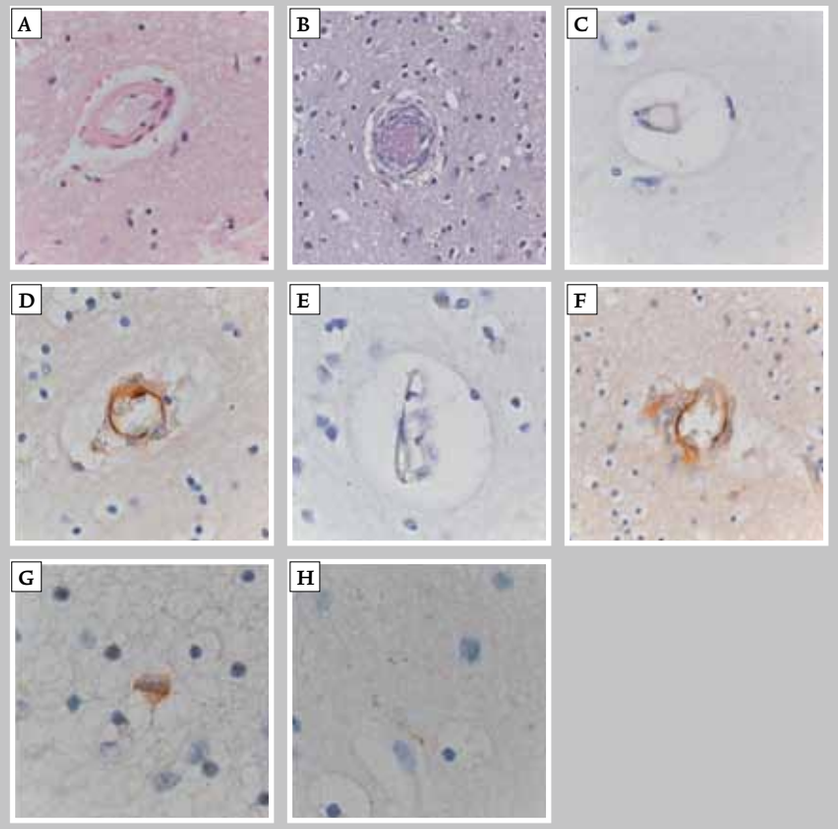


Figure 3A-H shows different histopathological findings and immunohistochemical staining patterns (Magnification of all images: 40x). Figure 3A gives a typical example of vasculopathy with thickening of the vessel wall without an inflammatory infiltrate. Figure 3B shows a typical example of a cerebral microthrombus. An example of the observed linear intravascular deposition pattern for C1q is given in 3D (C1q positive staining in an NP-SLE patient). Figure 3E (negative C4d staining) and F (positive C4d staining) show that C4d has a similar deposition pattern as C1q. MBL never co-localized with C1q or C4d, but instead stained positive in cases and controls in star-shaped cells suggestive for astrocytes. Examples of MBL staining are shown in figure 3F and 3G.

FIG 4 STAINING PATTERNS OF C1Q AND C4D IN NP-SLE, SLE AND CONTROLS

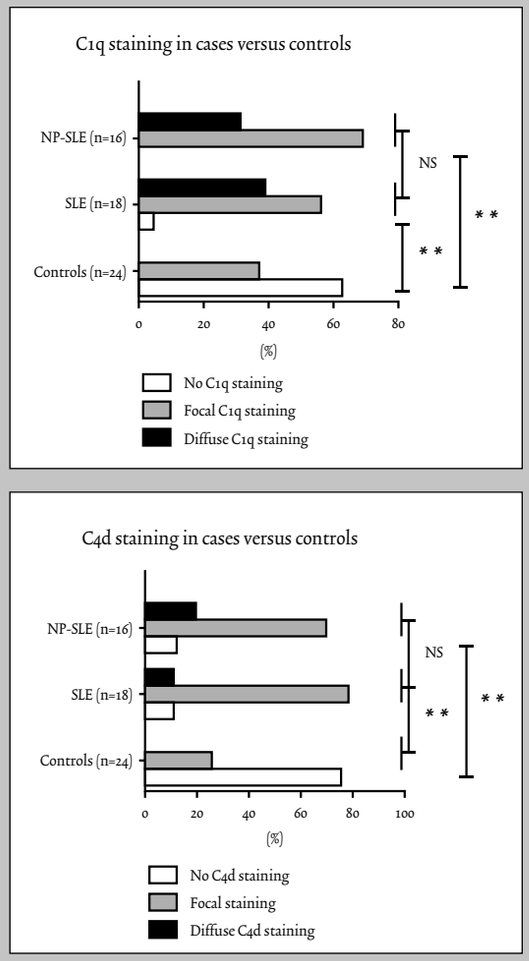
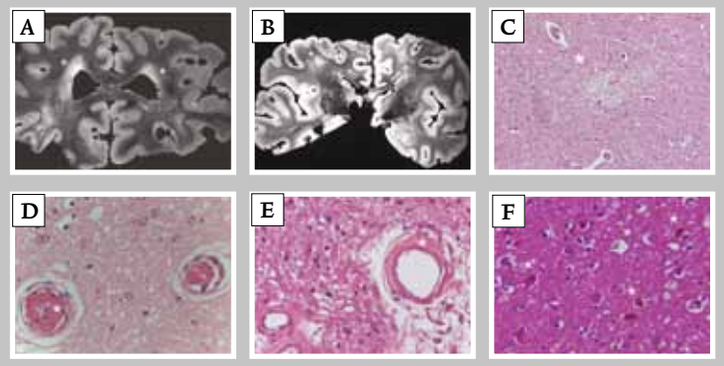


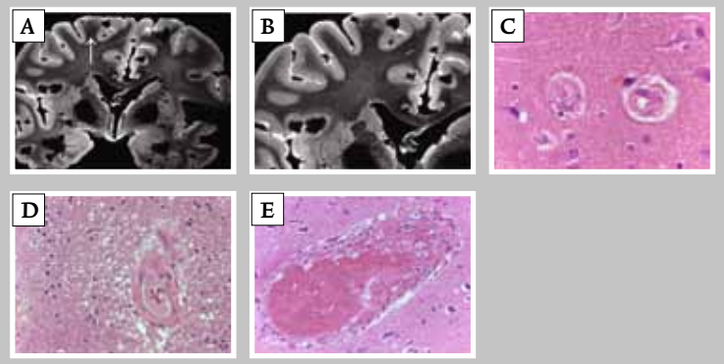
Figure 4 shows staining-patterns of C1Q and C4d. Both were scored semiquantitatively as 'no staining' (total absence of C1Q or C4d staining in all low powerfields), 'mild staining' (1-2 vessels showing C1Q or C4d positivity per low powerfield) or 'diffuse staining' (several vessels showing C1Q or C4d positivity in all low powerfields). Both C1Q (figure 4A) and C4d (figure 4B) were present equally frequent in SLE and NP-SLE, but significantly more often than in controls. (see also table 2)

FIG 5 POST MORTEM 7TESLA MRI AND CORRESPONDING HISTOPATHOLOGICAL LESIONS

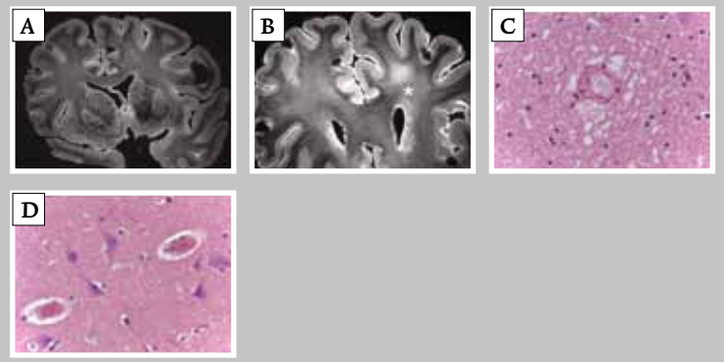
Figure 5 shows the findings of post mortem MRI and corresponding histopathological lesions in three SLE patients of whom whole formalin-fixed brains were available.



Patient 1: SLE and antiphospholipid syndrome, with epilepsy and multiple infarctions.
Figure 1A: Extensive confluent periventricular and deep white matter lesions (*). Figure 1B: Deep white matter lesions with sparing of U-fibers (*) and white matter lesions with a perivascular distribution (arrow). Figure 1C-F: Sections taken from both deep and periventricular white matter lesion showed areas of recent and older micro- and macroinfarction and thrombo-ischemic injury. Figure 1C shows an example of microinfarction and 1D shows examples of multiple microthrombi found in the vicinity of white matter lesions. Figure 1E: Prominent vasculopathy in both grey and white matter was found in all areas. Vasculopathy was seen within white matter hyperintensities and infarcted areas, but also in normal appearing white and grey matter. Figure 1F: Evidence of laminar necrosis found in vast areas of the cortex.



Patient 2: SLE with an acute confusional state.
Figure 2A-B. Normal grey and white matter differentiation. Several linear hyperintensities were characteristic of normal Virchow Robin spaces (2A, arrow). Figure 2C-E: As no abnormalities were identified on MRI, pathological sections were taken from various areas in the cortex. All sections revealed severe vasculopathy (2C-D). Figure 2E: Several venes and venules showed signs of venous vasculitis.



Patient 3: SLE without neuropsychiatric symptoms
Figure 3A shows a prominent Virchow Robin space, and normal grey and white matter differentiation. Figure 3B shows a white matter lesion that was identified in the frontal white matter. Sections taken from the frontal white matter lesion showed mild vasculopathy (Figure 3C). However, vasculopathy was seen throughout all other (normal appearing) sections as well in a similar focal distribution pattern (Figure 3D).

TABLE 1 PATIENT CHARACTERISTICS

	NP-SLE (N=16)	SLE (N=18)	CONTROLS (N=24)
Number of Females (%)	15 (94)	12 (67)	10 (42)
Mean age at death in years (SD)	44 (14)	46 (19)	47 (17)
Neuropsychiatric symptoms (ACR *99 criteria*)			
Cerebrovascular disease (%)	11 (69)	2 (11)	0 (0)
Movement Disorder (%)	2 (12)	0 (0)	0 (0)
Seizures and seizure disorders (%)	1 (6)	0 (0)	0 (0)
Acute confusional state (%)	2 (12)	1 (6)	0 (0)
Cognitive dysfunction (%)	1 (6)	0 (0)	0 (0)
Psychosis (%)	1 (6)	0 (0)	0 (0)
No Neuropsychiatric symptoms (%)	0 (0)	15 (83)	24 (100)
Primary versus secondary NP-SLE			
Primary NP-SLE (%)	16 (0)	0 (0)	0 (0)
Secondary NP-SLE (%)	0 (0)	3 (17)	0 (0)
Neurological infection (%)	3 (19)	5 (28)	0 (0)
Weight brain (SD)	1308 (177)	1290 (137)	1437 (171)

TABLE 2 C1Q AND C4D STAINING IN PATIENTS VERSUS CONTROLS

	NP-SLE (N=16)	SLE (N=18)	CONTROLS (N=24)	P VALUE
No C1q staining n (%)	0 (0)	1 (6)	15 (63)	
Focal C1q staining n (%)	11 (69)	10 (55)	9 (37)	
Diffuse C1q staining n (%)	5 (31)	7 (39)	0 (0)	P<0,001
No C4d staining n (%)	2 (12)	2 (11)	18 (75)	
Focal C4d staining n (%)	11 (69)	14 (78)	6 (25)	
Diffuse C4d staining n (%)	3 (19)	2 (11)	0 (0)	P<0,001

TABLE 3 CORRELATION BETWEEN C4D AND MICROTHROMBI IN NP-SLE AND SLE PATIENTS

	MICROTHROMBI ABSENT	MICROTHROMBI PRESENT*
No C4d deposition	4	0
Mild C4d deposition	21	4
Diffuse C4d deposition	2	3

*p = 0,029

TABLE 4 CORRELATION BETWEEN C1Q AND C4D IN SLE AND NP-SLE

SLE (N=18)	NO C4D STAINING	FOCAL C4D STAINING	DIFFUSE C4D STAINING
No C1q staining	0	1	0
Focal C1q staining	2	7	1
Diffuse C1q staining	0	6	1
NP-SLE (N=16)	**NO C4D STAINING**	**FOCAL C4D STAINING**	**DIFFUSE C4D STAINING**
No C1q staining	0	0	0
Focal C1q staining	2	7	2
Diffuse C1q staining	0	4	1

REFERENCES

1 Johnson AE, Gordon C, Palmer RG and Bacon PA. The prevalence and incidence of systemic lupus erythematosus in Birmingham, England. Relationship to ethnicity and country of birth. Arthritis Rheum. *1995*; 38:551-558.

2 D'Cruz DP, Khamashta MA, and Hughes GR. Systemic lupus erythematosus. Lancet. *2007*; 369:587-596.

3 Johnson RT and Richardson EP. The neurological manifestations of systemic lupus erythematosus. Medicine (Baltimore). *1968*; 47:337-369.

4 Bertsias GK, Ioannidis JP, Aringer M, Bollen E *et al.* EULAR recommendations for the management of systemic lupus erythematosus with neuropsychiatric manifestations: report of a task force of the EULAR standing committee for clinical affairs. Ann Rheum Dis. 2010; 69:2074-2082.

5 Joseph FG and Scolding NJ. Neurolupus. Pract Neurol. 2010; 10:4-15.

6 Luyendijk J, Steens SC, Ouwendijk WJ, Steup-Beekman GM *et al.* Neuropsychiatric systemic lupus erythematosus: lessons learned from magnetic resonance imaging. Arthritis Rheum. 2011; 63:722-732.

7 Devinsky O, Petito CK, and Alonso DR. Clinical and neuropathological findings in systemic lupus erythematosus: the role of vasculitis, heart emboli, and thrombotic thrombocytopenic purpura. Ann Neurol. *1988*; 23:380-384.

8 Ellis SG and Verity MA. Central nervous system involvement in systemic lupus erythematosus: a review of neuropathologic findings in 57 cases, 1955--1977. Semin Arthritis Rheum. *1979*; 8:212-221.

9 Hanly JG, Walsh NM, and Sangalang V. Brain pathology in systemic lupus erythematosus. J Rheumatol. *1992*; 19:732-741.

10 Sibbitt WL Jr., Brooks WM, Kornfeld M, Hart BL *et al.* Magnetic resonance imaging and brain histopathology in neuropsychiatric systemic lupus erythematosus. Semin Arthritis Rheum. 2010; 40:32-52.

11 Cohen D, Koopmans M, Kremer Hovinga I, Berger SP *et al.* Potential for glomerular C4d as an indicator of thrombotic microangiopathy in lupus nephritis. Arthritis Rheum. 2008; 58:2460-2469.

12 Cohen D, Berger SP, Steup-Beekman GM, Bloemenkamp KWM *et al.* Diagnosis and management of the antiphospholipid syndrome. BMJ. 2010; 340:c2541.

13 Pierangeli, SS, Girardi G, Vega-Ostertag M, Liu X *et al.* Requirement of activation of complement C3 and C5 for antiphospholipid antibody-mediated thrombophilia. Arthritis Rheum. 2005; 52:2120-2124.

14 Salmon JE, Girardi G, and Holers VM. Complement activation as a mediator of antiphospholipid antibody induced pregnancy loss and thrombosis. Ann Rheum Dis. 2002; 61 Suppl 2:ii46-ii50.

15 Noris M and Remuzzi G. Atypical hemolytic-uremic syndrome. N Engl J Med. 2009; 361:1676-1687.

16 Pierangeli SS, Colden-Stanfield M, Liu X, Barker JH *et al.* Antiphospholipid antibodies from antiphospholipid syndrome patients activate endothelial cells in vitro and in vivo. Circulation. *1999*; 99:1997-2002.

17 Pierangeli SS, Chen PP, Raschi E, Scurati S *et al.* Antiphospholipid antibodies and the antiphospholipid syndrome: pathogenic mechanisms. Semin Thromb Hemost. 2008; 34:236-250.

18 Casparie M, Tiebosch AT, Burger G, Blauwgeers H *et al.* Pathology databanking and biobanking in The Netherlands, a central role for PALGA, the nationwide histopathology and cytopathology data network and archive. Cell Oncol. *2007*; 29:19-24.

19 Tan EM, Cohen AS, Fries JF, Masi AT *et al.* The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. *1982*; 25:1271-1277.

20 Colvin RB. Antibody-mediated renal allograft rejection: diagnosis and pathogenesis. J Am Soc Nephrol. 2007; 18:1046-1056.

21 Shepherd TM, Thelwall PE, Stanisz GJ and Blackband SJ. Aldehyde fixative solutions alter the water relaxation and diffusion properties of nervous tissue. Magn Reson Med. 2009; 62:26-34.

22 van Roden S, Maat-Schieman ML, Nabuurs RJ, van der Weerd L *et al.* Cerebral amyloidosis: postmortem detection with human 7.0-T MR imaging system. Radiology. 2009; 253:788-796.

23 Cohen D, Buurma A, Goemaere NN, Girardi G *et al.* Classical complement activation as a footprint for murine and human antiphospholipid antibody-induced fetal loss. J Pathol. 2011; 225:502-511.

24 Holers VM, Girardi G, Mo L, Guthridge JM *et al.* Complement C3 activation is required for antiphospholipid antibody-induced fetal loss. J Exp Med. 2002; 195:211-220.

25 Girardi G, Berman J, Redecha P, Spruce L *et al.* Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. J Clin Invest. 2003; 112:1644-1654.

26 Ten VS, Sosunov SA, Mazer SP, Stark RI *et al.* C1q-deficiency is neuroprotective against hypoxic-ischemic brain injury in neonatal mice. Stroke. 2005; 36:2244-2250.

27 DeGiorgio LA, Konstantinov KN, Lee SC, Hardin JA *et al.* A subset of lupus anti-DNA antibodies cross-reacts with the NR2 glutamate receptor in systemic lupus erythematosus. Nat Med. 2001; 7:1189-1193.

28 Diamond B, Bloom O, Al Abed AY, Kowal C *et al.* Moving towards a cure: blocking pathogenic antibodies in systemic lupus erythematosus. J Intern Med. 2011; 269:36-44.

29 Faust TW, Chang EH, Kowal C, Berlin R *et al.* Neurotoxic lupus autoantibodies alter brain function through two distinct mechanisms. Proc Natl Acad Sci U S A. 2010; 107:18569-18574.

30 Morigi M, Galbusera M, Gastoldi S, Locatelli M *et al.* Alternative pathway activation of complement by Shiga toxin promotes exuberant C3a formation that triggers microvascular thrombosis. J Immunol. 2011; 187:172-180.

31 Atkinson C, He S, Morris K, Qiao F *et al.* Targeted complement inhibitors protect against posttransplant cardiac ischemia and reperfusion injury and reveal an important role for the alternative pathway of complement activation. J Immunol. 2010; 185:7007-7013.

32 Silver KL, Higgins SJ, McDonald CR and Kain KC. Complement driven innate immune response to malaria: fuelling severe malarial diseases. Cell Microbiol. 2010; 12:1036-1045.

33 Hess DC. Cerebral lupus vasculopathy. Mechanisms and clinical relevance. Ann N Y Acad Sci. *1997*; 823:154-168.

34 Jacob A, Hack B, Chiang E, Garcia JG *et al.* C5a alters blood-brain barrier integrity in experimental lupus. FASEB J. 2010; 24:1682-1688.

35 Jacob A, Hack B, Bai T, Brorson JR *et al.* Inhibition of C5a receptor alleviates experimental CNS lupus. J Neuroimmunol. 2010; 221:46-52.

36 The American College of Rheumatology nomenclature and case definitions for neuropsychiatric lupus syndromes. Arthritis Rheum. *1999*; 42:599-608.

37 Salmon JE, Heuser C, Triebwasser M, Liszewski MK *et al.* Mutations in complement regulatory proteins predispose to preeclampsia: a genetic analysis of the PROMISSE cohort. PLoS Med. 2011; 8:e1001013.

38 Ruiz-Irastorza G, Khamashta MA, Castellino G and Hughes GR. Systemic lupus erythematosus. Lancet. 2001; 357:1027-1032.

39 Roth A, Hock C, Konik A, Christoph S *et al.* Chronic treatment of paroxysmal nocturnal hemoglobinuria patients with eculizumab: safety, efficacy, and unexpected laboratory phenomena. Int J Hematol. 2011; 93:704-714.

GENERAL INTRODUCTION

PART ONE
C4D AND THROMBOTIC COMPLICATIONS

PART TWO
C4D AND ADVERSE PREGNANCY OUTCOME

CONCLUSION AND DISCUSSION

APPENDICES

VI

CLASSICAL COMPLEMENT ACTIVATION AS A FOOTPRINT FOR MURINE AND HUMAN ANTIPHOSPHOLIPID ANTIBODY-INDUCED FETAL LOSS

DANIELLE COHEN / ALETTA BUURMA / NATASCHA N GOEMAERE
GUILLERMINA GIRARDI / SASKIA LE CESSIE / SICCO SCHERJON
KITTY WM BLOEMENKAMP / EMILE DE HEER / JAN ANTHONIE BRUIJN
INGEBORG M BAJEMA

JOURNAL OF PATHOLOGY 2011; 225: 502–511

Abstract

INTRODUCTION Recurrent miscarriage, fetal growth restriction and intrauterine fetal death are frequently occurring complications of pregnancy in patients with systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS). Murine models show that complement activation plays a pivotal role in antiphospholipid antibodymediated pregnancy morbidity. However, the exact pathways of complement activation and their potential role in human pregnancy are insufficiently understood. Given the antibody-mediated nature of SLE and APS in which pregnancy losses are pertinent, we hypothesized that the classical pathway would play a major role in inducing fetal loss.

METHODS To gain more insight into the contribution of different complement pathways to fetal outcome, pregnant C57BL/6 mice and mice deficient in C1q and factor D were injected with antiphospholipid antibodies or normal human IgG. Mice-placentas were subsequently stained with an anti-C4 antibody and anti-normal human IgG to determine presence of classical complement activation and IgG binding. Findings in mice were validated in 88 human placentas from 83 women (SLE and APS cases versus controls), which were immunohistochemically stained for C4d, C1q, properdin and MBL. Staining patterns were compared to pregnancy outcome.

RESULTS In murine placentas of mice pre-treated with antiphospholipid antibodies, increased C4 deposition was observed, which was associated with adverse fetal outcome but not with IgG binding. In humans, diffuse C4d staining at the fetomaternal interface was present almost exclusively in patients with SLE and/or APS ($p < 0.001$) and was related to intrauterine fetal death ($p = 0.03$).

CONCLUSION Our data show that presence of C4d in murine and human placentas is strongly related to adverse fetal outcome in the setting of SLE and APS. The excessive deposition of C4d supports the concept of severe autoantibody-mediated injury at the fetomaternal interface. We suggest C4d as a potential biomarker of autoantibody-mediated fetal loss in SLE and APS.

