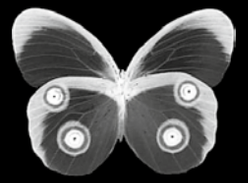
V

NATIONWIDE AUTOPSY STUDY LINKS COMPLEMENT TO THROMBO-ISCHEMIA IN NEUROLUPUS



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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

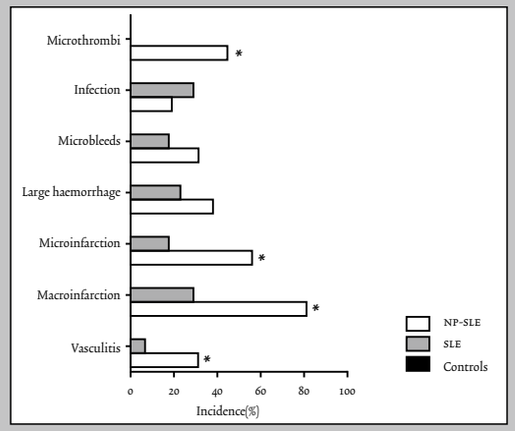


Figure 1 shows the presence of different histological parameters in brain tissue of patients with SLE, neuropsychiatric SLE (NP-SLE) and controls. Microthrombi, microinfarction, macroinfarction and vasculitis were present statistically significantly more often in NP-SLE compared to SLE. None of these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

FIG 2 VASCULOPATHY IN NP-SLE, SLE AND CONTROLS

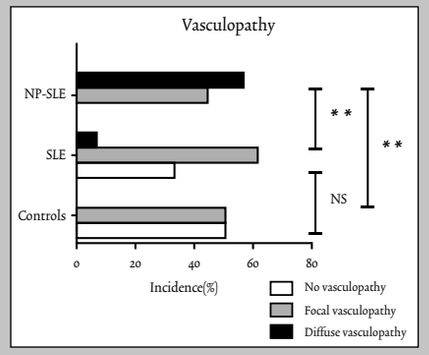


Figure 2 shows the results of semi-quantitative scoring of vasculopathy in brain tissue of patients with SLE, neuropsychiatric SLE (NP-SLE) and controls. Vasculopathy was defined as endothelial cell proliferation, thickening of the vessel wall and narrowing of the capillary lumen, and was scored semiquantitatively 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

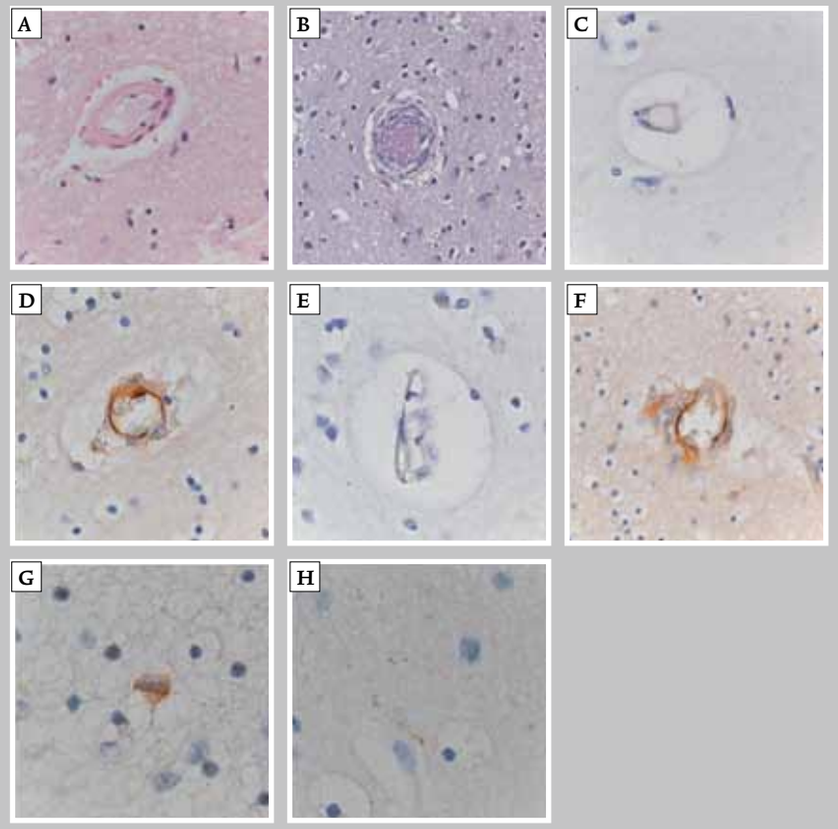


Figure 3A-H shows different histopathological findings and immunohistochemical staining patterns (Magnification of all images: 40x). Figure 3A gives a typical example of vasculopathy with thickening of the vessel wall without an inflammatory infiltrate. Figure 3B shows a typical example of a cerebral 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