Introduction

Recurrent miscarriage, fetal growth restriction and intrauterine fetal death (IUFD) are devastating complications of pregnancy that occur 20 to 40 times more often in patients with systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS) than in healthy pregnant women.[1,2] Presence of circulating antiphospholipid antibodies (aPL) which is a prerequisite for APS and occurs in 40% of SLE patients, is strongly associated with thrombosis and fetal loss.[3]

The increased risk of thrombosis in the presence of aPL suggests that thrombosis of the uteroplacental vasculature could be an important cause of pregnancy morbidity. However, thrombotic lesions are not significant and even not always detectable in placentas of patients with aPL-mediated fetal loss.[4] Recent studies in complement deficient murine models [5-8] suggested that complement activation plays a pivotal role in aPL-mediated fetal loss, shifting the focus more towards inflammation as the primary causative mechanism.[9;10] However, the exact pathways of complement activation and their potential role in human pregnancy are insufficiently understood. We hypothesized that classical complement activation is the main responsible pathway in SLE and APS related pregnancy morbidity, based on an antibody-mediated allo-response at the fetomaternal interface. We therefore investigated the role of classical, alternative and mannose binding lectin (MBL) pathway activation in a murine model of aPL-mediated fetal injury and in human placentas of pregnancies affected by SLE and APS. As a marker for classical pathway activation we used C4d, a marker for classical complement activation.

In transplantation pathology, C4d is widely used as a biomarker to diagnose antibody-mediated allograft rejection.[11] As a degradation product of C4, one of the main components of the classical complement cascade, C4d has the ability to bind covalently to cell surfaces and basement membranes near sites of C4 activation. Covalently bound C4d is anchored to the tissue, and remains attached much longer than the antibodies that originally activated the classical pathway. This makes C4d a highly stable marker, and has led to C4d being referred to as 'a footprint' of antibody-mediated tissue injury.[12]

In a mouse model of aPL-mediated fetal loss we first studied murine placentas with an anti-C4 antibody to determine presence of classical complement activation. Observations in mice were validated by investigating the presence C4d deposits in placentas of patients with SLE and/or APS in relation to pregnancy outcome, compared to healthy and disease controls. In additional studies we confirmed that placental C4d depositions reflect specific involvement of classical pathway activation, and are not derived from lectin or alternative pathway activation.

Materials and methods

MOUSE MODEL OF ANTIPHOSPHOLIPID ANTIBODY-INDUCED FETAL LOSS Adult mice (6-8 weeks), C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME, USA) and mice deficient in C1q and factor D (C1qfDKO) [13] (generously provided by Greg Stahl, Harvard University) were used in all experiments.

On days 8 and 12 of pregnancy, females were treated with I.P. injections of human IgG-containing aPL (aPL-IgG) (10 mg) or normal human IgG (NH-IgG) (10 mg) as previously described.[5;14] APL-IgG were obtained from patients with APS [characterized by high titer aPL antibodies (▸140 GPL units), NH-IgG was obtained from healthy non-autoimmune individuals.

Mice (C57BL/6 and C1qfDKO) were sacrificed on day 8, 2h after aPL-IgG injection (cases, n=5) or NH-IgG injection (controls, n=5), uteri were dissected and deciduas and placentas were harvested. Another group of cases (n=5) and controls (n=5) were euthanized on day 15 of pregnancy to harvest placentas. Resorption sites that result from loss of a previously viable embryo were counted as previously described.[5;14]

For immunohistochemical studies, deciduas from day 8 and placentas from day 15 of pregnancy were fixed in paraformaldehyde 4%, frozen in O.C.T. compound, and cut into 10 m sections. Sections

were stained for C4d with a rat monoclonal anti-C4 antibody (C4 antibody [16D2] (ab11863) Abcam, Cambridge, MA, USA) at a dilution 1/50. This antibody reacts with murine C4, C4b and C4d**.** As a positive control we used a kidney of a mouse injected with FB1 (mouse monoclonal-aPL that activates complement). As a positive control we used a kidney that belonged to a mouse injected with FB1 (mouse monoclonal antiphospholipid antibody from M. Monastier that activates complement). This antibody (specificity, isolation, etc) was described in studies in pregnancy and renal thrombotic microangiopathy. The control mouse received mouse IgG.

An HRP-labeled secondary antibody and DAB as substrate or a FITC-labeled secondary antibody were used to develop the reaction. For fluorescence staining gold antifade reagent with DAPI (Invitrogen, Carisbad, CA, USA) was used.

Day 15 placentas were also stained with anti-human IgG (Sigma-Aldrich, St Louis, MO) at a dilution 1/150.

All animal studies were approved by the institutional Animal Care and Use Committee of the Hospital for Special Surgery or Weill Medical College of Cornell University and York College, City University of New York, New York.

PATIENTS AND PLACENTAS We studied 88 placentas from 83 women who delivered at the Obstetrical Department of the Leiden University Medical Centre (LUMC) between 1995 and 2009. Women were subdivided into three groups: The case-group consisted of 21 patients with a confirmed diagnosis of SLE and/or APS, from which we selected all available placentas (26 placentas of 21 patients). Multiple placentas were available from 4 patients in the case group. For statistical analysis, only the placenta of the first pregnancy was taken into account. All patients in the case group were tested for antiphospholipid antibodies (lupus anticoagulant and anticardiolipin antibodies) according to the most recent guidelines.[15;16]

For a first control group (live-birth controls) we included 40 placentas of pregnancies that resulted in live births in the same period as above. These placentas represented pregnancies ranging from completely normal to relatively complicated, including both maternal and fetal morbidity. None of the patients had preeclampsia or Hemolysis Elevated Liver enzymes and Low Platelets (HELLP) syndrome.

For a second control group (IUFD-controls) we included 22 placentas of pregnancies that resulted in IUFD by various causes other than SLE or APS. Fetal losses in this group were mainly caused by fetal chromosomal abnormalities.

An overview of the clinical characteristics of all patients, derived from the case-records of the LUMC, is given in table 1.

All tissue samples were handled in a coded fashion, according to Dutch national ethical guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of Medical Scientific Societies).

ROUTINE HISTOPATHOLOGY AND IMMUNOHISTOCHEMISTRY Placentas were fixed in 4% buffered formalin and embedded in paraffin. Paraffin sections were routinely stained with HE. To study complement activation, immunohistochemical staining was performed for C4d (BI-RC4d, Biomedica Gruppe, Austria), C1q (Dako Cytomation, Denmark), MBL (Sigma-Aldrich Biotechnology) and properdin (primary antibody kindly provided by the department Nephrology, Leiden, the Netherlands). Optimal antibody dilutions and incubation times for the different antibodies were pre-determined by means of titration on positive control sections.

C4D Endogenous peroxidase activity was blocked. Antigen retrieval was performed with 10 mM citrate buffer (pH 6.0). A polyclonal rabbit anti-human C4d antibody (Biomedica Gruppe, Austria), was applied at a dilution of 1:80 in 1% BSA/PBS, and slides were incubated for one hour at room temperature. The slides were then incubated with a secondary antibody (anti-rabbit EnVision, K5007, Dako Cytomation, Denmark) for 30 minutes. Staining was visualized with diaminobenzidine (Dako Cytomation, Denmark) and counterstained with Haematoxylin.

A tissue sample from a renal biopsy of a patient with humoral rejection with C4d-positive staining served as a positive control.

C1q Endogenous peroxidase activity was blocked. Antigen retrieval was performed in EDTA-TRIS (pH 9.0). A polyclonal rabbit anti-human polyclonal C1q antibody (Dako Cytomation, Denmark) was applied at a dilution of 1:700 in 1%BSA/PBS, and slides were incubated for one hour at 37 C. The slides were then incubated with a secondary antibody (anti-rabbit EnVision, K5007, Dako Cytomation, Denmark) for 30 minutes. Staining was visualized with diaminobenzidine (Dako Cytomation, Denmark) and counterstained with Haematoxylin. Tonsil tissue served as a positive control.

MBL Endogenous peroxidase activity was blocked. Antigen retrieval was performed with 10 mM citrate buffer (pH 6.0). A polyclonal rabbit anti-human MBL antibody (Sigma-Aldrich Biotechnology) was applied at a dilution of 1:250 in 1% BSA/PBS, and slides were incubated for one hour at room temperature. The slides were then incubated with a secondary antibody (anti-rabbit EnVision, K5007, Dako Cytomation, Denmark) for 30 minutes. Staining was visualized with diaminobenzidine (Dako Cytomation, Denmark) and counterstained with Haematoxylin. Liver tissue served as a positive control.

PROPERDIN Endogenous peroxidase activity was blocked. Antigen retrieval was performed with 10 mM citrate buffer (pH 6.0). The slides were subsequently blocked with 5% heat-inactivated normal human serum in 1% bovine serum albumin/phosphate-buffered saline (BSA/PBS) for 45 min at room temperature. A polyclonal rabbit anti-human properdin antibody (kindly provided by the department Nephrology, Leiden, the Netherlands) was applied at a dilution of 1:800 in 1%BSA/PBS overnight. The slides were then incubated with a secondary antibody (anti-rabbit EnVision, K5007, Dako Cytomation, Denmark) for 30 minutes. Staining was visualized with diaminobenzidine (Dako Cytomation, Denmark) and counterstained with Haematoxylin. A kidney with membranous nephropathy was used as a positive control.

QUANTIFICATION OF PLACENTAL MORPHOLOGY AND IMMUNOHISTOPATHOLOGY Sections were evaluated by two experienced pathologists who scored the slides blinded to the patients' clinical data. Differences in scorings were resolved by re-reviewing the sections and coming to consensus. Each placenta was scored separately for the presence or absence of histopathological changes associated with decreased uteroplacental perfusion: Acute and/or chronic deciduitis, decidual necrosis, increased syncytial knots, accelerated villous maturity, accellerated maturity, avascular villi, villous infarcts, retroplacental hematomas, intervillous thrombi and decidual vasculopathy.[17;18]

Positivity for immunohistochemical stainings was scored semi-quantitatively. A random area of 1 x 1 cm was selected from each tissue sample for scoring. Staining intensity around syncytiotrophoblast was scored as 0, 1, or 2, with 0 representing the total absence of staining, 1 representing focal positive staining, and 2 representing diffuse staining of all syncytiotrophoblast cell- and basement membranes within this area. Intravillous endothelial staining was scored on a 0, 1, 2 scale, with similar definitions as above.

STATISTICAL ANALYSIS Categorical variables were compared using the Chi-square test. Differences in quantitative parameters between groups were assessed using one-way ANOVA (for data normally distributed) or Kruskal Wallis H one-way analysis (for data not normally distributed). All analyses were performed using SPSS statistical software package (version 16.0; Chicago, IL). A p-value less than 0.05 was considered statistically significant.

Results

MOUSE MODEL OF APL-IGG TREATED MICE

C4 DEPOSITION IN APL-IGG TREATED MICE VERSUS CONTROLS

Placentas from the surviving fetuses in aPL -treated mice showed increased C4 deposition in the labyrinth (Figure 1BII, III, IV) when

compared to placentas from NH-IgG treated mice (Figure 1AII, III, IV). The labyrinth is the area of active feto-maternal exchange in the murine placenta, equivalent to chorionic villi in humans.

Specifically, C4 deposition was observed on the trophoblast giant cells (TGC) (Figure 1Bi). These cells are crucial for implantation and invasion of the conceptus into maternal decidua of the uterus. Abnormalities in these cells can cause placental defects and compromised pregnancies [19;20] and can explain the high resorption frequency observed in embryos from aPL-treated mice.[5;14]

C4 deposition in C1q- and factor D-deficient mice

To rule out that C4d positivity was caused by lectin pathway activation we studied pregnancy outcomes in mice that are deficient for complement component C1q and factor D (Figure 1C). Mice deficient in the classical pathway component C1q and the alternative pathway component factor D were protected from aPL-induced fetal injury. The fetal resorption frequency in these mice was not different from that calculated in NH-IgG treated mice with uneventful pregnancies (Figure 1C). No C4 deposition was observed, neither in deciduas nor in placentas from C1qfDKO mice treated with aPL (data not shown).

IgG deposition To study if complement deposition coincidences with aPL-binding in aPL-treated mice with increased fetal loss, we stained for human IgG in day 15 placentas from surviving fetuses. In contrast with the robust C4a deposition observed in these placentas, no IgG staining was found.

Clinical data Patients in all groups were of similar age. In the case group of patients with SLE and/or APS, 15 patients met the American College of Rheumatology criteria for SLE[21], 12 patients met the classification criteria for APS[22], and 6 patients had both SLE and APS (see table 1).

None of the patients in the live-born control group had been tested for aPL. In the IUFD-group all patients with an IUFD of unknown etiology were tested for aPL. None of the patients met the laboratory criteria for APS. Other patient and pregnancy characteristics are shown in table 1.

Histopathological findings in cases versus controls

Retroplacental hematomas and intervillous thrombi were infrequent and not increased either in placentas from patients with SLE and/or APS, compared to both control groups. Villous infarction and accelerated maturity occurred more often in placentas from patients with SLE/APS than in both control groups. Figure 2 shows the incidence of various histopathological findings in all placentas.

Detection of complement components and their association with clinical parameters

C4d When present, C4d showed positivity at the fetomaternal interface, on the maternal side of the syncytiotrophoblast, either in a focal or a diffuse staining pattern. Typical examples of C4d staining patterns are shown in figure 3A-C. A diffuse C4d staining pattern in the placenta was strongly associated with SLE and/or APS ($p < 0.001$) (figure 3M). Within the case group, diffuse C4d staining was associated with IUFD ($p < 0.03$) (figure 3N). Detailed information on C4d staining in the cases versus controls is shown in table 2.

Diffuse C4d staining around all syncytiotrophoblast cell- and basement membranes (figure 3C) was found in 12 placentas: Ten were from patients with SLE and/or APS, of whom 5 had a pregnancy resulting in IUFD, 4 pregnancies were characterized by severe intrauterine growth retardation and/or preeclampsia and one of the patients had an uncomplicated pregnancy, which was her first live born child after 4 late miscarriages. Two of the 12 placentas that were diffusely positive for C4d were from patients from the control group with IUFD: Both patients had an unexplained IUFD. One patient was negative for aPL, the other appeared to have circulating aPL (anticardiolipin IgG antibodies) when she was tested three months after delivery. A diffuse or focal positive C4d staining pattern was never found in the control group with live-born children ($p<0.001$).

Detailed information on focal and absent C4d staining patterns is given in table 2.

C1q C1q was present in both intravillous endothelial cells and around the syncytiotrophoblast cells (Figure 3D-F), and was never completely negative, neither in cases nor controls (data not shown). In cases of diffuse C4d deposition around syncytiotrophoblast cells, C1q clearly co-localized with C4d depositions.

PROPERDIN Properdin was almost uniquely positive in intravillous endothelial cells, and showed minor positivity around syncytiotrophoblast cells in only 2 cases of live birth controls. It did not co-localize with C4d deposition (figure 3G-I). Diffuse properdin staining occurred almost at a similar rate in live born placentas and in placentas in the SLE/APS group (33% and 28% respectively). Properdin positivity was negatively associated with IUFD: In only 2 out of 22 IUFD cases a diffuse staining pattern was observed.

MBL In all cases MBL deposition was absent (figure 3J-L), whereas liver tissue that was used as a positive control was evidently positive. MBL also did not occur in C4d positive placentas, or in placentas of patients with prolonged rupture of membranes.

PATIENTS OF WHICH MULTIPLE PLACENTAS OF SUBSEQUENT PREGNANCIES WERE AVAILABLE Multiple placentas were available from 4 patients in the case group (see table 3)

The first patient was diagnosed with SLE and had four subsequent pregnancies, of which the first ended in an early miscarriage (no tissue available), the second and third ended in intrauterine fetal loss and the fourth was a live birth. For the first fetal loss an explanation or cause was never found, the second fetus died because of congenital heartblock in the presence of maternal anti-SSA and anti-SSB antibodies. In her fourth pregnancy she gave birth to a healthy child. This patient did not have antiphospholipid antibodies at any point in time and her placentas were negative for C4d every time. The next patient had SLE and secondary antiphospholipid syndrome. She had three pregnancies of which two placentas were available. All three pregnancies ended in live births, but with severely growth restricted children and severe maternal preeclampsia during the first and second pregnancy. In the third pregnancy she was treated with aspirin and heparin. Her first placenta was not available, she had diffuse C4d staining in the second, and focal C4d staining in the third placenta. The last two patients had primary antiphospholipid syndrome. One patient had three pregnancies of which two (the first and third pregnancy) ended in intrauterine fetal death, accompanied by severe growth retardation and maternal HELLP syndrome. Both placentas were diffusely positive for C4d. Her second pregnancy, of which no placenta was available, ended in a live birth but with maternal HELLP syndrome complicated by liver infarction. The other patient also had three pregnancies, all of which ended in intrauterine fetal death accompanied by severe fetal growth retardation. Only the last two placentas were available, which were both diffusely positive for C4d.

Discussion

APS and SLE are autoantibody mediated autoimmune diseases in which severe pregnancy morbidity is pertinent. The present study demonstrated that C4d deposits were present in areas of active fetomaternal exchange in the murine placenta of mice pre-treated with human aPL-IgG, and absent in those treated with NH-IgG. C4d deposits in murine placentas were associated with an increased fetal absorption rate (figure 1). Observations in mice were validated in human placentas, where placental C4d deposition was frequently and almost exclusively present around syncytiotrophoblast cells of placentas from patients with SLE and/or APS. Its presence in human placentas was strongly related to adverse pregnancy outcome in SLE and APS patients. In patients without SLE or APS but with IUFDs diffuse C4d staining was rare. C4d staining was always negative in placentas from patients with live births (figure 3, table 2). Our data

support the concept of a severe autoantibody-mediated immune response at the fetomaternal interface, leading to impaired fetal outcome.

To rule out that C4d deposition is a reflection of lectin pathway activation we studied the deposition patterns of MBL. In the mice-experiments, mice deficient in the classical pathway component C1q and the alternative pathway component factor D were protected from aPL-induced fetal injury. No C4 deposition was observed in placentas from these mice. MBL was not detectable in human placentas, and did not co-localize with C4d. These observations indicate that it is very unlikely that the lectin pathway is involved in the pathogenesis of fetal losses induced by aPL.

Direct exposure of trophoblast to the maternal blood puts these cells at risk of being attacked by complement activation products, both in the setting of maternal autoimmune disease as in normal pregnancies. Our data indicate that deposition of early classical pathway component C1q is most likely a non-pathological phenomenon. The observed presence of C1q in placentas of controls could reflect the physiological presence of maternal IgG and IgM. It is not surprising that this usually does not lead to full blown activation of the complement cascade, because of the many complement regulatory mechanisms normally present at the level of the syncytiotrophoblast.[6] Alternatively, it was previously shown that decidual endothelial cells can synthesize C1q and express it on their surface in physiological situations. [23;24] Bulla *et al* showed that deposition of C1q in the placenta was not necessarily associated with the presence of IgG, IgM or C4.[23] The clear association of C4d (a complement split-product more downstream than C1q) with SLE and APS related pregnancy morbidity as presented in this study, may be regarded as evidence of a severe antibody-mediated allo-response, where the complement inhibitory mechanisms are surpassed and fail to suppress the evolving events in the complement cascade.

Evidence from mouse-models suggested that a large part of the aPL-mediated placental damage is caused by amplification of the classical pathway by the alternative pathway.[25] However, we found properdin, a membrane bound marker of alternative pathway activation, in placentas of both patients with SLE and APS as well as in live-birth controls, making no distinction between a good or bad pregnancy outcome. Interestingly, properdin depositions are found within the vessels of the villi, and do not co-localize with C4d deposits at the maternal side of the syncytiotrophoblast. This means that properdin must originate from the fetal complement system[26], and may reflect a general state of fetal distress, rather than being directly related to the C4d depositions.

Although the clinical implications of aPL are well known, testing for their presence remains difficult and only highly specialized laboratories can provide reliable test results.[27;28] C4d stainings are performed in most clinical pathology laboratories and a diffuse C4d staining pattern in the placenta is easy to recognize (figure 3C). We therefore propose that placental C4d staining could be a useful additional tool to further strengthen the diagnosis of aPl-mediated fetal loss, for instance in the work-up of a first late miscarriage or fetal death. This might even have implications for treatment with heparin, as it was shown in animal models that heparin prevents aPL-induced fetal loss by inhibiting complement activation.[29] Alternatively, it would be interesting to explore whether targeted inhibition of complement activation would be beneficial for pregnancy outcome, similar to the findings in mice models.[5;7]

The analogy between pregnancy and transplantation was made as early as 1953, when Peter Medawar introduced the concept of 'the fetal allograft'.[30] In transplantation, humoral allograft rejection has gained much attention since the discovery of C4d.[11] Our data demonstrate that complicated pregnancies of patients with autoimmune diseases such as APS, share several pathofysiological aspects with humoral rejection. Both aPL and donor-specific antibodies to donor-HLA bind at the frontier where cells from the one individual (mother or host) meet the other (fetus or graft).

Interestingly, both in pregnancy and in transplantation we find no histological evidence for the binding of the antibodies themselves, whereas C4d remains attached and is easily detectable

both in mice placentas at day 8 and 15, and in human placentas of various gestational age. In day 15 placentas of aPL-treated mice, we did not find NH-IgG deposition, whereas it was previously published that NH-IgG can be detected in day 8 placentas of similarly treated mice.[31] This shows that these antibodies do bind at the fetomaternal interface initially, but do not remain attached longer than a few days, whereas C4d is detectable throughout the whole pregnancy. From the transplant setting, it is known that this phenomenon can be explained by the relatively weak binding capacity of antibodies, compared to the covalent binding of C4d which anchors to the damaged tissue.[11] In this study a parallel between the humoral rejection and pregnancy is now found in the form of C4d deposition, indicating that C4d might be a more reliable and long lasting marker of placental antibody-mediated tissue injury.

In human placentas, we demonstrated that 62% of patients with SLE/APS have a focal or a diffuse C4d staining pattern. However 38% of these patients do not show any deposition of C4d, while their pregnancy outcomes are also impaired, either by growth restriction, preeclampsia or even fetal death. There are two important explanations for this situation. Firstly, as we take only a small part of the placenta for analysis, C4d positivity present in a focal, patchy pattern through the whole placenta can be missed due to sampling error. In future studies, the effect of more extensive tissue sampling could be investigated. Secondly, it is evident that in any pregnancy, many more than C4d-related causes can lead to IUFD or impaired pregnancy outcome. For instance, anti-SSA or anti-SSB antibodies can cause a congenital heart block and subsequently, fetal death, by travelling through the placenta into the fetal circulation. This is a totally different mechanism for SLE-related IUFD in which no trophoblastic C4d deposition is expected. Furthermore, there is always a chance for fetal chromosomal abnormalities, and in such cases no diffuse C4d deposition is expected as we have illustrated in our IUFD control group.

In conclusion, we have shown that classical complement activation plays a major role in aPL- mediated fetal injury, and that placental C4d deposition is a reflection of classical complement activation.
C4d is strongly associated to impaired fetal outcome, both in a mouse model of aPL- mediated fetal loss and in human pregnancy affected by SLE and APS. Especially in women with a first pregnancy resulting in IUFD, placental C4d staining has potential as a diagnostical tool
to detect aPL-mediated fetal loss. Further prospective studies need
to confirm if C4d positivity in a previous IUFD or late miscarriage
can be considered as a biomarker of a future complicated pregnancy.

FIG 1A-B INCREASED C4 DEPOSITION IN APL-IGG TREATED MICE VERSUS NH-IGG CONTROLS

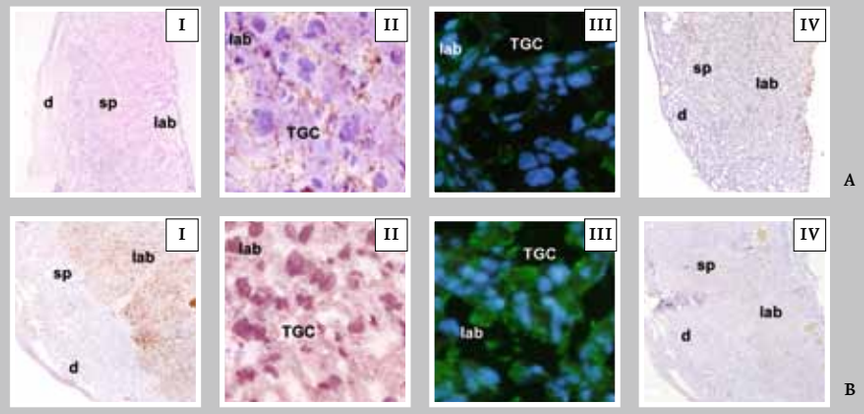


Figure 1BI, II, and III show increased C4 deposition in the labyrinth (lab) compared to figure 1AI, I, and III. The labyrinth is the area of active feto-maternal exchange in the murine placenta. The equivalent to this area in humans is the chorionic villi. C4 deposition was observed on the trophoblast giant cells (TGC) of aPL-IgG treated mice (Figure 1BII and III). In contrast, minimal C4d staining was found in placentas from NH-IgG-treated mice (Figure 1AI, II, III).

FIG 1C

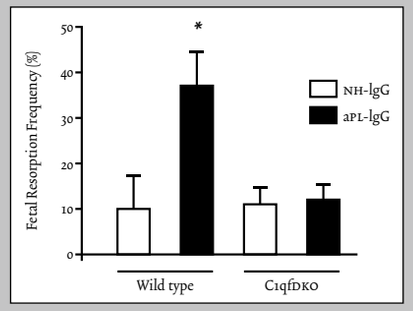
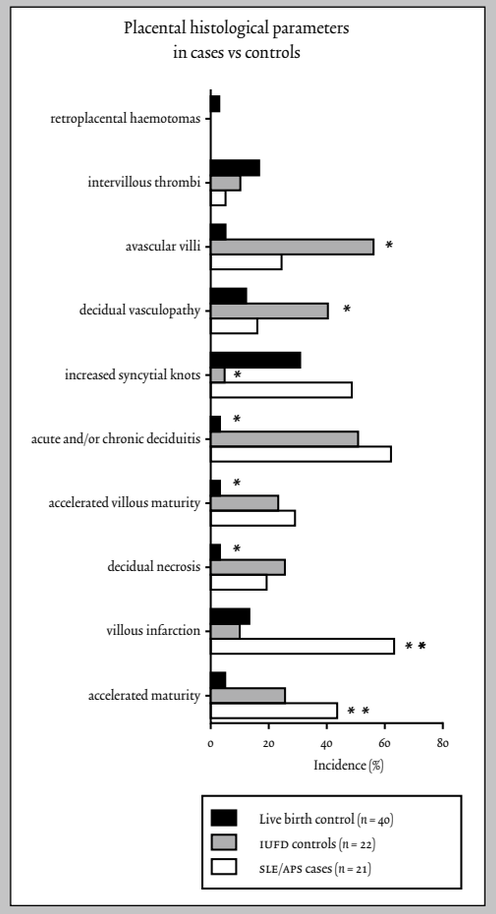


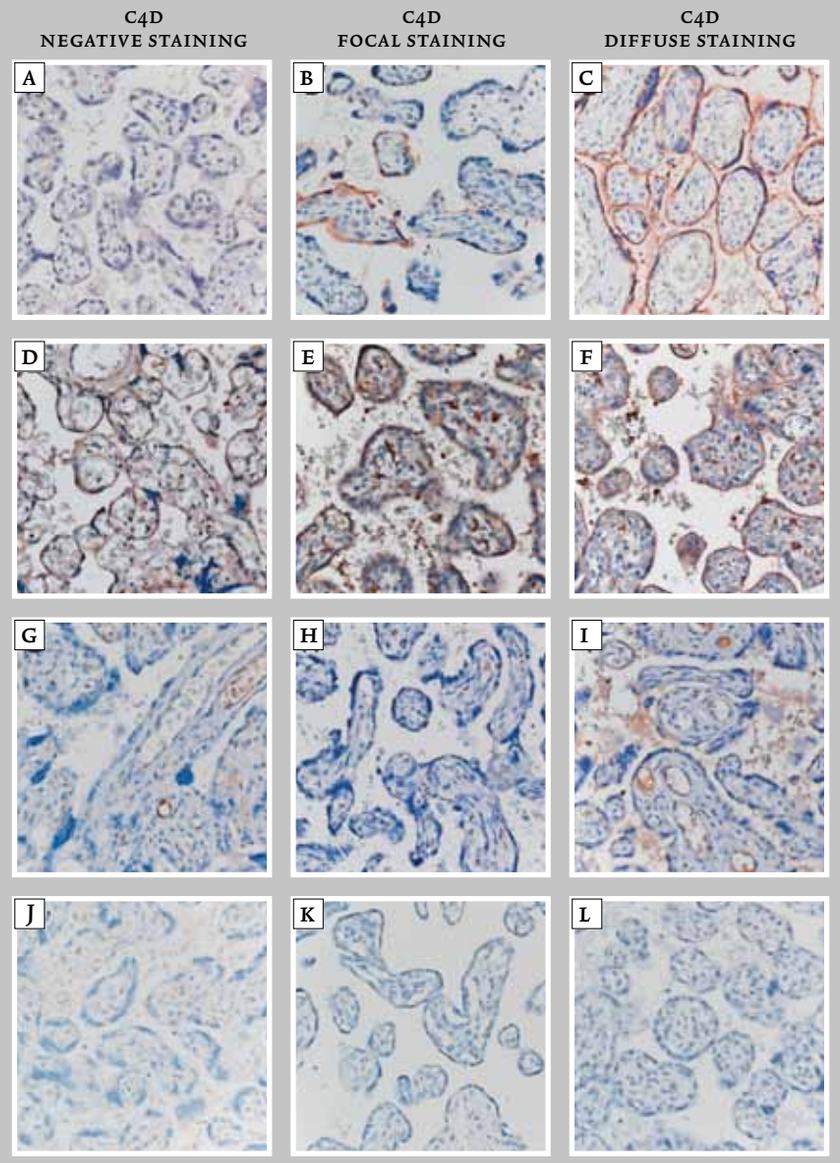
Figure 1C shows fetal resorption frequency in C1q- and factor D-deficient mice. Mice deficient in the classical pathway component C1q and the alternative pathway component factor D are protected from aPL-induced fetal injury. The fetal resorption frequency in these mice was not different from that calculated in NH-IgG treated mice with uneventful pregnancies. No C4 deposition was observed neither in deciduas nor in placentas from C1qfDKO mice treated with antiphospholipid antibodies (data not shown).

FIG 2 BAR GRAPH OF SPECIFIC PLACENTAL HISTOLOGICAL SCORES AS DESCRIBED IN THE RESULT SECTION



This figure gives an indication of the abundance of various lesions in different groups. The differences between groups is represented with (*) indicating a p-value of ◂ 0.05 and (**) indicating a p-value of ◂ 0.01.

FIG 3 **IMMUNOHISTOCHEMICAL STAINING PATTERNS IN HUMAN PLACENTAS**



Panels 3A-L: typical examples of immunohistochemical staining patterns of C4d, C1q, Properdin and MBL of placentas. Vertically the panels are organized in such a way that each column represents the same placenta. Horizontally the different immunohistochemical stainings are shown. The first column represents a C4d negative placenta of a patient with a live birth. The middle column represents a placenta of a patient with an IUFD, with a focal C4d staining pattern. In the third column a placenta of a patient with SLE and secondary APS is shown which is diffusely positive for C4d. The pregnancy was accompanied by severe maternal preeclampsia and severe fetal growth retardation.

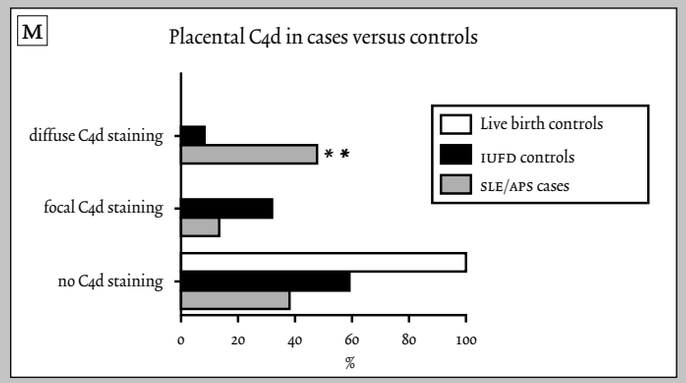
Panels 3A-C: Typical examples of different C4d staining intensities of villous syncytiotrophoblast cell and basement membranes observed in patients with SLE and/or APS compared to controls. Panels demonstrate the different staining intensities by which the placentas were scored: (A) 'no placental C4d staining', (B) 'focal placental C4d staining', and (C) 'diffuse placental C4d staining'. In (C) it is clearly shown that C4d depositions are found on the maternal side of the placental syncytiotrophoblast, and not within the fetal vasculature.

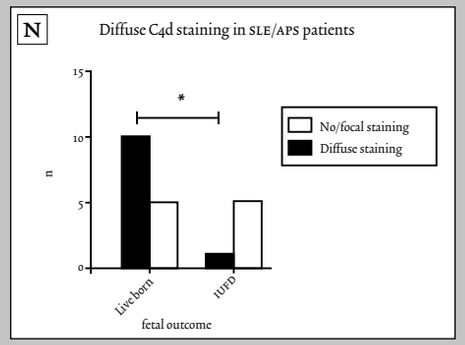
Panels 3D-F: Typical examples of placental C1q staining. Interestingly, C1q was not unique for C4d positive placentas, but was observed frequently in non-C4d positive placentas too. This phenomenon is illustrated in figure (D), where a C4d negative placenta is evidently C1q positive. However, panel (F) shows that C1q does co-localization with C4d in a C4d positive placenta, confirming that C4d originates from classical pathway activity.

Panels 3G-I: Typical examples of properdin staining, in which properdin deposits on endothelial cells of the fetal vessels. Properdin deposition is not corresponding with the sites of C4d deposition, suggesting that classical complement activation does not necessarily lead to properdin deposition – i.e. alternative pathway activation.

Panels J-L: In all cases MBL deposition was absent, further confirming that the MBL pathway does not contribute to the deposition of C4d in the setting of SLE and aPL-mediated fetal loss.

FIG 3





M: Shows the association of diffuse C4d staining with IUFD in SLE/APS cases. The differences between groups is represented with (*) indicating a p-value of ‹0.05 and (**) indicating p-value of ‹ 0.01.

N: Bar graph of C4d staining in SLE/APS cases versus control groups. Figure 3 gives an indication of the abundance of diffuse C4d staining in the SLE/APS case group and the striking absence of diffuse C4d in the live birth controls.

TABLE 1 PATIENT CHARACTERISTICS

	SLE/APS* CASES (N=21)	IUFD** CONTROLS (N=22)	LIVE BIRTH CONTROLS (N=40)
Mean maternal age in years (SD)	29,7(4)	30,5 (6)	31,5 (6)
Mean gravidity (SD)	2,3 (1,5)	2,6 (1,6)	2,2 (0,87)
Mean parity (SD)	0,6 (1,1)	1,3 (1,4)	0,8 (0,8)
Previous live birth (%)	5 (24)	11 (50)	15 (38)
Previous miscarriage or fetal loss (%)	8 (38)	6 (27)	14 (35)
Gestational age at delivery (wk + day) (SD in days)	32 + 6 (4)	25 + 3 (8)	37 + 1 (7)
Delivery at ‹ 24 wk (%)	4 (19)	14 (64)	0
Delivery at 24-38 wk (%)	17 (81)	6 (27)	20 (50)
Delivery at 38-42 (%)	5 (24)	2 (9)	20 (50)
Intrauterine fetal death (%)	6(29)	22(100)	0
Fetal distress (%)	6(29)	0	11(27)
Mean birth weight (grams) (SD)	1639,3 (1060)	901,3 (1203)	2534,5 (981)
Placental weight (grams) (SD)	306,8 (173)	230,2 (205)	472,9 (139)
Heparin therapy during pregnancy (%)	8 (38)	0	0
Comorbidity			
SLE (%)	15 (71)	0	0
SLE and APS (seconday APS) (%)	6 (28)	0	0
APS, no SLE (primary APS) (%)	6 (28)	0	0
Lupus anticoagulant (%)	8 (38)	0	0
Anticardiolipin antibodies, IgG (%)	10 (48)	0	0
Preeclampsia (%)	5 (24)	0	0
HELLP syndrome (%)	2 (10)	0	0
Normal pregnancy and delivery (%)	2 (10)	0	14 (35)
Unexplained intrauterine fetal death (%)	0	5 (23)	0
Infant congenital abnormalities (%)	0	12 (55)	3 (8)

* Systemic Lupus Erythematosus and antiphospholipid syndrome

** Intrauterine fetal death

TABLE 2 C4D AND PROPERDIN IN SLE AND APS PLACENTAS VERSUS CONTROLS

	SLE/APS CASES* (N=21)	IUFD** CONTROLS (N=22)	LIVE BORN CONTROLS (N=40)	P-VALUE SLE/APS VS CONTROLS
No C4d deposition (%)	8 (38)	13 (59)	40(100)	
Focal C4d deposition (%)	3 (14)	7 (32)	0	
Diffuse C4d deposition (%)	10 (48)	2 (9)	0	P < 0.001
No properdin deposition (%)	8 (38)	19 (86)	17 (43)	
Focal properdin deposition (%)	6 (29)	2 (9)	12 (30)	
Diffuse properdin deposition (%)	7 (33)	1 (5)	11 (28)	P < 0.918

** Systemic Lupus Erythematosus and APS

* IUFD control group

TABLE 3 PATIENTS WITH MULTIPLE PLACENTAS

CASE NR	PATIENT DIAGNOSIS	GRAVIDITY	C4D STAINING	FETAL OUTCOME	WEEKS & DAYS
1	SLE*	G1	no tissue available	early miscarriage	< 10
		G2	no C4d staining	IUFD*** of unknown etiology	18
		G3	no C4d staining	IUFD, congenital heart block in the presence of anti-SSA and anti-SSB antibodies	31
		G4	no C4d staining	Live birth	38 + 1
2	SLE + APS**	G1	no tissue available	Live birth, IUGR†	37 + 2
		G2	diffuse C4d staining	Live birth, IUGR and severe Preeclampsia	38 + 4
		G3	focal C4d staining	Live birth, IUGR	38 + 3
3	Primary APS	G1	diffuse C4d staining	IUFD, severe IUGR and maternal HELLP ‡ syndrome.	23 + 2
		G2	no tissue available	live birth, HELLP syndrome and maternal liver infarction	37
		G3	diffuse C4d staining	IUFD, severe HELLP syndrome	28 + 2
4	Primary APS	G2	no tissue available	IUFD of unknown etiology	21 + 4
		G3	diffuse C4d staining	IUFD, severe IUGR	18 + 1
		G4	diffuse C4d staining	IUFD, severe IUGR	26 + 1

* Systemic lupus erythematosus

** Antiphospholipid syndrome

*** Intrauterine fetal death

† Intrauterine growth retardation

‡ Hemolysis Elevated Liver enzymes and Low Platelets

REFERENCES

1 Opatrny L, David M, Kahn SR, Shrier I, Rey E. Association between antiphospholipid antibodies and recurrent fetal loss in women without autoimmune disease: a metaanalysis. *J Rheumatol* 2006; 33(11):2214-2221.

2 Cohen D, Berger SP, Steup-Beekman GM, Bloemenkamp KW, Bajema IM. Diagnosis and management of the antiphospholipid syndrome. *BMJ* 2010; 340:c2541.

3 Ruiz-Irastorza G, Crowther M, Branch W, Khamashta MA. Antiphospholipid syndrome. *Lancet* 2010.

4 Sebire NJ, Backos M, El Gaddal S, Goldin RD, Regan L. Placental pathology, antiphospholipid antibodies, and pregnancy outcome in recurrent miscarriage patients. *Obstet Gynecol* 2003; 101(2):258-263.

5 Girardi G, Berman J, Redecha P, Spruce L, Thurman JM, Kraus D *et al.* Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. *J Clin Invest* 2003; 112(11):1644-1654.

6 Girardi G, Bulla R, Salmon JE, Tedesco F. The complement system in the pathophysiology of pregnancy. *Mol Immunol* 2006; 43(1-2):68-77.

7 Holers VM, Girardi G, Mo L, Guthridge JM, Molina H, Pierangeli SS *et al.* Complement C3 activation is required for antiphospholipid antibody-induced fetal loss. *J Exp Med* 2002; 195(2):211-220.

8 Di SN, Meroni PL, de PN, Raschi E, Caliandro D, De Carolis CS *et al.* Antiphospholipid antibodies affect trophoblast gonadotropin secretion and invasiveness by binding directly and through adhered beta2-glycoprotein I. *Arthritis Rheum* 2000; 43(1):140-150.

9 Cavazzana I, Manuela N, Irene C, Barbara A, Sara S, Orietta BM *et al.* Complement activation in anti-phospholipid syndrome: a clue for an inflammatory process? *J Autoimmun* 2007; 28(2-3):160-164.

10 Salmon JE, Girardi G. Antiphospholipid antibodies and pregnancy loss: a disorder of inflammation. *J Reprod Immunol* 2008; 77(1):51-56.

11 Colvin RB. Antibody-mediated renal allograft rejection: diagnosis and pathogenesis. *J Am Soc Nephrol* 2007; 18(4):1046-1056.

12 Nickeleit V, Mihatsch MJ. Kidney transplants, antibodies and rejection: is C4d a magic marker? *Nephrol Dial Transplant* 2003; 18(11):2232-2239.

13 Shannon JG, Cockrell DC, Takahashi K, Stahl GL, Heinzen RA. Antibody-mediated immunity to the obligate intracellular bacterial pathogen Coxiella burnetii is Fc receptor- and complement-independent. *BMC Immunol* 2009; 10:26.

14 Redecha P, Franzke CW, Ruf W, Mackman N, Girardi G. Neutrophil activation by the tissue factor/Factor VIIa/PAR2 axis mediates fetal death in a mouse model of antiphospholipid syndrome. *J Clin Invest* 2008; 118(10):3453-3461.

15 Pengo V, Tripodi A, Reber G, Rand JH, Ortel TL, Galli M *et al.* Update of the guidelines for lupus anticoagulant detection. Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis. *J Thromb Haemost* 2009; 7(10):1737-1740.

16 Pierangeli SS, Harris EN. A protocol for determination of anticardiolipin antibodies by ELISA. *Nat Protoc* 2008; 3(5):840-848.

17 Magid MS, Kaplan C, Sammaritano LR, Peterson M, Druzin ML, Lockshin MD. Placental pathology in systemic lupus erythematosus: a prospective study. *Am J Obstet Gynecol* 1998; 179(1):226-234.

18 Out HJ, Kooijman CD, Bruinse HW, Derksen RH. Histopathological findings in placentae from patients with intra-uterine fetal death and anti-phospholipid antibodies. *Eur J Obstet Gynecol Reprod Biol* 1991; 41(3):179-186.

19 Cross JC. How to make a placenta: mechanisms of trophoblast cell differentiation in mice--a review. *Placenta* 2005; 26 Suppl A:S3-S9.

20 Kraut N, Snider L, Chen CM, Tapscott SJ, Groudine M. Requirement of the mouse I-mfa gene for placental development and skeletal patterning. *EMBO J* 1998; 17(21):6276-6288.

21 Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997; 40(9):1725.

22 Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R *et al.* International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost* 2006; 4(2):295-306.

23 Bulla R, Agostinis C, Bossi F, Rizzi L, Debeus A, Tripodo C *et al.* Decidual endothelial cells express surface-bound C1q as a molecular bridge between endovascular trophoblast and decidual endothelium. *Mol Immunol* 2008; 45(9):2629-2640.

24 Bulla R, Bossi F, Agostinis C, Radillo O, Colombo F, De SF *et al.* Complement production by trophoblast cells at the feto-maternal interface. *J Reprod Immunol* 2009; 82(2):119-125.

25 Thurman JM, Kraus DM, Girardi G, Hourcade D, Kang HJ, Royer PA *et al.* A novel inhibitor of the alternative complement pathway prevents antiphospholipid antibody-induced pregnancy loss in mice. *Mol Immunol* 2005; 42(1):87-97.

26 Kohler PF. Maturation of the human complement system. I. Onset time and sites of fetal C1q, C4, C3, and C5 synthesis. *J Clin Invest* 1973; 52(3):671-677.

27 Devreese K, Hoylaerts MF. Challenges in the diagnosis of the antiphospholipid syndrome. *Clin Chem* 2010; 56(6):930-940.

28 Pengo V, Biasiolo A, Gresele P, Marongiu F, Erba N, Veschi F *et al.* Survey of lupus anticoagulant diagnosis by central evaluation of positive plasma samples. *J Thromb Haemost* 2007; 5(5):925-930.

29 Girardi G, Redecha P, Salmon JE. Heparin prevents antiphospholipid antibody-induced fetal loss by inhibiting complement activation. *Nat Med* 2004; 10(11):1222-1226.

30 Billingham RE, Brent l, Medawar PB. Actively acquired tolerance of foreign cells. *Nature* 1953; 172(4379):603-606.

31 Berman J, Girardi G, Salmon JE. TNF-alpha is a critical effector and a target for therapy in antiphospholipid antibody-induced pregnancy loss. *J Immunol* 2005; 174(1):485-490.

VII

C4D AS A FOOTPRINT OF MATERNAL ANTI-FETAL IMMUNITY IN RECURRENT MISCARRIAGE

A novel pathogenic mechanism

DANIELLE COHEN / JANTIEN VISSER / TESS MEULEMAN / FRANS CLAAS / DAVE ROELEN
JAN ANTHONIE BRUIJN / INGEBORG M. BAJEMA / KITTY W.M. BLOEMENKAMP

SUBMITTED

Abstract

BACKGROUND The conceptus represents a foreign body to the maternal immune system. This 'natural' allograft is usually not rejected. In analogy with solid organ transplantation, we hypothesized that antibody-mediated rejection, characterized by activation of the classical complement system, could play a role in women with unexplained recurrent miscarriage. We therefore investigated the presence of placental C4d deposition, a marker for classical complement activation, as well as the presence of anti-HLA class I and II antibodies in women with recurrent miscarriage compared to control subjects.