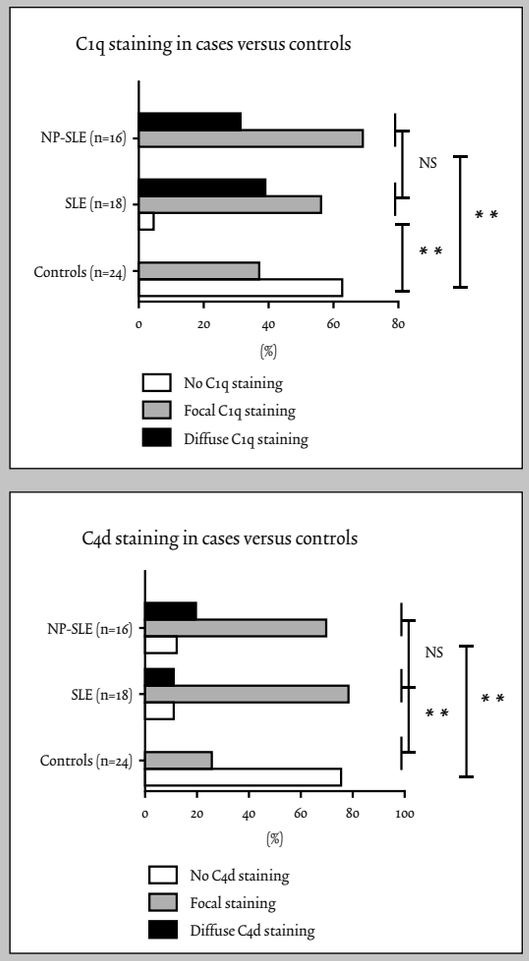
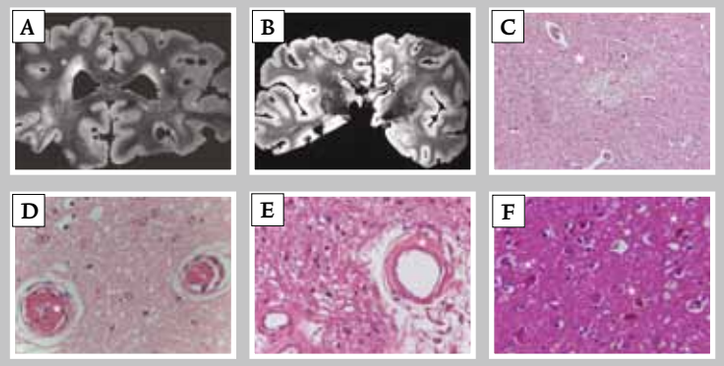


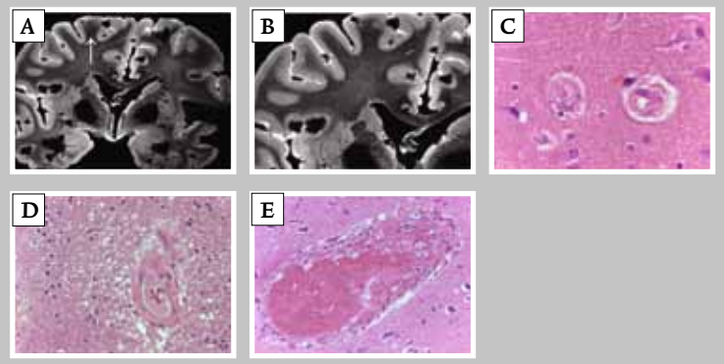
Figure 4 shows staining-patterns of C1Q and C4d. Both were scored semiquantitatively as 'no staining' (total absence of C1Q or C4d staining in all low powerfields), 'mild staining' (1-2 vessels showing C1Q or C4d positivity per low powerfield) or 'diffuse staining' (several vessels showing C1Q or C4d 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

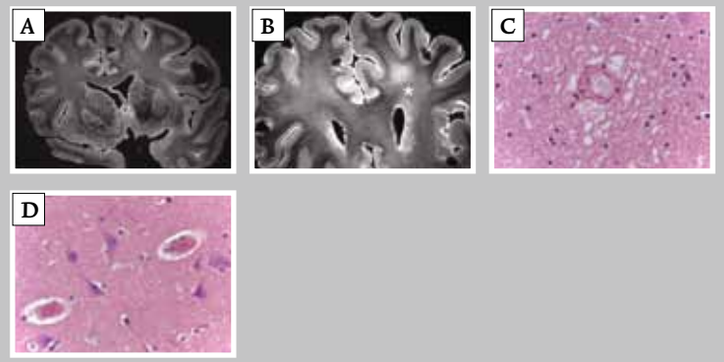
Figure 5 shows the findings of post mortem MRI and corresponding histopathological lesions in three SLE patients of whom whole formalin-fixed brains were available.



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

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