METHODOLOGY AND PRINCIPLE FINDINGS We studied placental C4d deposition in 38 women with unexplained recurrent miscarriage (cases), a first control group of 22 women who experienced one spontaneous miscarriage but subsequently had live births and a second control group of 67 women who underwent elective termination of pregnancy. C4d depositions were found at the maternal side of the syncytiotrophoblast and a diffuse staining pattern was strongly associated with recurrent miscarriage (p=0,004) when compared tot control subjects. Presence of anti-HLA class I and II antibodies was determined in 28 out of 38 women in the recurrent miscarriage-group, of whom 11 (39%) had a positive titer for anti-HLA class I or II antibodies. Of those, 9 of 11(82%) had focal or diffuse placental C4d depositions, whereas in the 17 patients without anti-HLA antibodies diffuse placental C4d was present in only 4 patients (23%) (p=0,003).

CONCLUSIONS Placental C4d is present significantly more often in patients with unexplained recurrent miscarriage compared to control subjects. C4d deposition is found at the fetal-maternal interface, and may be interpreted as a footprint of antibody mediated trophoblast injury. We identified 9 out of 39 patients with unexplained recurrent miscarriage who had both placental C4d deposits and positive titers

for anti-HLA antibodies. This combination strongly suggests an antibody-mediated immune response, and might embody a new pathophysiologic mechanism responsible for recurrent miscarriages.

Introduction

About 1-3% of all couples will be confronted with recurrent miscarriage, which is defined as ▸3 consecutive miscarriages within 20 weeks of gestation.[1] In any recognized pregnancy there is a chance of miscarriage of about 10-15%. The large majority of these sporadic miscarriages are caused by fetal chromosomal aneuploidies.[2] In recurrent miscarriage, maternally derived underlying causes can be identified in a substantial proportion of women. Examples of such causes are uterine anomalies, endocrine disorders, autoimmune disorders such as SLE or antiphospholipid syndrome, thrombophilia or balanced translocations in the maternal (and/or paternal) DNA. However, in more than 50% of woman suffering from recurrent miscarriage, no causal factor can be identified.[1] This burden of continuous uncertainty has major impact on the lives of women and their partners. For clinicians, the lack of both etiological insight and evidence based therapeutic interventions makes the management of these patients complex and sometimes frustrating.

In analogy with solid organ transplantation it has been hypothesized that recurrent miscarriage of unknown etiology is a form of maternal anti-fetal allograft rejection. In the transplant world, allo-antibody mediated rejection (humoral rejection) has gained much attention since the discovery of the biomarker C4d in the early nineties.[3,4] C4d is a tissue-biomarker for classical complement activation, a powerful component of human innate immunity that plays an essential role in inducing tissue injury in many allo- and autoimmune settings. When antibodies (allo- or autoantibodies) bind or deposit, the classical complement pathway is activated via a cascade of enzymatic reactions. The formation of potent anaphylactoxins C5a and C3a and the formation of the membrane attack complex are the main results of this process.

C4d is a non-functional split product of classical complement activation that covalently attaches to cells and tissues. While antibodies dissociate over time, C4d stays anchored to the tissue, thereby acting as a footprint of recent antibody mediated tissue injury. Nowadays C4d is routinely used by transplantation pathologists all over the world.[5]

We have recently demonstrated that C4d is abundantly present in placentas of women with autoimmune mediated pregnancy losses caused by SLE and antiphospholipid syndrome.[6] Placental C4d was found at the fetal-maternal interface, and was strongly associated with intrauterine fetal death and severe forms of preeclampsia.[7-10] The concept of excessive complement activation as an important mediator of maternal anti-fetal immunity has shown to be relevant in settings other than autoimmune disease too. Lee *et al* recently published that C4d in fetal cord endothelium was associated with circulating maternal anti-HLA I antibodies in a setting of spontaneous preterm birth.[11] Furthermore, a recent cohort study of patients with severe preeclampsia demonstrated that 19% of women had mutations in complement regulatory genes. It was shown that such mutations are responsible for inadequate inhibition of complement activation at the fetal-maternal interface, serving as a basis for impaired trophoblast functioning and placental development.

In recurrent miscarriage of unknown etiology both auto- and alloantibodies could theoretically be involved. Auto-antibodies could for instance be antiphospholipid-like antibodies that are not picked up by current assays but have a similar effect on trophoblast cells. Allo-antibodies could be anti-HLA antibodies directed against fetal inherited paternal HLA antigens. We hypothesized that if an ongoing antibody-mediated process is responsible for miscarriage, C4d should be present at the fetal-maternal interface. We therefore aimed to investigate the presence of C4d deposition on trophoblast tissue of patients with unexplained recurrent miscarriage compared to control subjects. To further unravel the disease mechanism, we related placental C4d with the presence of circulating anti-HLAantibodies in

a subgroup of women, with the hypothesis that antibody-mediated rejection of the fetal allograft may indeed be responsible for a proportion of women with unexplained recurrent miscarriage.

Material and methods

ETHICS STATEMENT All tissue and serum samples were handled in a coded and anonymized fashion, according to the Dutch National Ethical guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of Medical Scientific Societies). This national guideline or code makes it possible to perform research with human material that came available within the framework of patient care. Subsequently, this human material can be used for research purposes when properly coded and anonymized.

PATIENTS We studied products of conception of 127 women, who were divided into three groups: A case-group of 38 patients with recurrent miscarriage of unknown etiology, a first control group of 22 healthy women with one spontaneous miscarriage who subsequently had normal pregnancies and live births, and a second control group of 67 women who underwent an elective termination of pregnancy due to medical reasons (i.e. fetal chromosomal anomalies) or social reasons.

The case-group consisted of 38 women diagnosed with recurrent miscarriage of unknown etiology, who were selected from a population of women enrolled in a clinical trial performed at the Leiden University Medical Center (Habenox trial, trial register number NVT0095962).[12] The Habenox trial investigated the effect of anticoagulant treatment on pregnancy outcome in women with unexplained recurrent miscarriage or thrombophilia. Recurrent miscarriage was defined as three or more consecutive first trimester miscarriages (<13 weeks), two or more second trimester miscarriages (13-24 weeks) or one third trimester miscarriage combined with at least one first trimester miscarriage. Patients with thrombophilia, defined as factor V Leiden mutation, prothrombin gene mutation, protein C or S deficiency, high factor VIII or presence of antiphospholipid antibodies were excluded for the current study. Other exclusion criteria were history of thromboembolism or bleeding disorders, allergy to aspirin or enoxaparin, uterine anomalies, cervical insufficiency, untreated thyroid disease, poorly treated diabetes mellitus, parental chromosomal abnormalities and pregnancies achieved by assisted reproductive techniques. We included all women of whom tissue samples of miscarriages were available in the archives of the pathology department of the Leiden University Medical Center, Leiden, the Netherlands.

For a first control group (sporadic miscarriage group) we included 22 tissue samples of miscarriages from women who experienced one spontaneous miscarriage, but subsequently had live births and uneventful pregnancies. None of the patients had a history of preeclampsia or Hemolysis Elevated Liver enzymes and Low Platelets (HELLP) syndrome, or any of the other exclusion criteria used in the case group.

For a second control group (elective termination of pregnancy group) we included 67 tissue samples of elective terminations of pregnancy, of which 21 terminations were performed for medical reasons (i.e. fetal chromosomal anomalies) and 46 for social reasons. Of 13 cases in this group we received tissue samples from an abortion clinic, therefore, of these patients we have no clinical information other than the gestational age of the pregnancy.

An overview of the clinical characteristics of all patients, derived from the case-record files, is given in table 1.

TISSUE SAMPLES OF MISCARRIAGES AND ELECTIVE TERMINATIONS OF PREGNANCY Products of conception were fixed in 4% buffered formalin and embedded in paraffin. Paraffin sections were routinely stained with HE. To study classical complement activation, immunohistochemical staining was performed for C4d (BI-RC4d, Biomedica Gruppe, Austria). Optimal antibody dilutions and incubation times for the different antibodies were pre-determined by means of titration on positive control sections. Endogenous peroxidase activity was blocked. Antigen retrieval was performed with 10 mM

citrate buffer (pH 6.0). A polyclonal rabbit anti-human C4d antibody (Biomedica Gruppe, Austria), was applied at a dilution of 1:80 in 1% BSA/PBS, and slides were incubated for one hour at room temperature. The slides were then incubated with a secondary antibody (anti-rabbit EnVision, K5007, Dako Cytomation, Denmark) for 30 minutes. Staining was visualized with diaminobenzidine (Dako Cytomation, Denmark) and counterstained with Haematoxylin.

QUANTIFICATION OF MORPHOLOGY AND IMMUNOHISTOPATHOLOGY Sections were evaluated by two experienced observers (IMB and DC) who scored the slides blinded to the patients' clinical data. Differences in scorings were resolved by re-reviewing the sections and coming to consensus. Positivity for C4d was scored semi-quantitatively. Staining intensity around syncytiotrophoblast was scored as 0, 1, or 2, with 0 representing the total absence of staining, 1 representing focal positive staining, and 2 representing diffuse staining of all syncytiotrophoblast cell- and basement membranes. Typical examples of staining patterns are given in figure 1.

SEROLOGY: ANTI-HLA ANTIBODIES AND ANTIBODY SPECIFICITY Serum samples from 28 patients from the recurrent miscarriage group were available for analysis. The samples were procured from 80 C storage and a Lambda Antigen Tray class I & II ELISA (One Lambda, Canoga Park, CA) was carried out to detect the presence of HLA class I and class II IgG antibodies. The ELISA was conducted according to protocol with OD readouts at 630 nm.

STATISTICAL ANALYSIS Categorical variables were compared using the Chi-square test and its trend version (linear-by-linear analysis). Differences in quantitative parameters between groups were assessed using one-way ANOVA (for data normally distributed) or the non-parametric Kruskall Wallis-test (for non-normally distributed data). All analyses were performed using SPSS statistical software package (version 16.0; Chicago, IL). A p-value less than 0,05 was considered statistically significant.

Results

CLINICAL CHARACTERISTICS OF CASES AND CONTROL SUBJECTS Table 1 shows the clincial characteristics of the study population. Differences between women with unexplained recurrent miscarriages and control subjects were observed in maternal age and gravidity (both p‹0.05). Women from the recurrent miscarriage group were treated with anticoagulant therapy during pregnancy in 25 out of 38 cases (67%). The medication used was a prophylactic dose of low molecular weight heparin (LMWH) in 15 cases (40%), aspirin in 10 cases (27%) or a combination of both in 6 cases (16%). None of the women with sporadic miscarriage used any medication during pregnancy. In the elective termination of pregnancy-group this information was not available.

IMMUNOHISTOCHEMICAL C4D STAINING IN CASES VERSUS CONTROL SUBJECTS Immunohistochemistry was performed on trophoblast tissue from miscarriage material from the three study-groups. When C4d was present on trophoblast tissue, positivity was detected at the fetal-maternal interface, on the maternal side of the syncytiotrophoblast, either in a focal or a diffuse staining pattern. Typical examples of C4d staining patterns are shown in figure 1A-C.

The presence of placental C4d and its distribution in either a focal or diffuse pattern differed significantly among the three groups in a chi-square linear by linear association analysis (p‹0.004). A diffuse C4d staining pattern in the placenta was present in 10 of 38 of women with unexplained recurrent miscarriage (26%), compared to 3 out of 22 in the sporadic miscarriage group (13%) and 7 out of 67 in elective abortions (10,4%)(p‹0,004). Detailed information on C4d staining in cases versus control subjects is given in table 2.

ANTI-HLA ANTIBODIES AND THEIR RELATION WITH C4D Table 3 shows the relationship between anti-HLA seropositivity and presence of C4d in placental tissue. In total, serum samples of 28 women with unexplained recurrent miscarriage were analysed.

Seropositivity for anti-HLA class I or II IgG antibodies was detected in 11 cases. Of those, 9 women (82%) also had placental C4d deposits in a focal or diffuse pattern. In 17 women without detectable anti-HLA antibodies, only 4 (23%) had focal or diffuse placental C4d staining (P=0,004).

Conclusion and discussion

Recurrent miscarriage is a devastating complication of pregnancy, affecting a large population of women worldwide. Many disease mechanisms for this multifactorial disorder have been identified, but in 50% of couples no underlying cause can be found.[1] For many years it has been questioned whether the fetus can indeed be interpreted as an 'allograft' and thus, miscarriage as 'rejection'.[1;13] In this study we demonstrate that antibody-mediated rejection of the fetal allograft may indeed be present in a subgroup of women with so far unexplained recurrent miscarriages.

C4d, a biomarker of classical complement activation and a footprint of antibody-mediated injury, was present in areas of active fetal-maternal exchange at the maternal side of the syncytiotrophoblast. Placental C4d in a diffuse staining pattern was present significantly more often in women with unexplained recurrent miscarriages compared to two control groups. Moreover, presence of C4d was associated to the presence of anti-HLA class I or II antibodies in the recurrent miscarriage group. Taken together, our data support the concept of an antibody-mediated immune response at the fetal-maternal interface, leading to miscarriage in a certain subgroup of patients with so far unexplained recurrent miscarriages.

Antibody deposition is present in the placenta under physiological conditions but because the placenta is strongly protected from spontaneous complement activation by regulatory mechanism such as Decay Accelerating Factor (DAF), Membrane Cofactor Protein (MCP) and CD59, this usually does not lead to extensive tissue damage.[14-16] Therefore, excessive complement deposition as we observed in certain women with recurrent miscarriage can be interpreted as a sign of local dysregulation of the placental complement system. In other words, there must be either 'excessive complement activation', or 'inadequate complement regulation'.

Too much complement activation may be caused by excessive antibody deposition.[6;17] Antiphospholipid antibodies are likely candidates, as they are associated with placental complement activation and impaired pregnancy outcome.[6] However, all women in our recurrent miscarriage population were tested for anticardiolipin IgG, IgM and lupus anticoagulant and were excluded from the study if any of these antibodies were positive.[12] Allo-antibodies could also be involved. Recently, Nielsen *et al* described that anti-HLA antibodies are related to a reduced live birth rate in women with recurrent miscarriage.[18] Interestingly, we found anti-HLA class I and II antibodies in serum samples of women with unexplained recurrent miscarriage, and demonstrated their potential trace at the fetal-maternal interface via C4d deposition. These antibodies are most likely directed against inherited paternal antigens expressed on trophoblast cells. Up to 30% of women have circulating anti-HLA antibodies during pregnancy, which usually do not predispose to higher risk of adverse pregnancy outcome, miscarriage or pre-eclampsia.[19;20] However, from transplantation settings we know that only some allo-antibodies cause rejection, depending on their antigenicity, their ability to activate complement and their avidity for the antigenic target. Furthermore, presence and detection of anti-HLA antibodies could also be a marker for a broader antibody response. This was shown previously in HLA identical family transplantations, where the presence of anti-HLA antibodies was a risk factor for worse outcome, although clearly anti-HLA antibodies themselves could not have caused any harm.[21] The specific allo-antibodies involved in recurrent miscarriage should be subject for further studies.

Apart from excessive activation, inadequate complement regulation at the fetal-maternal interface may also play a role. It was recently shown in a group of women with SLE and antiphospholipid syndrome, that up to 19% of patients who develop severe preeclampsia

have genetic mutations in genes encoding for complement regulatory proteins that are necessary to prevent damage of host tissue due to uncontrolled activation of complement.[22] These mutations were first described in populations with atypical Hemolytic Uremic Syndrome (aHUS) where they lead to widespread microthrombotic injury. In the study by Salmon *et al*, patients with these mutations developed severe forms of preeclampsia, intrauterine growth restriction and even third trimester intrauterine fetal death. They did not have signs of microthrombotic injuries in organs other than the placenta and did not present as HUS cases. It is possible that genetic defects in complement regulation may cause recurrent miscarriages. The excessive deposition in placental tissue of some of our patients is in line with this concept.

At present there is no evidence based treatment for women with unexplained recurrent miscarriage. None of the published randomized controlled trials investigating the effect of LMWH and aspirin in this population could detect a beneficial effect of these interventions on live birth rate.[12;23;24] In our population a substantial proportion of women was using LMWH, aspirin, or a combination of both at time of miscarriage. In our study, we could not find a relation between use of anticoagulation and presence of C4d, or presence of anti-HLA antibodies (data not shown). Clearly the current group is too small to draw definite conclusions.

Recurrent miscarriage, as is demonstrated by this study, is not a condition with a single cause. Progress in understanding the many different mechanisms that may lead to recurrent miscarriage is urgently needed. This is not a condition with a single cause, and the trials described above illustrate that searching for a single treatment for all patients is likely futile. Unraveling possible pathofysiological mechanisms for recurrent miscarriage in order to define patient tailored treatment strategies is essential. Our findings possibly identify a subgroup of patients in which complement activation plays an important role. This is especially interesting in the light of animal studies by Girardi *et al*, showing that heparin is beneficial in patients with antiphospholipid antibodies because it inhibits complement activation, and not because of its effects on the coagulation cascade.[25] Patients with recurrent miscarriage and evidence for excessive complement activation at the fetal-maternal interface could be the 'positive responders' to treatment with heparin or LMWH. Whether a positive C4d stain in placental tissue of a patient with multiple miscarriages may guide treatment is an interesting subject for further investigations.

TABLE 1 PATIENT CHARACTERISTICS

	RECURRENT MISCARRIAGE (CASES) (N=38)	SPORADIC MISCARRIAGE (CONTROLS) (N=22)	ELECTIVE ABORTION (CONTROLS) (N=67)
Mean maternal age in years (SD)	33,5 (5,4)	31,9 (6,7)	28,6 (7,7)
Mean gravidity (SD)	4,2 (2,1)	2,1 (1,0)	NA
Mean parity (SD)	0,8 (0,8)	1 (1,1)	NA
Previous miscarriage or fetal loss (%)	100	0	NA
Gestational age at miscarriage or abortion (wks) (SD in wks)	10,7 (3,8)	10,5 (2,0)	9,149 (3,1)
▸ 4 miscarriages n(%)	26 (68)	0	NA
Heparin therapy during pregnancy n(%)	15 (40)	0	NA
Aspirin during pregnancy n(%)	10 (27)	0	NA
Aspirin and heparin during pregnancy n(%)	6 (16)	0	NA

NA = No information available

TABLE 2 RECURRENT MISCARRIAGE AND C4D STAINING

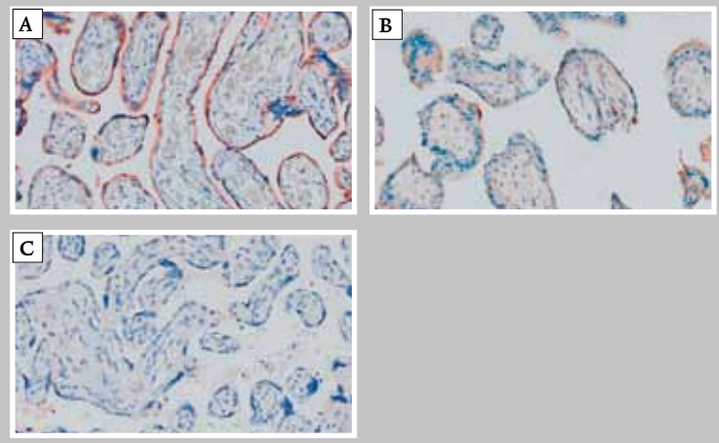
	NO C4D DEPOSITION	FOCAL C4D DEPOSITION	DIFFUSE C4D DEPOSITION	TOTAL
Recurrent miscarriage n(%)	16 (42,1)	12 (31,6)	10 (26,3)	38
Spontaneous miscarriage n(%)	7 (31,8)	12 (54,6)	3 (13,6)	22
Abortion on request n(%)	45 (67,2)	15 (22,4)	7 (10,4)	67
P ◂ 0,004 (Chi squared linear by linear analysis)				

TABLE 3 C4D AND ANTI-HLA ANTIBODIES IN 28 PATIENTS WITH UNEXPLAINED RECURRENT MISCARRIAGE

	NEGATIVE ANTI-HLA CLASS I OR II	POSITIVE ANTI-HLA CLASS I OR II
No or focal C4d deposition	13	2
Diffuse C4d deposition	4	9
	17	11

P = 0,004

FIG 1 EXAMPLES OF IMMUNOHISTOCHEMICAL STAINING PATTERNS OF PLACENTAL C4D



A. Diffuse C4d staining on trophoblast tissue derived from miscarriage material. C4d stains red and is positive at the fetal-maternal interface. Every fetal villus is fully covered with C4d deposits. No staining is visible within fetal villi, suggesting a maternal origin of complement activation.
B. Focal C4d staining. Between 10% and 50% of fetal villi show signs of C4d deposition. Some parts of the trophoblast layer stain positive, but other parts remain negative.
C. Negative C4d staining.

REFERENCES

1 Branch DW, Gibson M, Silver RM. Clinical practice. Recurrent miscarriage. *N Engl J Med* 2010; 363(18):1740-1747.

2 Sullivan AE, Silver RM, LaCoursiere DY, Porter TF, Branch DW. Recurrent fetal aneuploidy and recurrent miscarriage. *Obstet Gynecol* 2004; 104(4):784-788.

3 Collins AB, Schneeberger EE, Pascual MA, Saidman SL, Williams WW, Tolkoff-Rubin N *et al.* Complement activation in acute humoral renal allograft rejection: diagnostic significance of C4d deposits in peritubular capillaries. *J Am Soc Nephrol* 1999; 10(10):2208-2214.

4 Colvin RB. Dimensions of antibody-mediated rejection. *Am J Transplant* 2010; 10(7):1509-1510.

5 Sis B, Mengel M, Haas M, Colvin RB, Halloran PF, Racusen LC *et al.* Banff '09 meeting report: antibody mediated graft deterioration and implementation of Banff working groups. *Am J Transplant* 2010; 10(3):464-471.

6 Cohen D, Buurma A, Goemaere NN, Girardi G, le CS, Scherjon S *et al.* Classical complement activation as a footprint for murine and human antiphospholipid antibody-induced fetal loss. *J Pathol* 2011.

7 Salmon JE, Girardi G, Holers VM. Complement activation as a mediator of antiphospholipid antibody induced pregnancy loss and thrombosis. *Ann Rheum Dis* 2002; 61 Suppl 2:ii46-ii50.

8 Pierangeli SS, Vega-Ostertag M, Liu X, Girardi G. Complement activation: a novel pathogenic mechanism in the antiphospholipid syndrome. *Ann N Y Acad Sci* 2005; 1051:413-420.

9 Girardi G, Salmon JB. The role of complement in pregnancy and fetal loss. *Autoimmunity* 2003; 36(1):19-26.

10 Cohen D, Berger SP, Steup-Beekman GM, Bloemenkamp KW, Bajema IM. Diagnosis and management of the antiphospholipid syndrome. *BMJ* 2010; 340:c2541.

11 Lee J, Romero R, Xu Y, Kim JS, Topping V, Yoo W *et al.* A signature of maternal anti-fetal rejection in spontaneous preterm birth: chronic chorioamnionitis, anti-human leukocyte antigen antibodies, and c4d. PLoS One 2011; 6(2):e16806.

12 Visser J, Ulander VM, Helmerhorst FM, Lampinen K, Morin-Papunen L, Bloemenkamp KW *et al.* Thromboprophylaxis for recurrent miscarriage in women with or without thrombophilia. HABENOX: a randomised multicentre trial. *Thromb Haemost* 2011; 105(2):295-301.

13 Guleria I, Sayegh MH. Maternal acceptance of the fetus: true human tolerance. *J Immunol* 2007; 178(6):3345-3351.

14 Grennan DM, McCormick JN, Wojtacha D, Carty M, Behan W. Immunological studies of the placenta in systemic lupus erythematosus. *Ann Rheum Dis* 1978; 37(2):129-134.

15 Johnson PM, Natvig JB, Ystehede UA, Faulk WP. Immunological studies of human placentae: the distribution and character of immunoglobulins in chorionic villi. *Clin Exp Immunol* 1977; 30(1):145-153.

16 Bulla R, Bossi F, Agostinis C, Radillo O, Colombo F, De SF *et al.* Complement production by trophoblast cells at the feto-maternal interface. *J Reprod Immunol* 2009; 82(2):119-125.

17 Shamonki JM, Salmon JE, Hyjek E, Baergen RN. Excessive complement activation is associated with placental injury in patients with antiphospholipid antibodies. *Am J Obstet Gynecol* 2007; 196(2):167-5.

18 Nielsen HS, Witvliet MD, Steffensen R, Haasnoot GW, Goulmy E, Christiansen OB *et al.* The presence of HLA-antibodies in recurrent miscarriage patients is associated with a reduced chance of a live birth. *J Reprod Immunol* 2010; 87(1-2):67-73.

19 Harris RE, Lordon RE. The association of maternal lymphocytotoxic antibodies with obstetric complications. *Obstet Gynecol* 1976; 48(3):302-304.

20 Balasch J, Ercilla G, Vanrell JA, Vives J, Gonzalez-Merlo J. Effects of HLA antibodies on pregnancy. *Obstet Gynecol* 1981; 57(4):444-446.

21 Opelz G. Non-HLA transplantation immunity revealed by lymphocytotoxic antibodies. *Lancet* 2005; 365(9470):1570-1576.

22 Salmon JE, Heuser C, Triebwasser M, Liszewski MK, Kavanagh D, Roumenina L *et al.* Mutations in complement regulatory proteins predispose to preeclampsia: a genetic analysis of the PROMISSE cohort. PLoS Med 2011; 8(3):e1001013.

23 Clark P, Walker ID, Langhorne P, Crichton L, Thomson A, Greaves M *et al.* SPIN (Scottish Pregnancy Intervention) study: a multicenter, randomized controlled trial of low-molecular-weight heparin and low-dose aspirin in women with recurrent miscarriage. *Blood* 2010; 115(21):4162-4167.

24 Kaandorp SP, Goddijn M, van der Post JA, Hutten BA, Verhoeve HR, Hamulyak K *et al.* Aspirin plus heparin or aspirin alone in women with recurrent miscarriage. *N Engl J Med* 2010; 362(17):1586-1596.

25 Girardi G, Redecha P, Salmon JE. Heparin prevents antiphospholipid antibody-induced fetal loss by inhibiting complement activation. *Nat Med* 2004; 10(11):1222-1226.

GENERAL INTRODUCTION

PART ONE
C4D AND THROMBOTIC COMPLICATIONS

PART TWO
C4D AND ADVERSE PREGNANCY OUTCOME

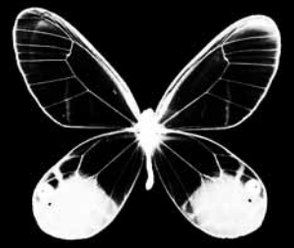
CONCLUSION AND DISCUSSION

APPENDICES

VIII

GENERAL DISCUSSION

Pros and Cons for C4d as a biomarker in auto- and alloimmunity



DANIELLE COHEN / ROBERT B. COLVIN / MOHAMED DAHA
CINTHIA BESKOW DRACHENBERG / MARK HAAS / VOLKER NICKELEIT / JANE E. SALMON
BANU SIS / MING-HUI ZHAO / JAN A. BRUIJN / INGEBORG M. BAJEMA

ADAPTED FROM: PROS AND CONS FOR C4D AS A BIOMARKER. KIDNEY INTERNATIONAL, 2012

The introduction of C4d in daily clinical practice in the late nineties aroused an ever increasing interest in the role of antibody mediated mechanisms in allograft rejection. As a marker of classical complement activation, C4d made it possible to visualize the direct link between anti-donor antibodies and tissue injury at sites of antibody binding in a graft. In this thesis C4d has been studied outside the field of solid organ transplantation. The most important findings described in the different chapters can be summarized as follows: (1) C4d deposition is associated with microthrombotic injury in kidneys and brains of patients with SLE. (2) Placental C4d is associated with adverse pregnancy outcome in SLE and antiphospholipid syndrome such as miscarriage, severe preeclampsia, HELLP syndrome and intrauterine fetal death. (3) In recurrent miscarriage of unknown etiology C4d may be helpful to unravel a possible subgroup of patients in which complement activation and antibody mediated fetal rejection plays a pathophysiological role.

In conclusion, the studies in this thesis have contributed to the fact that C4d is now increasingly being recognized as a potential biomarker in several fields where antibodies can cause tissue damage, such as systemic autoimmune diseases and pregnancy. C4d holds promise to detect patients at risk for the consequences of antibody-mediated disease. Moreover, the emergence of new therapeutics that block complement activation makes C4d a marker that can potentially identify patients who may possibly benefit from these drugs.

This final chapter provides an overview of the past, present, and future perspectives of C4d as a biomarker, focusing on its role in solid organ transplantation and discussing its possible new roles in autoimmunity and pregnancy. For this purpose, a group of experts were interviewed about the role of C4d within their fields of expertise and challenged to think about the following issues:

* Will we still be using C4d in 10 years time, and if not, what alternatives would you suggest?
* What would you like to investigate if you would receive funding to be spent on research in the field of C4d?
* What is your take home message for readers and listeners?

* The interviews form the backbone of this chapter, together with a review of the recent literature on C4d. We would like to motivate readers to listen to the audio-files that can be found online, which include highlights, quotes and authors' comments on both the state of the art and controversies in the field of C4d. A summary of this chapters most important points is given in BOX 1.

Biology of C4d

THE HUMAN COMPLEMENT SYSTEM The complement system is an ancient component of the innate immune system. Complement activation is a non-specific, potent force. Once activated, it makes no distinction between self and non-self. Therefore its activation is as tightly controlled as its natural regulation.[1] The three pathways by which the complement system can become activated, namely the classical, lectin and alternative pathway, converge at the level of C3 and proceed into the formation of the membrane attack complex (MAC) on complement activating surfaces, causing direct tissue injury by perforation of the cell membrane. Additionally, potent anaphylatoxins C3a and C5a are being formed in the process, which elicit the recruitment of other inflammatory cells to the site of activation. (figure 1A)

THE CLASSICAL PATHWAY AND GENERATION OF C4D The classical pathway of complement is initiated via binding of its recognition molecule C1q to immune complex deposits, antibody-antigen binding or charged molecules. When C1q becomes activated, it subsequently activates its natural substrate C4. C4d is a split product of C4 activation, without a biological function.[2;3] Although C4d is mainly interpreted as a trace of classical pathway activation, it must be kept in mind that C4 can also be generated via the lectin pathway. MBL or Ficolins binding to carbohydrate ligands on the surface of a wide variety of pathogens results in activation of the lectin pathway and cleavage of C4.[4;5] Consequently, C4d may be generated without prior antibody binding. (Figure 1A)

– BOX 1 –

SUMMARY AND TAKE HOME POINTS

C4d is a widely used marker for antibody mediated rejection in kidney, heart, pancreas and possibly lung allografts.
In abo incompatible grafts, c4d is not a useful test, and may even indicate graft-accomodation.
In pregnancy, C4d at the fetal-maternal interface indicates antibody mediated rejection of 'the fetal allograft', as was demonstrated in antiphospholipid antibody-induced fetal loss.
C4d shows that the complement system is involved: If complement targeted therapies will be part of our future treatment options, a marker like c4d will be needed to identify patients susceptible for those kind of (expensive) treatments.
Alternatives for C4d are emerging (genomics, molecular diagnostics and endothelial transcripts) and if proven useful, effort will be made to transform these techniques or their progeny to practical tests.

C4D AS A FOOTPRINT OF ANTIBODY-MEDIATED CELL INJURY It is interesting that C4d is a biomarker even though it is an inactive split product of the complement cascade. C4d has been called 'a footprint' of antibody-mediated tissue injury. This nickname is based on the unusual phenomenon that C4b, the larger molecule that C4d is derived from, has an internal thio-ester in the molecule, giving it the ability to form a covalent bond with any free hydrogen group on target cells. When C4d is cleaved from C4b, the covalent bond between C4d and the tissue remains intact. Covalently bound C4d has a much longer half-life, and therefore remains at the site of complement activation whereas antibodies bind to tissue by hydrostatic, van der Waals type of interactions. The 'footprint-effect' of the internal thio-ester of C4d (figure 1B) becomes strikingly apparent when the blood stream can clear all soluble/weakly bound molecules quickly, as happens with antibodies at endothelial surfaces. Covalently bound C4d will not be affected, because it is anchored tightly to the tissue and therefore serves as a footprint of antibody mediated tissue injury.

– BOX 2 –

STATE OF THE ART: BANFF GUIDELINES FOR THE DIAGNOSIS OF AMR

The diagnosis of AMR in renal allografts is currently based on criteria established during the Banff conference on Allograft Pathology in 2007 which include the three following cardinal features:

- Morphologic evidence of acute or chronic tissue injury
- Immunopathologic staining for c4d in peritubular capillaries
- Presence of circulating antibodies to donor HLA or other antigens expressed on donor endothelial cells
- It is recommended that every renal, cardiac and pancreas allograft biopsy should be stained for C4d

C4d staining is considered positive only when depositions are found in the following anatomical locations:

- Kidney: Peritubular capillaries (figure 3a)
- Pancreas: Interacinar capillaries (figure 3b)
- Heart: Endomyocardial capillaries

C4d is scored semiquantitatively in four categories:

- No C4d staining (0% of (peritubular) capillaries)
- Minimal C4d staining (0-10% of (peritubular) capillaries),
- Focal C4d staining (10-50% of (peritubular) capillaries)
- Diffuse C4d staining (›50% of (peritubular) capillaries)

Immunofluorescence is a more sensitive method to detect C4d than immunohistochemistry, by approximately one grade of the scoring system. Although a diffuse C4d staining is defined as positive, the definition and clinical significance of 'minimal' and 'focal' C4d staining remain debated issues. Most experts consider a focal staining pattern as a red flag, especially when detected on paraffin-embedded tissue or in the presence of DSA and/or suspicious histopathological features.

The discovery of C4d as a clinical marker for amr

From hyper-acute rejection episodes it was known that donor specific antibodies (DSA), either anti-HLA or anti-ABO, had the capacity to destroy a graft.[6-8] However, although there was speculation about a role for allo-antibodies in other forms of rejection apart from the hyperacute form, it was unclear what fraction of acute rejection episodes had a humoral component and how to recognize that an AMR was present. The publications of Helmut Feucht and colleagues in the early nineties marked a turning point in the history of solid organ transplantation.[9;10] Feucht showed that patients with suspected antibody-mediated injury in the renal graft had a linear C4d staining pattern in peritubular capillaries and that the presence of C4d was associated with impaired graft function. Remarkably, these initial publications received relatively little attention in the transplant community.[9;11;12] At the turn of the century the group of Collins *et al* tested for presence of C4d along with other markers of endothelial activation or injury in renal transplant biopsies suspected of AMR. C4d was found in each of 10 renal biopsies of patients with circulating DSA and morphologic signs suspicious of AMR, and in none of 14 controls with acute cellular rejection without detectable DSA.[13] This work embodied the connection of dots between C4d, the presence of DSA and a selection of histomorphologic features of AMR, which after 4 decades of an intensive search for a marker was nothing short of revolutionary. A few years later the correlation with graft survival that Feucht *et al* had already reported on in 1993, was confirmed by other groups.[2;14] This led to general acceptance of the usefulness of C4d in the identification of acute AMR. In 2003 'C4d' was incorporated in the Banff classification.[15] (BOX 2)

C4d in current clinical practice

For the kidney a consensus was reached that a diagnosis of AMR requires the simultaneous presence of *de novo* DSA, distinguishable histopathological findings and deposition of C4d in peritubular capillaries (figure 2 and figure 3a). Most centers involved in the management of transplant-recipients have incorporated routine C4d staining in diagnostic pathology evaluation of all renal allograft biopsies.[15] A solid base for regular C4d staining of biopsied allograft tissue is now established for heart transplantation and pancreas transplantation.[16-18;18] For other transplanted organs such as the lung, the usage of C4d staining is still controversial.[19;20] In liver and short bowel transplantation C4d seems to have no additional diagnostic value.[21]

In BOX 3, guidelines are given on how to interpret various test outcomes of C4d staining and DSA test results that clinicians who work with C4d commonly encounter in daily clinical practice. Finally, BOX 4 elaborates on current treatment options for AMR.[22-25]

– BOX 3 –

ISSUES FOR CLINICIANS WORKING WITH C4D IN DAILY PRACTICE

Focal staining

In paraffin-embedded tissue combined with a positive test for DSA and histopathological features: Most laboratories consider this as 'positive for AMR'. It is advised to treat the patient. In frozen tissue combined with a positive test for DSA and histopathological features: This is sometimes called a 'probable AMR'. Most experts would consider treatment, however, prospective studies should investigate this group further.

C4D positivity without detectable DSA

Possible explanations:

Allo-antibodies are present, but they are not detected by standard anti-HLA assays (for example anti-endothelial antibodies).

Allo-antibodies are absorbed by the graft, as is sometimes shown by reappearance of allo-antibodies in the blood after graft nephrectomy.

Allo-antibodies are not present and C4d deposition is caused by something else than DSA (For instance autoimmune disease i.e. lupus nephritis or any form of lectin pathway activation)

How to proceed?

In case of diffuse C4d staining in peritubular capillaries and histological evidence for AMR: Most experts would advise to treat the patient for AMR.

In case of focal C4d staining and histological evidence for AMR : Check for other possible underlying diseases. If no other cause can be found: Treat the patient for AMR.

In case of focal or diffuse C4d staining and absence of histologic changes te decision as to wether to treat for AMR is more uncertain. Treating or close follow-up are bothe suitable options.

C4D positivity in grafts without histological abnormalities

In ABO incompatible grafts: In case of no histopathology and no graft dysfunction: It is suggested to interpret this as 'graft accomodation'. No treatment is necessary, but close follow-up is strongly advised, as it is unknown what happens with these grafts during long term follow-up.

In positive cross-matched patients (presensitized patient) this should not be seen as graft-accomodation and is a rare and more worrying situation than the above: In case of normal histology and no graft dysfunction: Interpret as probable AMR and consider treating the patient. Prospective studies on the long term follow-up of this group of patients are awaited.

Debated issues 1: c4d in chronic rejection episodes

Soon after the introduction of C4d in daily clinical practice, the phenomenon of a diffuse C4d staining pattern was frequently observed years after transplantation and was associated with chronic changes in the graft.[26;27] This was in contrast with the idea that antibodies

– BOX 4 –

THERAPY-OPTIONS FOR AMR

Although much progress has been made in understanding the etiology of AMR and diagnosing the condition, it remains to be elucidated what treatment option is most beneficial. AMR is relatively unresponsive to therapies targeting T-lymphocytes used in acute cellular rejection such as steroids, cyclosporine, tacrolimus and sirolimus. This issue has not been addressed in a randomized controlled fashion so far. Therapeutic strategies reported in the literature in case-reports, case-series and cohort studies are the following:[23]

The suppression of the T-cell dependent antibody response (steroids, cyclosporine, tacrolimus, sirolimus). The removal of donor reactive antibody (plasmapheresis) The blockade of the residual alloantibody (IvIg) . The depletion of naive and memory B-cells (Rituximab). Inactivation of plasma cells (Bortezomib)[24;25;26]. The blockade of complement component C5 by monoclonal antibodies (Eculizumab)

There is large variability between transplant centers around the world in their specific therapeutic approach but generally a combination of IvIg, plasmapheresis and recently, Rituximab is used. Trials (especially randomized controlled trials) investigating the effects of targeted complement blockade are awaited.

were only involved in hyper-acute and acute rejection episodes. The presence of C4d in late and chronic rejection episodes prompted clinicians and researchers to the hypothesis that an antibody component was present in forms of chronic rejection.

The concrete arguments that underline the role of antibodies in chronic rejection are as follows: Firstly, in experimental models of non-human primates with transplanted kidneys (with no immunosuppressive drugs) progression to chronic graft injury and loss consistently goes through four stages: alloantibody production, deposition of C4d in peritubular capillaries and sometimes glomeruli, chronic histopathologic changes and finally, graft loss. [28]

Secondly, several large (prospective) studies showed that presence of circulating anti-HLA antibodies are associated with late graft failure.[29-31] Thirdly, histologic changes associated with late graft loss, such as glomerular double contours, peritubular capillary basement membrane multilayering, interstitial fibrosis and fibrous intimal thickening in arteries, are found in close association with

C4d deposition in peritubular capillaries and presence of anti-HLA antibodies: In about 30-40% of biopsies with late graft dysfunction, C4d can be detected in peritubular capillaries. [26;31-35].

Based on current understanding, criteria were proposed to diagnose chronic AMR in 2007. To establish this diagnosis, three elements should be present:

* Histologic evidence of chronic injury
* Immunopathologic evidence of antibody mediated graft injury (C4d deposition)
* Evidence of antibodies reactive against the donor

However, there are several controversies surrounding chronic AMR. Most importantly there is no clear cut definition of what is meant by 'chronic'. For some it simply means burned-out scar formation. Others use the word in a broader sense, thinking that there are chronic changes with some kind of activity so that chronicity still has an ongoing active component (and thus, a potential for treatment). Here the advantage of C4d is that it indicates recent (weeks) activity of an active immunological process.

It is unknown why donor-specific antibodies cause acute rejection in one patient and chronic rejection in another, or even both sequentially in the same patient. Factors such as antibody-titer, antibody-avidity, and the extent of resistance (or accommodation) of the graft endothelium to complement activation could be responsible for this phenomenon. More research in this field is certainly needed, as the lack of insight into the natural history of chronic AMR now entails that the optimal therapy for chronic AMR remains undetermined.

Debated issues 2: C4d negative amr

Antibodies mainly damage a graft by targeting the endothelium of the graft's microcirculation. This concept is the basis for the molecular studies performed by Sis *et al* since 2007. These studies have elegantly uncovered a possible new form of AMR, namely, C4d negative AMR (which has been described in a chronic, but not in acute settings). This important finding is currently the most serious challenge for the concept of C4d as a biomarker, as the first studies using endothelial transcripts combined with DSA show excellent sensitivities for AMR (although less specificity than C4d), for chronic AMR.[36] As a marker of antibody interaction with the tissue, it is not inconceivable that this or a simpler derivative method will partly or fully replace C4d in future. In 2007 a retrospective study of biopsies from 1320 transplanted patients showed that more than 40% of cases with transplant glomerulopathy –a histological lesion considered relatively characteristic for chronic AMR- were C4d negative despite the fact that anti-HLA antibodies were detected in 73% of patients.[32] This work was followed by studies looking at mRNA levels of genes involved in endothelial activation and injury. Interestingly, biopsies with high expression of these endothelial transcripts in combination with circulating DSA, showed concurrent histopathological lesions of AMR (such as capillaritis, glomerulitis, transplant glomerulopathy, and fibrosis/atrophy) and had poor outcomes.[36;37] Many of these active AMR cases would have been missed otherwise: Only 40% of kidneys with high endothelial gene expression and histopathologic signs of chronic AMR were C4d positive.[38]

So far, two groups have confirmed the concept of C4d negative AMR. In sensitized recipients Loupy *et al* showed that C4d or capillaritis in 3 month protocol biopsies were risk factors for later transplant glomerulopathy, and capillaritis was predictive even in the absence of C4d.[38;39] The other evidence came from Haas and Mirocha, who investigated patients with donor specific antibodies who had a biopsy during the first 3 months after transplantation. Patients with a C4d negative biopsy who were not treated for AMR had a higher rate of progression to transplant glomerulopathy than those who were treated for AMR post-biopsy.[40]

Because of these complexities a working group was established at the 2011 Banff Conference to refine criteria for diagnosis of chronic AMR in the kidney, and to investigate whether C4d negative AMR should be incorporated in the Banff classification.

If there is indeed a C4d negative form of AMR, it must be based on a pathophysiological mechanism which is complement independent.

There are two types of experimental studies that recently provided some insight into this mechanism: Reed *et al* have set up an *in vitro* model of cultured endothelial cells, to which allo-antibodies can be added. The authors were able to show that allo-antibodies themselves can alter the state of the endothelium in the absence of complement or other inflammatory cells. In response to allo-antibodies, endothelial cells started expressing proinflammatory molecules, increased growth factor and adhesion molecules like E-selectin, P-selectin, ICAM-1, VCAM-1, CX3CL1.[41] Subsequently, it was demonstrated that adding NK cells or macrophages together with antibodies to cultured endothelial cells could damage the endothelial cells even more severely, through Fc receptor interactions.[42;43] Apparently, antibodies can induce injury through interaction with leukocytes like NK cells, without complement as a mediator. In vivo, this has been recently confirmed in mouse heart allografts (Hirohashi *et al*. A novel pathway of chronic allograft rejection mediated by NK cells and alloantibody [Abstract] *Am.J.Transplant* 2011) and within glomerular and peritubular capillaries in human biopsies showing AMR.[44] New diagnostic and therapeutic approaches are warranted to approach these cases in future.

Debated issues 3: C4d positivity as a sign of graft accommodation

Despite the experience that preformed antibodies against HLA- or blood group antigens due to pregnancy, blood transfusion or prior transplants are a major cause of hyperacute rejection of renal allografts, the ever expanding deceased-donor waiting list led to the development of protocols enabling transplantation across these immunologic barriers. Japan and North America were the first to successfully transplant ABO-incompatible grafts in patients who had been pretreated with improved immunosuppressive regimens and plasmapheresis to remove preexisting antibodies.[45;46] The follow-up of these cases revealed some unexpected phenomena, which served as the basis of a new and exiting concept: Stable graft accommodation.

As it soon became apparent that recurrence of low levels of anti-blood group antibodies occurred frequently in patients transplanted with an ABO-incompatible graft, there was concern about the development of AMR in these grafts. Not quite unexpectedly, diffuse C4d staining of peritubular capillaries in these biopsies was commonly found, even in protocol biopsies. However, the fact that more than 70-80% of ABO-incompatible grafts showed diffuse C4d positivity was a surprising finding, especially when compared to the marginal 30-40% diffuse positives observed in the group of patients with a positive cross match for anti-HLA antibodies.[47-49]

Strikingly, in contrast to conventional transplants where a diffuse C4d stain is strongly associated with histological abnormalities such as capillaritis and transplant glomerulopathy, the ABO-incompatible kidneys as a rule show diffuse C4d positivity without histological tissue injury.[50;51] A recent retrospective case-control study by Haas *et al* indicated that persistent C4d positive ABO-incompatible grafts without histological abnormalities are not subject to increased graft scarring, transplant glomerulopathy or reduced renal function within the first year after transplantation.[52] Moreover, ABO-incompatible grafts with persistent diffuse C4d positivity had significantly less chronic damage after one year. These puzzling observations can possibly be understood in the light of accommodation, in which a graft acquires resistance to humoral injury and continues to function well despite the constant presence of low levels of antibodies against the ABO- antigens on the endothelium.

An underlying mechanism that might explain in part the development of graft accommodation as suggested by Park *et al* [53], could be the upregulation of complement regulatory proteins in endothelial cells, by which the initial activation of complement due to antibody binding is blocked at a point later in the cascade. This could explain the persistent presence of C4d without signs of microvascular injury[54], although why this happens frequently in the setting of ABO incompatibility but at most rarely in the setting of HLA-mis-

matched patients is poorly understood at the moment. Still, just as antibody-mediated graft injury cannot be completely accounted for by complement activation[37], complement inhibition alone does not appear to prevent chronic antibody-mediated graft injury. (Cornell LD *et al*. Chronic humoral rejection despite C5 inhibition after positive-crossmatch kidney transplantation [Abstract]. *Am J Transplant* 2010)

In conclusion, the common finding of C4d positivity in ABO incompatible grafts without histologic abnormalities currently forces pathologists to look more closely into the histology when trying to diagnose AMR in an ABO-incompatible graft, as a C4d stain in this group appears to signify something different than 'rejection' and cannot be reliably used as a diagnostic tool.

New fields 1 – C4d and amr in other transplanted solid organs

After the recognition of C4d as a tool to detect AMR in the transplanted kidney, this concept was soon translated to virtually all other transplanted solid organs. The transplanted organs in which the significance of C4d deposition has been most studied are the heart, lung, liver, and pancreas.

C4D IN CARDIAC TRANSPLANTATION Many groups have shown that linear C4d deposition along myocardial capillaries is a reliable specific marker for antibody-mediated cardiac allograft rejection.[16;55;56] Moreover, in line with earlier studies in the kidney it was also shown that C4d positivity in the heart is an independent predictor of cardiac dysfunction and of cardiac mortality.[18;57] The International Society for Heart and Lung Transplantation (ISHLT) recommends that a diagnosis of AMR in a cardiac allograft can be justified when there is clinical evidence of graft dysfunction, histological evidence of acute capillary injury and immunopathologic evidence for C4d capillary positivity on endomyocardial biopsies.

According to the ISHLT, positive histologic features indicative of AMR are necessary to warrant C4d staining.[58] However, a recent publication by Fedrigo *et al* casted doubt upon this approach, and suggested that C4d should instead be performed routinely on endomyocardial graft biopsies: The authors investigated 985 endomyocardial biopsies from 107 heart transplant recipients by staining them immunohistochemically for C4d. Intragraft C4d capillary deposition was present in 34%, but only 7% had AMR based on the ISHLT criteria. Interestingly, C4d positivity, even without the presence of DSA, impaired graft function, or histological features of AMR, was independently associated to a higher mortality risk (unadjusted hazard ratio in patients with positive C4d staining, without DSA or loss of graft function).[18] This study supports the concept of routine C4d staining, as no correlation between histology alone and clinical status could be elicited. In response to the emerging data, the Banff group reached consensus recommending specific time points to monitor DSA as well as C4d staining on every cardiac allograft biopsy, interpreting C4d staining only in myocardial capillaries and scoring as diffuse (▸50% of capillaries), focal(◂50%) or negative, but accepting only diffuse staining as positive.[59]

C4D IN LUNG TRANSPLANTATION Hyperacute and acute AMR episodes are well documented in lung transplantation: In such cases, diffuse alveolar damage, neutrophilic infiltrates and post-transplant pulmonary capillary injury are typical histological findings that are distinct from cellular rejection and less responsive to corticosteroid treatment. C4d deposition in such cases was detected in several studies, mainly in septal capillaries, and was associated to parenchymal injury, clinical status and the presence of DSA or anti-endothelial antibodies.[20;60;61]

However, a consistent anatomical deposition pattern of C4d in the lung was more problematic to identify than in the kidney (peritubular capillaries), the heart (endomyocardial capillaries) or even the pancreas (interacinar capillaries). A study by Wallace *et al* could not describe any specific staining pattern in 68 lung allograft

biopsy specimens using currently available techniques. Focal non-specific staining occurred just as often in cases with suspected AMR compared to more chronic forms of rejection.[62] Probably, the anatomy of the lung complicates pattern recognition, since the frequent occurrence of alveolar hemorrhage and septal damage give rise to non-specific staining patterns, which makes it hard to score pulmonary allograft biopsies.

Compared to kidney transplants, the role of AMR and C4d in chronic pulmonary allograft rejection (bronchiolitis obliterans syndrome, BOS) is heavily debated. Magro *et al* reported on evident C4d deposition in a series of 13 single-lung transplant patients with BOS, who had circulating anti-endothelial antibodies.[63] Westall *et al* investigated septal capillary C4d staining early after lung transplantation. Complement staining was not associated with acute cellular or chronic rejection, or with morphologic features of AMR, but in a sub-group analysis the authors identified 9 cases who developed early bronchiolitis obliterans syndrome (BOS). Interestingly, these cases showed significant lung allograft C3d/C4d deposition along with light-microscopic features suggestive of AMR, suggesting that C4d staining could potentially play a role in the identification of patients at risk of developing a chronic humoral form of pulmonary allograft rejection.[64] Although these results point into the direction of antibody-mediated processes in chronic pulmonary graft rejection, these results should be replicated in larger cohorts to make any definitive statement.

In conclusion, the presence in one patient of both anti-HLA antibodies or anti-endothelial antibodies and pathologic findings suspicious for AMR (including C4d staining) should be seen as strong evidence for AMR of the pulmonary allograft, but there is not enough evidence for C4d as a marker for AMR in the lung graft to perform routine C4d staining on all pulmonary allograft biopsies.

C4D IN PANCREAS TRANSPLANTATION Few studies are available describing the histological and immunohistochemical features of rejection episodes of the pancreas, compared to other transplanted solid organs. The first large cohort appeared in 2009 including 27 pancreas biopsies, showing that C4d deposition in interacinar capillaries (figure 3c) is associated with *de novo* DSA and impaired graft outcome, suggestive of AMR. These results were followed by a study that reported on a correlation between interacinar C4d staining with several serum and urine pancreas rejection markers. A third study discussing the role of AMR in simultaneous pancreas-kidney transplantation was performed in 2010, confirming that presence of C4d was associated with impaired pancreas survival.[17]

In all studies, only C4d staining in interacinar capillaries of the pancreas was demonstrated to correlate with circulating DSA. Coinciding histological parameters included capillaritis, edema, active septal inflammation, acinar inflammation, and acinar cell injury/necrosis. These findings led to the inclusion of C4d staining in the Banff classification for pancreas transplant pathology.[59] However, to date no prospective studies have been performed evaluating the effect of treatment targeted at antibody-mediated injury, or reporting on long-term follow up of C4d positive versus C4d negative pancreas grafts. These will be future challenges. Meanwhile, it is advised to stain all pancreas biopsies for C4d, with diffuse positive staining as indicative of AMR and focal positivity as suspected for AMR.

C4D IN LIVER TRANSPLANTATION In the liver there are several excellent studies available, but results are variable as well as the C4d staining pattern: In different studies, emphasis is being put on sinusoidal staining, portal vein staining, central vein staining and even stromal staining in the portal tract. There seems to be no agreement.[21] And even beyond that, studies have reported significant C4d staining in cases that are not directly related to rejection, such as auto-immune hepatitis, or viral hepatitis. There might be a different role for complement in rejection of the liver, since many complement components are produced in this organ. The endothelium of the liver could thus be more resistant to complement-induced damage.

In fact, this may partly explain the relatively low frequency of liver rejection in general, as well as the possibility of ABO-incompatible transplantation. All in all, in liver transplantation C4d is not a useful diagnostic marker to detect AMR.

New Fields 2 – C4d in native renal disease

The detection of capillary C4d in kidney transplants was the logical consequence of previous studies of the classical complement cascade in normal and diseased native kidneys[65] including also other mammalian kidneys.[66] After the discovery of C4d as a biomarker in transplantation, many studies have sought evidence for C4d deposition in native kidneys, mainly in the setting of autoimmunity.

In native kidney disease, peritubular capillary C4d staining was investigated in many forms of glomerulonephritis [65;67-71] where peritubular capillary C4d staining was virtually never observed. The only exception was lupus nephritis, in which granular peritubular capillary staining has been rarely described, which should be kept in mind when a diagnosis of AMR in a transplanted SLE patient is considered. Recurrence of the original disease should then be ruled out.

Glomerular C4d deposition on the other hand, is a relatively common finding in native diseased kidneys. Zhao *et al* recently investigated which complement pathways were involved in ANCA-associated vasculitis. Interestingly, they detected glomerular C4d only in a small subgroup of patients with ANCA negative pauci-immune GN, whereas in the ANCA positive patients, it was absent.[71] The authors could not identify glomerular deposition of C1q and most C4d positive cases were also MBL positive. This is an example of C4d positivity that does not seem to be linked primarily to classic pathway activation. MBL positivity may instead be associated with exposure of carbohydrate (sugar) moieties in damaged glomeruli or GBM, an infectious pathogenesis, or just a consequence of tissue damage or remodeling. Although interesting from an etiological point of view, it is not likely that C4d will be used as a diagnostic marker in ANCA associated vasculitis in the near future.

In lupus nephritis, glomerular C4d deposition can be detected in the majority of cases with a full-house immunofluorescence pattern, as a result of immune complex deposition (figure 3b).[65;72] In chapter 3 of this thesis biopsies of patients with lupus nephritis with prominent diffuse glomerular C4d staining had detectable glomerular microthrombi significantly more often than biopsies of patients with focal or mild C4d staining.[69] This relation between thrombotic microangiopathy and glomerular C4d has been confirmed in renal biopsies of patients with antiphospholipid syndrome, a similar antibody-mediated autoimmune disease leading to endothelial damage and thrombosis in all vascular beds.[73] Apparently, uncontrolled or abundant complement activation can cause severe damage to the glomerular endothelium to such an extent that a thrombotic microangiopathy can develop. This is in line with the occasionally observed thrombotic microangiopathy in cases of C4d positive acute AMR.[74] Furthermore, this mechanism also plays a role in atypical Hemolytic Uremic Syndrome (aHUS), where a genetic defect in complement regulation causes widespread microthrombosis.[75]. In the setting of thrombotic microangiopathies independent of the underlying disease, performing a C4d stain might help clinicians understand the mechanisms of renal microvascular thrombosis. A positive C4d stain could indicate that complement is involved, and could even guide future treatment, for instance with complement inhibitors. However, this concept needs further basic study, and its clinical utility must await trials of complement inhibitory therapies.

New Fields 3 – C4d in pregnancy: Antibody-mediated pregnancy loss?

The analogy between pregnancy and transplantation was made as early as 1953, when Peter Medawar introduced the concept of 'the fetal allograft'.[76] The conceptus represents a foreign body to the maternal immune system. This 'natural' allograft is usually not

rejected. Failure of placentation, which may be triggered by immune mechanisms, underlies a spectrum of common pregnancy disorders.[77] Defective placentation is known to occur in a substantial proportion of cases of early pregnancy loss, with reduced trophoblast invasion into both the decidua and spiral arteries.[78] As sporadic miscarriages are most often caused by chromosomal anomalies of the fetus, this is generally not regarded as an immune-mediated process. However, in settings of recurrent miscarriage (›3 consecutive miscarriages) this might be different: The more miscarriages a women experiences, the higher the chance of an underlying maternal condition.[79]

In certain autoimmune diseases such as SLE and antiphospholipid syndrome, recurrent early and late miscarriage occur up to 20 times more often than in the normal population, and placental insufficiency leading to preeclampsia and fetal growth restriction are also of increased prevalence.[80;81] In antiphospholipid syndrome, it has been established that pathogenic antibodies bind to trophoblast.[82] The question is: Can these pregnancy losses and other complications be interpreted as 'antibody-mediated'? Chapter 5 of this thesis demonstrated that complicated pregnancies of patients with SLE and antiphospholipid syndrome share several pathophysiological aspects with AMR (figure 4). [83] Interestingly, placental C4d was detectable in the majority of SLE and antiphospholipid syndrome cases (›60%) in a diffuse staining pattern at the fetal-maternal interface (figure 3d), whereas in normal pregnancies C4d was always negative. Excessive placental C4d was related to impaired fetal outcome due to fetal loss or due to prematurity in the setting of preeclampsia. These studies extend previous work showing increased C4d in placentas from patients with antiphospholipid syndrome [84], and argue that C4d is associated with clinical outcomes. Both antiphospholipid antibodies and DSA seem to bind at the interface where cells from the one individual (mother or host) meet the other (fetus or graft), and C4d functions as a footprint for antibody-mediated tissue injury. Chapter 6 further elaborates on this theme by investigating C4d in women with recurrent miscarriage of unknown etiology.

Recurrent miscarriage can be interpreted as a multifactorial disorder in wich pregnancies are repeatedly lost before 13 weeks of gestation. In a subgroup of women extensive deposits of C4d were detected, and more than 80% of these women also had detectable anti-HLA antibodies. The combination of C4d, presence of anti-HLA antibodies and placental insufficiency points to a similar disease mechanism as in humoral rejection of a solid organ.

These results point to a role for complement in disease pathogenesis and possible role for C4d as a biomarker to verify that this pathway is activated in pregnancy complications. Identification of patients with C4d and antibody-mediated pregnancy morbidity, for instance after a late pregnancy loss or following multiple miscarriages, might direct their future therapy.

To take this concept further, a similar mechanism could play a role in other pregnancy-related disorders with a possible immunological background and a clinical course of miscarriages, fetal death or early delivery. Indeed, reports are slowly emerging investigating the role of complement and C4d in preeclampsia and HELLP syndrome[85] and spontaneous early delivery.[86] In a cohort of women with severe preeclampsia with and without underlying SLE/antiphospholipid syndrome (the PROMISSE-study) Salmon *et al* recently showed that not only excessive activation of complement, but also inadequate regulation could contribute to preeclampsia.[85] The authors identified several mutations in complement regulatory genes in a subgroup of preeclamptic women, among which a new mutation involved in the regulation of C4 activation. These striking observations support the concept that C4 activation at the fetal-maternal interface is an essential mediator of both fetal loss and preeclampsia and that insufficient regulation of C4 leads to a more severe phenotype.

Although the diagnostic value of placental C4d has not been tested in a prospective manner, these studies would be helpful to investigate whether a positive placental C4d staining pattern is a risk factor of a future complicated pregnancy.

New fields 4 – Erythrocyte C4d as a possible biomarker for complement mediated disease activity

C4d has been extensively used as a histological biomarker, and is interpreted as a footprint of antibody-mediated tissue injury. However, C4b, the molecule from which C4d is derived, does not discriminate between fixed tissue or circulating cells when it is generated in the process of MBL or classical complement activation, but instead just forms a covalent bond with the nearest free hydrogen-bond available. Thus, C4d may remain behind on whichever surface that C4b first attached to, and can therefore also be found on circulating blood-cells, such as platelets and erythrocytes. A group from Pittsburgh took this as a basis for several studies in which they investigated the value of platelet-bound C4d (P-C4d)[87] and erythrocyte-bound C4d (E-C4d) [88] to monitor disease activity in Systemic Lupus Erythematosus. They measured levels of E-C4d by flow cytometry in 157 patients with SLE, 290 patients with other diseases and 256 healthy individuals and correlated the findings with disease activity scores for SLE (SLEDAI, SELENA, SLAM).[88] In a multivariable analysis, E-C4d was significantly associated with SLAM and SELENA-SLEDAI, after adjustment for C3, C4 and anti-dsDNA antibodies. The authors suggest using E-C4d for monitoring disease activity in lupus.

These findings are convincing and the concept could possibly be translated to other fields. For instance, in transplant settings it would be interesting to investigate if E-C4d levels correlate with C4d deposition in the transplanted graft, and with titers of DSA. Another option would be to explore whether E-C4d could be an alternative marker in cases where complement activity needs to be measured at the tissue level, but taking a biopsy is risky or impossible (for instance in patients on anti-coagulation or in case of a pancreas biopsy).

Finally, if C4d on erythrocytes is a good marker for disease activity in lupus, other cell-rich fluids could theoretically be used for similar measurements. Interestingly, in one study, Miller *et al* have investigated cell-bound C4d in bronchoalveolar lavage fluid to investigate whether this was related to antibody mediated rejection of the lung.[89]

Conclusion – Pros and Cons for C4d as a biomarker in transplantation, autoimmunity and pregnancy

The introduction of C4d as a biomarker into the standard work-up of renal transplant biopsies has provided an enormous amount of insight into the role of antibodies in different forms of allograft-rejection. C4d is now one of the core diagnostic tools to indentify AMR, and is being used in virtually all transplant centers around the world. The vast amount of research into the deposition patterns of C4d in different clinical settings such as in kidney, heart, pancreas and lung transplantation, has taught us that antibodies contribute largely to both acute and chronic rejection episodes.

In analogy with solid organ transplantation, C4d has recently been demonstrated in this thesis in pregnancy, in particular in the setting of 'antiphospholipid antibody-mediated fetal loss' and recurrent miscarriage. This thesis demonstrated that in auto-immune settings C4d might play a role in identifying patients at risk of developing thrombotic complications. More research will nevertheless be needed to discover the full extent of C4d as a biomarker in these new fields.

This final chapter has shown that there are certain drawbacks of using C4d. The difficulties of interpreting focal staining patterns, the relatively low sensitivity of C4d as a marker for AMR in late renal allograft biopsies, and its lack of utility as a marker for antibody-mediated injury in biopsies of ABO-incompatible allografts suggests that C4d has lost some of its magic during the past decade. However, most experts agree that if complement targeted therapies will be part of our future treatment options, a marker like C4d will be needed to identify patients susceptible for those kind of expensive treatments. Taken together, with its unique ability to act as a footprint for antibody-mediated injury, C4d will likely remain to play a prominent role in transplantation, and possibly in pregnancy and autoimmunity as well.

PROS AND CONS FOR C4D AS A BIOMARKER

Pro C4D

C4d staining is relatively inexpensive
C4d staining is easy to perform in basic laboratories
A diffuse staining pattern is relatively easy to interpret
C4d gives very few false positives (it is relatively specific)
C4d shows that the complement system is involved: If complement targeted therapies (Eculizumab) will be part of our future treatment options, a marker like C4d will be needed to identify patients susceptible for those kind of (expensive) treatments.
There are currently no reliable (prospectively tested) alternatives available

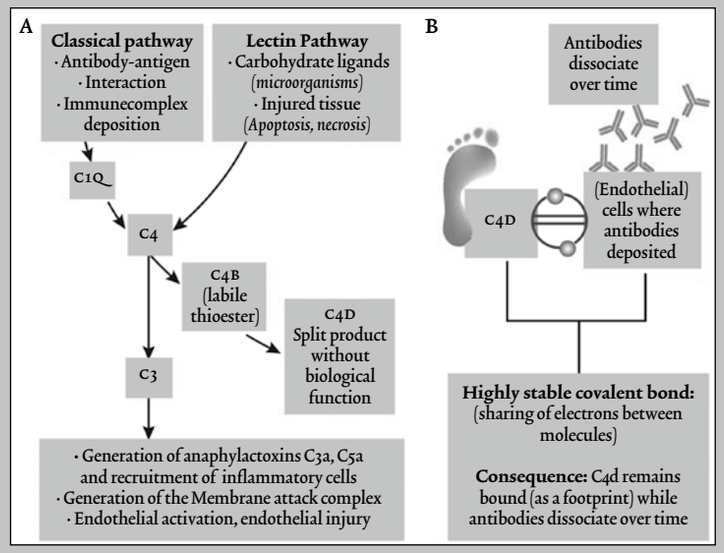
Contra C4D

C4d scoring is subjective and the issue of focal staining and C4d/DSA discrepancies will not be solved
C4d is not sensitive for chronic (or chronic/active) AMR
C4d is not helpful in ABO incompatible grafts
Alternatives for C4d are emerging (genomics, molecular diagnostics and endothelial transcripts) and if proven useful, great effort will be made to transform these techniques or their progeny to practical tests.

WHAT WOULD YOU LIKE TO INVESTIGATE IF YOU WOULD RECEIVE FUNDING TO BE SPENT ON RESEARCH IN THE FIELD OF C4D?

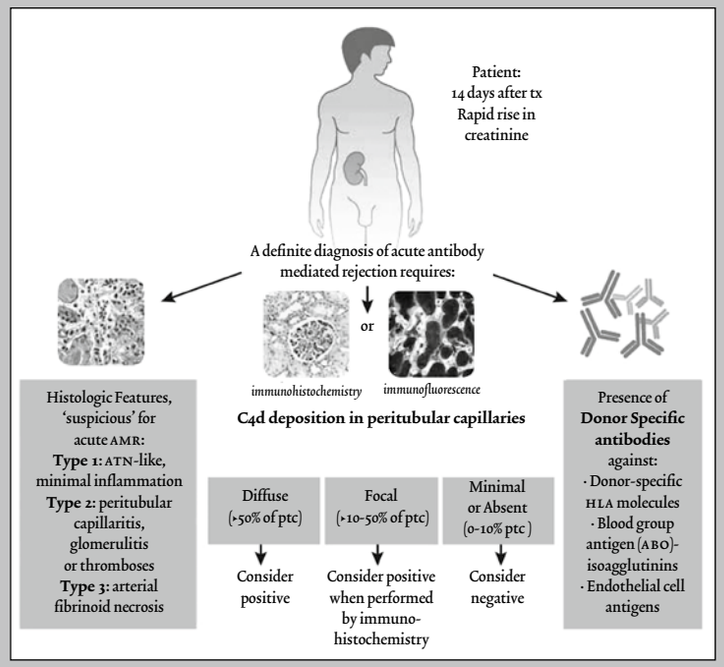
Robert Colvin: "Understand the mechanism of accommodation. There must be a mechanism that we can intervene with. And then I would like to try to find out if it is possible to induce the same endothelial state in the presence of anti-HLA antibodies".
Mohamed Daha: "I want to understand the effect of modulated and injured tissue to complement activation. In addition, I would like to know what local production of factors that control the complement system do, and see if we can influence them to control disease."
Cynthia Drachenberg: "One of the things that I find extremely puzzling is the contrast between acute AMR and chronic AMR. How do endothelial cells cope with this in the chronic setting and why do they behave so differently than in the acute form of AMR?"
Mark Haas: "What would be nice to do is to look at the genomics of ABO incompatible grafts with no histologic signs of rejection, and see how this differs from AMR meeting current Banff criteria, from C4d positivity without histologic findings of rejection in non ABO-incompatible settings, and also what genomic changes have occurred compared to ABO incompatible base-line biopsies.'
Volker Nickeleit: "I want to do a large prospective clinico-pathological study with thousands of patients, protocol biopsies and regular monitoring of anti-HLA antibodies to find out answers to many unanswered questions we struggle with in daily clinical practice.
Banu Sis: "I want to study the molecular phenotypes of early acute AMR in presensitized patients and C4d positive ABO incompatible kidneys and compare molecular mechanisms of acute AMR to chronic AMR. Subtle molecular changes may help us to intervene before irreversible tissue injury takes place."
Ming-hui Zhao: 'Recent advances on complement research provide us a chance to rethink the role and mechanism of complement played in many native kidney diseases. C4d is not always a reflection of classical pathway of complement activation, but can be MBL derived too. I would like to investigate the different roles for classical and MBL pathway-activation further in the setting of autoimmune renal disease'.
Jane Salmon: 'The most elegant approach would be to look at C4d in pregnancy from all possible directions, which would mean to look at the genetics of complement regulatory proteins, to investigate C4d deposition in placental tissues, to look at C4d on erythrocytes, and both complement and complement regulatory protein whole exome sequencing. And all in the context of previous clinical history and well phenotyped patients'

FIG 1 THE COMPLEMENT SYSTEM AND C4D



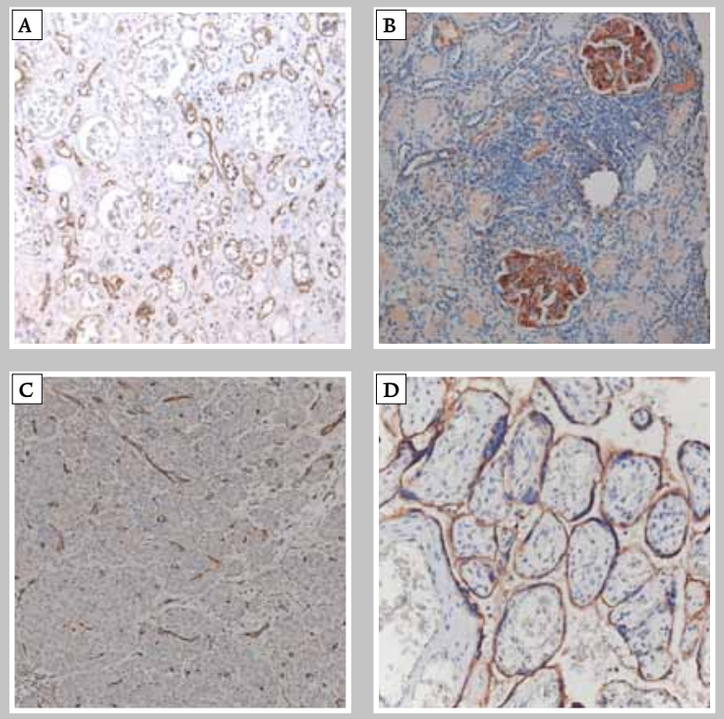
(A) The classical pathway of complement is initiated via binding of its recognition molecule C1q to immune complex deposits, antibody-antigen binding or charged molecules. When C1q becomes activated, it subsequently activates its natural substrate C4. C4d is a split product of C4 activation, without a biological function. Although C4d is mainly interpreted as a trace of classical pathway activation, it must be kept in mind that C4 can also be derived from the lectin pathway. Mannose-binding lectin (MBL) or ficolins binding to carbohydrate ligands on the surface of a wide variety of pathogens results in activation of the lectin pathway and cleavage of C4. Consequently, C4d may be generated without prior antibody binding. Classical complement activation converges with other pathways at the level of C3 and proceeds into the formation of the membrane attack complex on complement-activating surfaces, causing direct tissue injury by perforation of the cell membrane. In addition, potent anaphylatoxins C3a and C5a are being formed in the process, which elicit the recruitment of other inflammatory cells to the site of activation. (B) C4d as a footprint for antibody-mediated tissue injury. C4b, the larger molecule that C4d is derived from, has an internal thioester in the molecule, giving it the ability to form a covalent bond with target cells. When C4d is cleaved from C4b, the covalent bond between C4d and the tissue remains intact. Covalently bound C4d has a higher chance to remain at the site of complement activation than the antibodies themselves, which dissociate over time. C4d is anchored tightly to the tissue and therefore acts as a footprint of antibody-mediated tissue injury.

FIG 2 DIAGNOSING ACUTE ANTIBODY-MEDIATED REJECTION



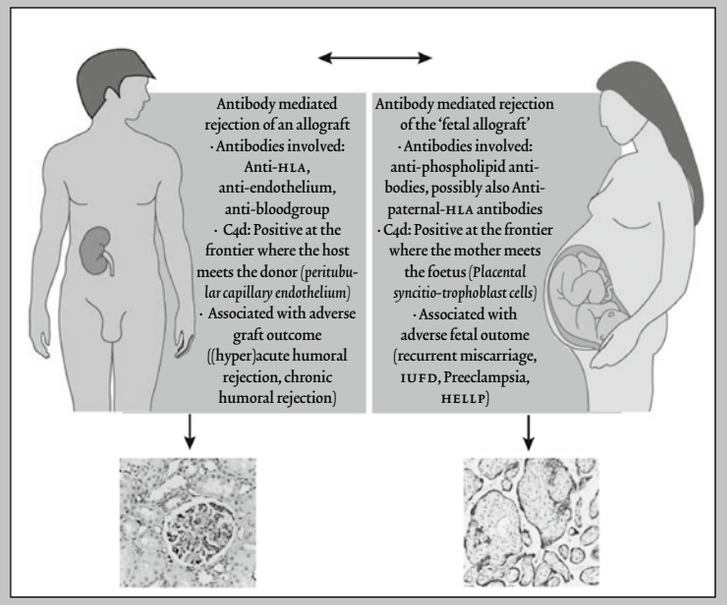
This flowchart shows that the diagnosis of acute antibody-mediated rejection requires the presence of histological features, a positive C4d stain, and the presence of donor-specific antibodies.

FIG 3 C4D STAINING PATTERNS IN DIFFERENT CLINICAL SETTINGS



(A) Acute AMR* of a kidney graft with typical peritubular capillary staining of C4d on paraffin-embedded tissue. (B) Glomerular C4d in a native kidney biopsy of a patient with lupus nephritis and thrombotic microangiopathy. (C) C4d in a pancreas graft with typical staining of C4d in interacinar capillaries, suggestive for AMR. (D) Placental C4d in a placenta from a patient with antiphospholipid syndrome and an intrauterine fetal death in this pregnancy. C4d is positive at the fetal–maternal interface on the maternal side of the syncytiotrophoblast, suggesting severe antibody-mediated injury leading to impaired placental development, impaired nutrient exchange, intrauterine fetal growth restriction, and finally, fetal death. *AMR: antibody-mediated rejection.

FIG 4 ANALOGY BETWEEN AMR AND ANTIBODY-MEDIATED PREGNANCY LOSS



In this scheme the analogy between antibody-mediated rejection of a transplanted graft and 'antibody-mediated pregnancy loss' is schematically shown.

REFERENCES

1 Sarma JV, Ward PA. The complement system. *Cell Tissue Res* 2011; 343(1):227-235.

2 Regele H, Bohmig GA, Habicht A, Gollowitzer D, Schillinger M, Rockenschaub S *et al.* Capillary deposition of complement split product C4d in renal allografts is associated with basement membrane injury in peritubular and glomerular capillaries: a contribution of humoral immunity to chronic allograft rejection. *J Am Soc Nephrol* 2002; 13(9):2371-2380.

3 Nickeleit V, Mihatsch MJ. Kidney transplants, antibodies and rejection: is C4d a magic marker? *Nephrol Dial Transplant* 2003; 18(11):2232-2239.

4 Berger SP, Roos A, Daha MR. Complement and the kidney: what the nephrologist needs to know in 2006? *Nephrol Dial Transplant* 2005; 20(12):2613-2619.

5 Daha MR, van KC, Roos A. Compliments from complement: A fourth pathway of complement activation? *Nephrol Dial Transplant* 2006; 21(12):3374-3376.

6 Williams GM, Hume DM, Hudson RP, Jr., Morris PJ, Kano K, Milgrom F. 'Hyperacute' renal-homograft rejection in man. *N Engl J Med* 1968; 279(12):611-618.

7 Kissmeyer-Nielsen F, Olsen S, Petersen VP, Fjeldborg O. Hyperacute rejection of kidney allografts, associated with pre-existing humoral antibodies against donor cells. *Lancet* 1966; 2(7465):662-665.

8 Porter KA. Morphological aspects of renal homograft rejection. *Br Med Bull* 1965; 21:171-175.

9 Feucht HE, Schneeberger H, Hillebrand G, Burkhardt K, Weiss M, Riethmuller G *et al.* Capillary deposition of C4d complement fragment and early renal graft loss. *Kidney Int* 1993; 43(6):1333-1338.

10 Feucht HE, Felber E, Gokel MJ, Hillebrand G, Nattermann U, Brockmeyer C *et al.* Vascular deposition of complement-split products in kidney allografts with cell-mediated rejection. *Clin Exp Immunol* 1991; 86(3):464-470.

11 Feucht HE, Lederer SR, Kluth B. Humoral alloreactivity in recipients of renal allografts as a risk factor for the development of delayed graft function. *Transplantation* 1998; 65(5):757-758.

12 Kluth-Pepper B, Schneeberger H, Lederer SR, Albert E, Land W, Feucht HE. Impact of humoral alloreactivity on the survival of renal allografts. *Transplant Proc* 1998; 30(5):1772.

13 Collins AB, Schneeberger EE, Pascual MA, Saidman SL, Williams WW, Tolkoff-Rubin N *et al.* Complement activation in acute humoral renal allograft rejection: diagnostic significance of C4d deposits in peritubular capillaries. *J Am Soc Nephrol* 1999; 10(10):2208-2214.

14 Herzenberg AM, Gill JS, Djurdjev O, Magil AB. C4d deposition in acute rejection: an independent long-term prognostic factor. *J Am Soc Nephrol* 2002; 13(1):234-241.

15 Racusen LC, Colvin RB, Solez K, Mihatsch MJ, Halloran PF, Campbell PM *et al.* Antibody-mediated rejection criteria - an addition to the Banff 97 classification of renal allograft rejection. *Am J Transplant* 2003; 3(6):708-714.

16 Crespo-Leiro MG, Veiga-Barreiro A, Domenech N, Paniagua MJ, Pinon P, Gonzalez-Cuesta M *et al.* Humoral heart rejection (severe allograft dysfunction with no signs of cellular rejection or ischemia): incidence, management, and the value of C4d for diagnosis. *Am J Transplant* 2005; 5(10):2560-2564.

17 de Kort H., Munivenkatappa RB, Berger SP, Eikmans M, van der WA, de Koning EJ *et al.* Pancreas allograft biopsies with positive c4d staining and anti-donor antibodies related to worse outcome for patients. *Am J Transplant* 2010; 10(7):1660-1667.

18 Fedrigo M, Gambino A, Tona F, Torregrossa G, Poli F, Benazzi E *et al.* Can C4d immunostaining on endomyocardial biopsies be considered a prognostic biomarker in heart transplant recipients? *Transplantation* 2010; 90(7):791-798.

19 Glanville AR. Antibody-mediated rejection in lung transplantation: myth or reality? *J Heart Lung Transplant* 2010; 29(4):395-400.

20 Magro CM, Pope HA, Klinger D, Orosz C, Adams P, Waldman J *et al.* Use of C4d as a diagnostic adjunct in lung allograft biopsies. *Am J Transplant* 2003; 3(9):1143-1154.

21 Neil DA, Hubscher SG. Current views on rejection pathology in liver transplantation. *Transpl Int* 2010; 23(10):971-983.

22 Lucas JG, Co JP, Nwaogwugwu UT, Dosani I, Sureshkumar KK. Antibody-mediated rejection in kidney transplantation: an update. *Expert Opin Pharmacother* 2011; 12(4):579-592.

23 Sberro-Soussan R, Zuber J, Suberbielle-Boissel C, Candon S, Martinez F, Snanoudj R *et al.* Bortezomib as the sole post-renal transplantation desensitization agent does not decrease donor-specific anti-HLA antibodies. *Am J Transplant* 2010; 10(3):681-686.

24 Wahrmann M, Haidinger M, Kormoczi GF, Weichhart T, Saemann MD, Geyeregger R *et al.* Effect of the proteasome inhibitor bortezomib on humoral immunity in two presensitized renal transplant candidates. *Transplantation* 2010; 89(11):1385-1390.

25 Walsh RC, Everly JJ, Brailey P, Rike AH, Arend LJ, Mogilishetty G *et al.* Proteasome inhibitor-based primary therapy for antibody-mediated renal allograft rejection. *Transplantation* 2010; 89(3):277-284.

26 Mauiyyedi S, Pelle PD, Saidman S, Collins AB, Pascual M, Tolkoff-Rubin NE *et al.* Chronic humoral rejection: identification of antibody-mediated chronic renal allograft rejection by C4d deposits in peritubular capillaries. *J Am Soc Nephrol* 2001; 12(3):574-582.

27 Bohmig GA, Exner M, Habicht A, Schillinger M, Lang U, Kletzmayr J *et al.* Capillary C4d deposition in kidney allografts: a specific marker of alloantibody-dependent graft injury. *J Am Soc Nephrol* 2002; 13(4):1091-1099.

28 Smith RN, Kawai T, Boskovic S, Nadazdin O, Sachs DH, Cosimi AB *et al.* Four stages and lack of stable accommodation in chronic alloantibody-mediated renal allograft rejection in Cynomolgus monkeys. *Am J Transplant* 2008; 8(8):1662-1672.

29 Hourmant M, Cesbron-Gautier A, Terasaki PI, Mizutani K, Moreau A, Meurette A *et al.* Frequency and clinical implications of development of donor-specific and non-donor-specific HLA antibodies after kidney transplantation. *J Am Soc Nephrol* 2005; 16(9):2804-2812.

30 Terasaki PI, Ozawa M. Predictive value of HLA antibodies and serum creatinine in chronic rejection: results of a 2-year prospective trial. *Transplantation* 2005; 80(9):1194-1197.

31 Worthington JE, Martin S, Al-Husseini DM, Dyer PA, Johnson RW. Posttransplantation production of donor HLA-specific antibodies as a predictor of renal transplant outcome. *Transplantation* 2003; 75(7):1034-1040.

32 Sis B, Campbell PM, Mueller T, Hunter C, Cockfield SM, Cruz J *et al.* Transplant glomerulopathy, late antibody-mediated rejection and the ABCD tetrad in kidney allograft biopsies for cause. *Am J Transplant* 2007; 7(7):1743-1752.

33 Regele H, Exner M, Watschinger B, Wenter C, Wahrmann M, Osterreicher C *et al.* Endothelial C4d deposition is associated with inferior kidney allograft outcome independently of cellular rejection. *Nephrol Dial Transplant* 2001; 16(10):2058-2066.

34 Sijpkens YW, Joosten SA, Wong MC, Dekker FW, Benediktsson H, Bajema IM *et al.* Immunologic risk factors and glomerular C4d deposits in chronic transplant glomerulopathy. *Kidney Int* 2004; 65(6):2409-2418.

35 Vongwiwatana A, Gourishankar S, Campbell PM, Solez K, Halloran PF. Peritubular capillary changes and C4d deposits are associated with transplant glomerulopathy but not IgA nephropathy. *Am J Transplant* 2004; 4(1):124-129.

36 Sis B, Halloran PF. Endothelial transcripts uncover a previously unknown phenotype: C4d-negative antibody-mediated rejection. *Curr Opin Organ Transplant* 2010; 15(1):42-48.

37 Sis B, Jhangri GS, Bunnag S, Allanach K, Kaplan B, Halloran PF. Endothelial gene expres-

sion in kidney transplants with alloantibody indicates antibody-mediated damage despite lack of C4d staining. *Am J Transplant* 2009; *9*(10):2312-2323.
38 Loupy A, Suberbielle-Boissel C, Hill GS, Lefaucheur C, Anglicheau D, Zuber J *et al.* Outcome of subclinical antibody-mediated rejection in kidney transplant recipients with preformed donor-specific antibodies. *Am J Transplant* 2009; *9*(11):2561-2570.
39 Loupy A, Hill GS, Suberbielle C, Charron D, Anglicheau D, Zuber J *et al.* Significance of C4d Banff scores in early protocol biopsies of kidney transplant recipients with preformed donor-specific antibodies (DSA). *Am J Transplant* 2011; 11(1):56-65.
40 Haas M, Mirocha J. Early Ultrastructural Changes in Renal Allografts: Correlation With Antibody-Mediated Rejection and Transplant Glomerulopathy. *Am J Transplant* 2011.
41 Zhang X, Reed EF. Effect of antibodies on endothelium. *Am J Transplant* 2009; *9*(11):2459-2465.
42 Lee CY, Lotfi-Emran S, Erdinc M, Murata K, Velidedeoglu E, Fox-Talbot K *et al.* The involvement of FcR mechanisms in antibody-mediated rejection. *Transplantation* 2007; 84(10):1324-1334.
43 Uehara S, Chase CM, Kitchens WH, Rose HS, Colvin RB, Russell PS *et al.* NK cells can trigger allograft vasculopathy: the role of hybrid resistance in solid organ allografts. *J Immunol* 2005; 175(5):3424-3430.
44 Hidalgo LG, Sis B, Sellares J, Campbell PM, Mengel M, Einecke G *et al.* NK cell transcripts and NK cells in kidney biopsies from patients with donor-specific antibodies: evidence for NK cell involvement in antibody-mediated rejection. *Am J Transplant* 2010; 10(8):1812-1822.
45 Montgomery RA, Zachary AA, Racusen LC, Leffell MS, King KE, Burdick J *et al.* Plasmapheresis and intravenous immune globulin provides effective rescue therapy for refractory humoral rejection and allows kidneys to be successfully transplanted into cross-match-positive recipients. *Transplantation* 2000; *70*(6):887-895.
46 Uchida K, Tominaga Y, Haba T, Katayama A, Ichimori T, Yamada K *et al.* ABO-incompatible renal transplantation--dissociation of ABO antibodies. *Transplant Proc* 1998; 30(5):2302-2303.
47 Fidler ME, Gloor JM, Lager DJ, Larson TS, Griffin MD, Textor SC *et al.* Histologic findings of antibody-mediated rejection in ABO blood-group-incompatible living-donor kidney transplantation. *Am J Transplant* 2004; *4*(1):101-107.
48 Haas M, Ratner LE, Montgomery RA. C4d staining of perioperative renal transplant biopsies. *Transplantation* 2002; *74*(5):711-717.
49 Kanetsuna Y, Yamaguchi Y, Horita S, Tanabe K, Toma H. C4d and/or immunoglobulins deposition in peritubular capillaries in perioperative graft biopsies in ABO-incompatible renal transplantation. *Clin Transplant* 2004; 18 Suppl 11:13-17.
50 Toki D, Ishida H, Setoguchi K, Shimizu T, Omoto K, Shirakawa H *et al.* Acute antibody-mediated rejection in living ABO-incompatible kidney transplantation: long-term impact and risk factors. *Am J Transplant* 2009; *9*(3):567-577.
51 Haas M, Rahman MH, Racusen LC, Kraus ES, Bagnasco SM, Segev DL *et al.* C4d and C3d staining in biopsies of ABO- and HLA-incompatible renal allografts: correlation with histologic findings. *Am J Transplant* 2006; *6*(8):1829-1840.
52 Haas M, Segev DL, Racusen LC, Bagnasco SM, Locke JE, Warren DS *et al.* C4d deposition without rejection correlates with reduced early scarring in ABO-incompatible renal allografts. *J Am Soc Nephrol* 2009; 20(1):197-204.
53 Park WD, Grande JP, Ninova D, Nath KA, Platt JL, Gloor JM *et al.* Accommodation in ABO-incompatible kidney allografts, a novel mechanism of self-protection against antibody-mediated injury. *Am J Transplant* 2003; 3(8):952-960.
54 Haas M. The significance of C4d staining with minimal histologic abnormalities. *Curr Opin Organ Transplant* 2010; 15(1):21-27.
55 Hammond EH, Yowell RL, Nunoda S, Menlove RL, Renlund DG, Bristow MR *et al.* Vascular (humoral) rejection in heart transplantation: pathologic observations and clinical implications. *J Heart Transplant* 1989; 8(6):430-443.
56 Lones MA, Czer LS, Trento A, Harasty D, Miller JM, Fishbein MC. Clinical-pathologic features of humoral rejection in cardiac allografts: a study in 81 consecutive patients. *J Heart Lung Transplant* 1995; 14(1 Pt 1):151-162.
57 Fedson SE, Daniel SS, Husain AN. Immunohistochemistry staining of C4d to diagnose antibody-mediated rejection in cardiac transplantation. *J Heart Lung Transplant* 2008; 27(4):372-379.
58 Stewart S, Winters GL, Fishbein MC, Tazelaar HD, Kobashigawa J, Abrams J *et al.* Revision of the 1990 working formulation for the standardization of nomenclature in the diagnosis of heart rejection. *J Heart Lung Transplant* 2005; 24(11):1710-1720.
59 Sis B, Mengel M, Haas M, Colvin RB, Halloran PF, Racusen LC *et al.* Banff '09 meeting report: antibody mediated graft deterioration and implementation of Banff working groups. *Am J Transplant* 2010; 10(3):464-471.
60 Ionescu DN, Girnita AL, Zeevi A, Duquesnoy R, Pilewski J, Johnson B *et al.* C4d deposition in lung allografts is associated with circulating anti-HLA alloantibody. *Transpl Immunol* 2005; 15(1):63-68.
61 Magro CM, Deng A, Pope-Harman A, Waldman WJ, Bernard CA, Adams PW *et al.* Humorally mediated posttransplantation septal capillary injury syndrome as a common form of pulmonary allograft rejection: a hypothesis. *Transplantation* 2002; *74*(9):1273-1280.
62 Wallace WD, Reed EF, Ross D, Lassman CR, Fishbein MC. C4d staining of pulmonary allograft biopsies: an immunoperoxidase study. *J Heart Lung Transplant* 2005; 24(10):1565-1570.
63 Magro CM, Ross P, Jr., Kelsey M, Waldman WJ, Pope-Harman A. Association of humoral immunity and bronchiolitis obliterans syndrome. *Am J Transplant* 2003; 3(9):1155-1166.
64 Westall GP, Snell GI, McLean C, Kotsimbos T, Williams T, Magro C. C3d and C4d deposition early after lung transplantation. *J Heart Lung Transplant* 2008; 27(7):722-728.
65 Zwirner J, Felber E, Herzog V, Riethmuller G, Feucht HE. Classical pathway of complement activation in normal and diseased human glomeruli. *Kidney Int* 1989; 36(6):1069-1077.
66 Zwirner J, Felber E, Burger R, Bitter-Suermann D, Riethmuller G, Feucht HE. Classical pathway of complement activation in mammalian kidneys. *Immunology* 1993; 80(2):162-167.
67 Chen M, Xing GQ, Yu F, Liu G, Zhao MH. Complement deposition in renal histopathology of patients with ANCA-associated pauci-immune glomerulonephritis. *Nephrol Dial Transplant* 2009; 24(4):1247-1252.
68 Chen M, Daha MR, Kallenberg CG. The complement system in systemic autoimmune disease. *J Autoimmun* 2010; 34(3):J276-J286.
69 Cohen D, Koopmans M, Kremer H, I, Berger SP, Roos van GM, Steup-Beekman GM *et al.* Potential for glomerular C4d as an indicator of thrombotic microangiopathy in lupus nephritis. *Arthritis Rheum* 2008; 58(8):2460-2469.
70 Kusunoki Y, Itami N, Tochimaru H, Takekoshi Y, Nagasawa S, Yoshiki T. Glomerular deposition of C4 cleavage fragment (C4d) and C4-binding protein in idiopathic membranous glomerulonephritis. *Nephron* 1989; 51(1):17-19.
71 Xing GQ, Chen M, Liu G, Zheng X, E J, Zhao MH. Differential deposition of C4d and MBL in glomeruli of patients with ANCA-negative pauci-immune crescentic glomerulonephritis. *J Clin Immunol* 2010; 30(1):144-156.
72 Kim SH, Jeong HJ. Glomerular C4d deposition indicates in situ classic complement pathway activation, but is not a marker for lupus nephritis activity. *Yonsei Med J* 2003; 44(1):75-80.
73 Shen Y, Chen XW, Sun CY, Dai M, Yan YC, Yang CD. Association between anti-beta2 glycoprotein I antibodies and renal glomerular C4d deposition in lupus nephritis patients with glomerular microthrombosis: a prospective

study of 155 cases. *Lupus* 2010; 19(10):1195-1203.
74 Noris M, Remuzzi G. Thrombotic microangiopathy after kidney transplantation. *Am J Transplant* 2010; 10(7):1517-1523.
75 Noris M, Remuzzi G. Atypical hemolytic-uremic syndrome. N *Engl J Med* 2009; 361(17):1676-1687.
76 Medawar PB. Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates. Symp.Soc.Exp.Biol. [7], 320-338. 1953.
77 Hiby SE, Apps R, Sharkey AM, Farrell LE, Gardner L, Mulder A *et al.* Maternal activating KIRs protect against human reproductive failure mediated by fetal HLA-C2. *J Clin Invest* 2010; 120(11):4102-4110.
78 Burton GJ, Jauniaux E. Placental oxidative stress: from miscarriage to preeclampsia. *J Soc Gynecol Investig* 2004; 11(6):342-352.
79 Branch DW, Gibson M, Silver RM. Clinical practice. Recurrent miscarriage. N *Engl J Med* 2010; 363(18):1740-1747.
80 Ruiz-Irastorza G, Crowther M, Branch W, Khamashta MA. Antiphospholipid syndrome. *Lancet* 2010; 376(9751):1498-1509.
81 Cohen D, Berger SP, Steup-Beekman GM, Bloemenkamp KW, Bajema IM. Diagnosis and management of the antiphospholipid syndrome. *BMJ* 2010; 340:c2541.
82 Rote NS, Vogt E, DeVere G, Obringer AR, Ng AK. The role of placental trophoblast in the pathophysiology of the antiphospholipid antibody syndrome. *Am J Reprod Immunol* 1998; 39(2):125-136.
83 Cohen D, Buurma A, Goemaere NN, Girardi G, le CS, Scherjon S *et al.* Classical complement activation as a footprint for murine and human antiphospholipid antibody-induced fetal loss. *J Pathol* 2011.
84 Shamonki JM, Salmon JE, Hyjek E, Baergen RN. Excessive complement activation is associated with placental injury in patients with antiphospholipid antibodies. *Am J Obstet Gynecol* 2007; 196(2):167-5.
85 Salmon JE, Heuser C, Triebwasser M, Liszewski MK, Kavanagh D, Roumenina L *et al.* Mutations in complement regulatory proteins predispose to preeclampsia: a genetic analysis of the PROMISSE cohort. *PLoS Med* 2011; 8(3):e1001013.
86 Lee J, Romero R, Xu Y, Kim JS, Topping V, Yoo W *et al.* A signature of maternal anti-fetal rejection in spontaneous preterm birth: chronic chorioamnionitis, anti-human leukocyte antigen antibodies, and C4d. *PLoS One* 2011; 6(2):e16806.
87 Navratil JS, Manzi S, Kao AH, Krishnaswami S, Liu CC, Ruffing MJ *et al.* Platelet C4d is highly specific for systemic lupus erythematosus. *Arthritis Rheum* 2006; 54(2):670-674.
88 Kao AH, Navratil JS, Ruffing MJ, Liu CC, Hawkins D, McKinnon KM *et al.* Erythrocyte C3d and C4d for monitoring disease activity in systemic lupus erythematosus. *Arthritis Rheum* 2010; 62(3):837-844.
89 Miller GG, Destarac L, Zeevi A, Girnita A, McCurry K, Iacono A *et al.* Acute humoral rejection of human lung allografts and elevation of C4d in bronchoalveolar lavage fluid. *Am J Transplant* 2004; 4(8):1323-1330.

IX

NEDERLANDSE SAMENVATTING

Het complementsysteem

In het humane immuunsysteem neemt complement een bijzondere plaats in. Dit systeem werkt samen met antistoffen en fagocyten bij de afweer tegen micro-organismen en is waarschijnlijk evolutionair een van de oudste verdedigingssystemen in de biologie. In tegenstelling tot lymfocyten, hoeft complement niet te 'leren' hoe om te gaan met micro-organismen; het werkt direct vanaf de geboorte.

Normaal gesproken is het complementsysteem in een niet-geactiveerde staat aanwezig als een aantal verschillende plasma-eiwitten in het bloed. De verschillende complementeiwitten worden gecodeerd door letters en cijfers, zoals C1, C3 of C4, afhankelijk van hun plaats in het systeem. Activatie, en daarmee het ontstaan van een kettingreactie aan acties en interacties tussen deze eiwitten, kan plaatsvinden na binding van complementeiwitten aan antilichamen en immuuncomplexen. Dit wordt de klassieke route genoemd. Het complementsysteem kan ook rechtstreeks geactiveerd worden door directe interactie tussen complement eiwitten met het celoppervlak van pathogenen (de zogenaamde 'Mannose Binding Lectin' of MBL route). Uiteindelijk leidt deze kettingreactie tot de vorming van anaphylatoxinen, stoffen die fagocyterende cellen aantrekken, en de vorming van het zogenaamde 'membrane attack complex', een ingenieus complex van eiwitten dat binnen enkele seconden tot destructie van de celwand van de 'target-cell' kan leiden.

Als verdedigingsmechanisme is het complementsysteem uiterst effectief en onmisbaar. Echter, wanneer het immuunsysteem zich tegen het eigen organisme keert en auto-antistoffen gevormd worden, speelt het complementsysteem een fundamentele rol bij het ontstaan van ongewenste weefselschade. Zowel de fysiologie als de rol van complement in auto- en alloimmuniteit worden in meer detail besproken in de algemene inleiding van dit proefschrift (**hoofdstuk 2**).

C4d

C4d is een molecuul dat als afsplitsproduct overblijft na activatie van C4, een eiwit dat een belangrijke rol speelt in de klassieke route van het complementsysteem. Hoewel C4d voor zover bekend geen biologische functie heeft, beschikt het over een belangrijke eigenschap: Het molecuul C4d vormt een covalente verbinding (een zeer stabiele verbinding tussen twee moleculen waarbij electronen gedeeld worden) met celoppervlakken of celmembranen. Hierdoor blijft C4d langdurig gebonden –veel langer dan antilichamen zelf– en kan door immuunhistochemische technieken gemakkelijk in beeld gebracht worden. Onder de microscoop kan men doordat C4d als een soort spoor op de plaats delict achterblijft, 'zien' waar in het weefsel antistoffen complement hebben geactiveerd en schade hebben veroorzaakt. Het onderzoeken van de aanwezigheid van C4d in weefsel geeft daardoor in de eerste plaats inzicht in ziektemechanismen, maar kan ook als diagnosticum gebruikt worden. Als diagnostische test wordt C4d sinds de jaren '90 gebruikt om antilichaam gemedieerde afstoting van getransplanteerde organen aan te tonen.

In de algemene inleiding (**hoofdstuk 2**) wordt C4d geïntroduceerd aan de lezer. In de algemene discussie van het proefschrift (**hoofdstuk 8**) wordt de geschiedenis, de huidige stand van zaken en toekomst van C4d als biomarker verder uitgewerkt.

Dit proefschrift

In dit proefschrift wordt de rol van C4d uitgediept in andere klinische settings dan orgaantransplantatie - waar het tot nu toe onderzocht en gebruikt werd. Waar C4d in getransplanteerde organen een indicator is van schade door alloantistoffen (eigen antistoffen gericht tegen vreemd weefsel) berust dit proefschrift op de hypothese dat in ziekten waar autoantistoffen geproduceerd worden (eigen antistoffen gericht tegen eigen weefsel) C4d eveneens van betekenis zou kunnen zijn.

C4d in SLE en antifosfolipiden syndroom

DEEL 1: C4D EN THROMBOTISCHE COMPLICATIES De studies in dit proefschrift zijn daarom voornamelijk verricht in patiënten met Systemische Lupus Erythematosus (SLE) en antifosfolipiden syndroom, twee autoimmuunziekten waar autoantistoffen een cruciale rol spelen. De antistoffen die deze patiënten aanmaken, circuleren in het bloed en komen op allerlei plekken in het lichaam terecht. Daar waar ze binden of neerslaan, kan –onder andere via activatie van het complement systeem- schade ontstaan. Patiënten met deze ziekten hebben derhalve symptomen in alle orgaansystemen, waarvan de nieren, de huid, en waarschijnlijk ook de hersenen frequent aangedaan raken. De achtergrond, kliniek en epidemiologie van SLE en antifosfolipiden syndroom komen respectievelijk aan de orde in de algemene inleiding (**hoofdstuk 2**) en in een overzichtsartikel over antifosfolipiden syndroom (**hoofdstuk 3**).

In de daarop volgende hoofdstukken wordt de rol van C4d in de nieren (**hoofdstuk 4**) en hersenen (**hoofdstuk 5**) van patiënten met SLE en antifosfolipiden syndroom onderzocht. Het blijkt dat in beide organen de aanwezigheid van C4d sterk geassocieerd is met trombose in de kleine bloedvaten (microtrombotische schade). Wanneer C4d aanwezig is in glomeruli in de nier, is er een sterkere kans ook glomerulaire microthrombi aan te treffen. Een C4d-kleuring op een nierbiopt van een patiënt met SLE of antifosfolipiden syndroom zou in de toekomst mogelijk consequenties kunnen hebben voor de risico-inschatting van patiënten op het ontwikkelen van thrombotische microangiopathie, en zelfs op eventuele behandeling met antistolling en/of complementremmers.

In de hersenen van patiënten met SLE en antifosfolipiden syndroom blijkt C4d sterk aan te kleuren in arteriolen en venulen in de cortex. Deze vaatjes hebben vaak een verdikte wand, zonder de aanwezigheid van lymfocyten, wat een inflammatoir proces suggereert. Bij patiënten met veel C4d depositie, zijn vaker microthrombi te detecteren in vergelijkbare kleine vaatjes, en tevens zijn er vaker

aanwijzingen voor micro-infarcten en ischemische schade. Tot nu toe was het onbekend hoe auto-antistoffen in het brein van SLE patiënten zouden kunnen leiden tot deze vorm van thrombo-ischemische schade. **Hoofdstuk 5** geeft een mogelijke verklaring hiervoor, namelijk complement activatie op het cerebrale endotheel met vaatwandveranderingen en microthrombose als gevolg.

DEEL 2: C4D EN ZWANGERSCHAP In het volgende deel van het proefschrift ligt de nadruk op zwangerschap en herhaalde miskramen. Niet alleen omdat bekend is dat patiënten met SLE en antifosfolipiden syndroom gecompliceerde zwangerschappen doormaken, maar ook omdat de zwangerschap in het algemeen sommige immunologische overeenkomsten vertoont met transplantatie, waar C4d immers voor het eerst beschreven werd. Wanneer een zwangerschap resulteert in een vroege of latere miskraam, zou dit geïnterpreteerd kunnen worden als een vorm van 'afstoting' van de lichaamsvreemde foetus. Deze gedachtengang was het uitgangspunt om de rol van C4d te onderzoeken in placenta's van patiënten met SLE en antifosfolipiden syndroom (**hoofdstuk *6***) en in patiënten met herhaalde miskramen zonder duidelijke oorzaak (**hoofdstuk *7***).

De placenta is een orgaan dat ontstaat tijdens de zwangerschap en een cruciale rol speelt bij het in stand houden van de zwangerschap, het fasciliteren van groei van de foetus door voedingsstoffen naar de circulatie van het kind te transporteren (en afvalstoffen af te voeren) en het verschaffen van immunologische tolerantie van de foetus. De placentaire vlokken zijn bekleed door syncytiotrophoblast, de cellaag die het grensvlak vormt waarop het maternale bloed in contact komt met foetaal weefsel.

In **hoofdstuk *6*** werd duidelijk dat C4d in sommige placenta's van patiënten met SLE en antiphopsholipiden syndroom veelvuldig aanwezig was op het foetomaternale grensvlak . In sommige placenta's bleef geen enkele vlok vrij van C4d. De zwangerschappen waarin dit fenomeen werd geobserveerd liepen veelal niet goed af. De aanwezigheid van C4d was sterk geassocieerd met intrauterine vruchtdood in een groep patiënten met SLE en antifosfolipiden syndroom. In placenta's van patienten met een normale zwangerschap, of patienten die een intrauterine vruchtdood doormaakten ten gevolge van aangeboren afwijkingen waarbij waarschijnlijk geen afstoting optrad, kwam C4d vrijwel nooit voor in de placenta. **Hoofdstuk *6*** laat zien dat het waarschijnlijk is dat C4d in de placenta een uiting is van een sterke antilichaam-gemedieerde immuunrespons. Anti–fosfolipiden antistoffen zijn een zeer waarschijnlijke kandidaat, omdat bekend is dat deze een sterke affiniteit hebben voor trophoblast. Andere antistoffen die bij SLE patiënten aanwezig zijn zouden echter evengoed de veroorzaker kunnen zijn. Ook is het voorstelbaar dat inflammatie op het foetomaternale grensvlak leidt tot een dysfunctionele placenta, met slechte zwangerschapsuitkomsten tot gevolg. Toekomstig onderzoek zal uit moeten wijzen of C4d in een placenta van een patiënt met SLE of antiphospholipiden syndroom (therapeutische) consequenties heeft voor een volgende zwangerschap.

De bevindingen van **hoofdstuk *6*** gaven aanleiding om in **hoofdstuk *7*** in een populatie van patiënten met herhaalde miskramen zonder duidelijke oorzaak te inventariseren of een vergelijkbaar mechanisme hier eveneens een rol zou kunnen spelen. Hier bleken sterke aanwijzingen voor te bestaan – in een subgroep van patiënten met herhaalde miskramen was er sprake van sterk C4d positief miskraammateriaal. Omdat bij deze patiënten antifosfolipiden antistoffen uitgesloten waren, werd in serum gezocht naar de aanwezigheid van anti-HLA antistoffen. Deze bleken in een aantal vrouwen met C4d positief miskraammateriaal aanwezig te zijn. Deze combinatie doet erg denken aan 'antilichaam gemedieerde afstoting' bij transplantatie, waar eveneens een combinatie van anti-donor antistoffen en C4d in het donororgaan pathognomonisch is voor deze vorm van rejectie. **Hoofdstuk *7*** biedt voornamelijk een nieuw pathofysiologisch inzicht in een populatie waar zeer vaak geen oorzaak wordt gevonden voor het optreden van herhaalde miskramen.

Discussie en conclusie

In de algemene discussie van het proefschrift (**Hoofdstuk 8**) wordt allereerst de rol van C4d in een historisch kader geplaatst door middel van interviews met verschillende experts die een belangrijke bijdrage hebben geleverd aan het veld. Ontwikkelingen vanaf de eerste studies naar deze marker in de jaren '90 in getransplanteerde nieren, tot de voorzichtige stappen met C4d in nieuwe gebieden zoals autoimmuunziekten en zwangerschap zoals beschreven in dit proefschrift, komen aan de orde. Wellicht zijn nieuwe, sensitievere methoden denkbaar om antilichaam gemedieerde rejectie aan te tonen. Deze komen in dit hoofdstuk aan de orde, en zijn voor sommigen reden om aan een toekomst voor C4d als biomarker te twijfelen. Aan de andere kant opent de komst van verschillende complement-remmende medicijnen (eculizumab) nieuwe deuren voor C4d als marker: Zouden hiermee patiënten geïdentificeerd kunnen worden die baat zouden kunnen hebben bij deze (zeer kostbare) middelen?

De bevindingen in dit proefschrift laten zien dat ook buiten de transplantatiewereld C4d van nut kan zijn bij het ontrafelen van antilichaam-gemedieerde ziektemechanismen, en uiteindelijk wellicht ook gebruikt kan worden als voorspellende biomarker voor trombotische en obstetrische complicaties. Prospectieve studies zullen nodig zijn om de haalbaarheid hiervan in de dagelijkse klinische praktijk te toetsen.

CURRICULUM VITAE

Danielle Cohen werd op 1 juli 1983 geboren te Lambeth, Londen. In 2001 behaalde zij haar eindexamen gymnasium en het International Baccalaureate English A1 aan het Rijnlands Lyceum Oegstgeest. Datzelfde jaar slaagde zij voor het toelatingsexamen van de Academia de Belle Arti in Bologna, Italië, waar ze het eerste jaar schilderkunst en kunstgeschiedenis studeerde. Zij ving in 2002 aan met een studie Lucht en Ruimtevaart Techniek aan de Technische Universiteit te Delft, maar verruilde deze in 2003 voor een plek aan de geneeskunde faculteit van het Leids Universitair Medisch Centrum (LUMC).

Tijdens haar studie geneeskunde werd zij toegelaten tot het excellente studenten traject, en startte als onderzoeker aan de afdeling Pathologie van het LUMC (afdelingshoofd: prof. Dr. G.J. Fleuren). Bij de onderzoeksgroep van de nefro- en immunopathologie onder begeleiding van prof. dr. Jan Anthonie Bruijn en dr. Ingeborg Bajema werd de basis gelegd voor het huidige proefschrift. Tijdens de co-schappen maakte het onderzoek een wending richting de obstetrie, en werd dr. Kitty Bloemenkamp de begeleider van deze onderzoekstak. Zij ontving een beurs in het kader van het MD/PhD traject waarmee vanaf september 2009 twee jaar voltijd onderzoek verricht kon worden. In deze periode was zij tevens als een van de twee podcast-redacteuren werkzaam bij het Nederlands Tijdschrift voor Geneeskunde (hoofdredacteur: prof. dr. Peter de Leeuw).

Na twee jaar onderzoek begon in september 2011 een nieuwe fase, met een arts-assistentschap gynaecologie in het Bronovo Ziekenhuis te Den Haag (opleider: dr. C.Holleboom).

Danielle Cohen was born on the 1st of July 1983 in Lambeth, London. In 2001 she graduated from the Rijnlands Lyceum in Oegstgeest and received the International Baccalaureat English A1 certificate. The same year she was admitted to the Accademia de Belle Arti in Bologna, Italy, where she studied painting and history of art. In 2002 she started with Aerospace Engineering at the Technical University of Delft, but switched to medical school at Leiden University the year after. During her medical study she was awarded a research scholarship within the Excellent Student Program of the Leiden University Medical Center (lumc) and she started as a junior research student at the department of Pathology, lumc (head: Prof. Dr. G.J.Fleuren). Within the nefro- and immunopathology research group under the guidance of Prof. Dr. Jan Anthonie Bruijn and Dr. Ingeborg Bajema the foundation of the current thesis was built. During her clinical rotations a research collaboration was formed with Dr. Kitty Bloemenkamp of the department of obstetrics, lumc. She received a scholarship within the MD/PhD Program of the lumc with which she started as a full time PhD student at the department of Pathology in September 2009. During this time she also worked as Podcast-editor at the Dutch Journal of Medicine (editor: Prof. Dr. Peter de Leeuw).

After two years of research she entered a new phase as a clinical resident at the department of obstetrics and gynecology at the Bronovo Hospital (mentor: Dr. C. Holleboom) in The Hague.

BIBLIOGRAPHY

Cohen D, Colvin RB, Daha M, Drachenberg C, Haas M, Nickeleit V, Salmon JE, Sis B, Zhao MH, Bruiijn JA, Bajema IM. Pros and Cons for c4d as a biomarker. Kidney Int. 2012 Apr; 81(7):628-39 doi: 10.1038/ki.2011.497

Cohen D, Buurma AAJ, Goemaere NN, Girardi G, le Cessie S, Scherjon S, Bloemenkamp KWM, de Heer E, Bruijn JA, Bajema IM. Classical complement activation as a footprint for murine and human antiphospholipid antibody induced fetal loss. J Pathol. 2011 doi: 10.1002/path.2893

Visser J, **Cohen D**, Bloemenkamp KW. Antithrombotic medications and recurrent miscarriage. N Engl J Med 2010; 363:887-888 (correspondence)

Cohen D, Berger SP, Steup-Beekman GM, Bloemenkamp KWM, Bajema IM. Diagnosis and management of the antiphospholipid syndrome. BMJ 2010; 340:c2541

Cohen D, Koopmans M, Kremer H, I, Berger SP, van Groningen MR, Steup-Beekman GM, de Heer E, Bruijn JA, Bajema IM. Potential for glomerular c4d as an indicator of thrombotic microangiopathy in lupus nephritis. Arthritis Rheum 2008; 58(8):2460-2469

Meuleman T, **Cohen D**. Zwangeren met een verhoogd risico op preeclampsie hebben baat bij heparine en aspirine. Ned Tijdschr Geneeskd 2012;156:A4596

Samuels S, **Cohen D** Risico op 'borderline'-ovariumtumoren na ivf-behandeling verhoogd. Ned Tijdschr Geneeskd 2012;156: A4463

Cohen D, Paridaans N. Oxytocine-infuus tegen bloedverlies na een keizersnede: nuttig voor onervaren operateur. Ned Tijdschr Geneeskd 2011;155:A4008

Kesteren F, **Cohen D**. Plaatsen van een spiraal na curettage: wachten is niet nodig, Ned Tijdschr Geneeskd. 2011;155:A3804

Buurma A, **Cohen D**. Ovariectomie of ovaria in situ laten bij hysterectomie? Ned Tijdschr Geneeskd 2011;155:A3729

Penning M, **Cohen D**. Moment van Epiduraal-anesthesie heeft geen invloed op partus. Ned Tijdschr Geneeskd. 2011;155:A3537

Paridaans N, **Cohen D**. Uterusextirpatie meest kosten-effectieve behandeling voor menorragie. Ned Tijdschr Geneeskd. 2011;155:A3673

Cohen D, Paridaans N. Behandeling menorragie blijft maatwerk. Ned Tijdschr Geneeskd. 2010;154:A2975

Cohen D, Visser J. Acetylsalicylzuur en heparine niet effectief bij recidiverende miskraam. Ned Tijdschr Geneeskd. 2010;154:A2289

Paridaans N, **Cohen D**. Electieve sectio caesarea vaak te vroeg uitgevoerd. Ned Tijdschr Geneeskd 2010;154:A2208

Cohen D, Paridaans N. Intra-uteriene druk-kathetersbij ingeleide bevalling onnodig. Ned Tijdschr Geneeskd 2010;154:A1873

"ALL YOU DO IS TO LOOK
AT A PAGE IN THIS BOOK
BECAUSE THAT'S WHERE WE ALWAYS WILL BE.
NO BOOK EVER ENDS
WHEN IT'S FULL OF YOUR FRIENDS
THE GIRAFFE AND THE PELLY AND ME."

Roald Dahl, *The Giraffe, the Pelly and me*