Systemic lupus erythematosus: pathogenesis, diagnosis, and treatment

Wilhelmus, S.

Systemic lupus erythematosus: pathogenesis, diagnosis, and treatment

Suzanne Wilhelmus

The research described in this thesis was performed at the Department of Pathology at Leiden University Medical Center, Leiden, The Netherlands. Part of this research was performed in collaboration with the Centre for Molecular and Cellular Biology of Inflammation, Division of Immunology, Infection, and Inflammatory Diseases, King's College London.

The work presented in this thesis was in part financially supported by the Ars Donandi - Schokkenkamp Wegener Lonzieme foundation.

The printing of this thesis was financially supported by the NVLE, Dutch Arthritis Foundation, The Dutch Kidney Foundation, and Chipsoft

Layout: E. Roos and W.J.V. van Est, Academic Medical Center Amsterdam
Cover design: W.J.V. van Est, Academic Medical Center Amsterdam

Printed by: Ridderprint BV
ISBN: 978-94-6299-539-0

Systemic lupus erythematosus: pathogenesis, diagnosis, and treatment

Proefschrift

ter verkrijging van

de graad van Doctor aan de Universiteit Leiden,

op gezag van de Rector Magnificus prof. mr. C.J.J.M. Stolker,

volgens besluit van het College voor Promoties

te verdedigen op woensdag 15 maart 2017

klokke 10.00 uur

door

Suzanne Wilhelmus

geboren te Zoetermeer

in 1982

Promotor

Prof. dr. J.A. Bruijn

Copromotor

Dr. I.M. Bajema

Promotiecommissie

Prof. dr. J.H.M. Berden, UMC Nijmegen
Prof. dr. R. Goldschmeding, UMC Utrecht
Prof. dr. V.T.H.B.M. Smit
Dr. C.J. Peutz-Kootstra, UMC Maastricht

Contents

1

General Introduction

Introduction

Systemic lupus erythematosus (SLE) is a potentially devastating autoimmune disease which can involve practically every organ system. SLE has an overall incidence ranging from 1.6 to 21.9 cases per 100 000 per year and a prevalence ranging from 7.4 to 159.4 cases per 100 000, varying considerably by ancestral group.[1] SLE affects mostly women of reproductive age. In contrast, in men, the incidence ranges from 0.14 to 2.5 cases per 100 000 per year and the prevalence from 0 to 52 cases per 100 000.[2] Up to 20% of all cases begin in childhood. The female predominance is not as outspoken in childhood-onset SLE as it is in adult-onset SLE.[3] Patients with childhood-onset SLE are more likely to have neurologic and renal involvement than patients with adult-onset SLE, and to accrue more renal damage.[4] Renal and neurological involvement are both considered to be severe manifestations of the disease. Approximately 20 to 60% of SLE patients develop renal involvement in the course of their disease[5] with the highest risk of renal disease and renal failure in young black women.[6 7] Lupus nephritis (LN) is associated with considerable morbidity and poor survival, in particular in patients who develop end-stage renal disease (ESRD) and require renal replacement therapy.

The diagnosis, treatment, and pathogenesis of SLE are intricately linked. The diagnosis of SLE can be difficult because of the many different faces of the disease. These many faces are also present within one of the disease manifestations: LN. The histological picture of LN varies greatly, but a correct diagnosis of the type of LN is essential for the choice of treatment. The question also is if different classes of LN have a different pathogenesis, or if they are part of the same spectrum. Familial LN often presents at an early age and appears more severe than sporadic LN. There may be an underlying difference in pathogenesis and possibly genetics between familial and sporadic LN, which can be used in studying the pathogenesis of LN. Further insight into the pathogenesis of SLE and LN may lead to new targets for therapy in the future.

Diagnosis

SLE

SLE can involve practically all organ systems. Therefore, patients can present with a wide range of symptoms. Not all symptoms are necessarily present at the same time. These factors can make diagnosing SLE complex. The first classification criteria were published by Cohen *et al.* in 1971.[8] The presence of four of 14 criteria was required to classify a patient as

having SLE. During the following decades new insights led to revisions by the Diagnostic and Therapeutic Criteria Committee of the American College of Rheumatology (ACR) in 1982[9] and 1997.[10] For the purpose of identifying patients in clinical studies, a patient was considered to have SLE if any four of 11 criteria were present, serially or simultaneously, during any interval of observation. These 11 criteria were a malar rash, discoid rash, photosensitivity, oral ulcers, arthritis, serositis, renal disorder, neurologic disorder, haematological disorder, immunological disorder and an abnormal anti-nuclear antibody (ANA) titer. Recently, the Systemic Lupus International Collaborating Clinics (SLICC) classification criteria were introduced, which were validated in a cohort of 690 patient scenarios including control patients with RA, undifferentiated connective tissue disease, primary antiphospholipid syndrome, vasculitis, chronic cutaneous lupus erythematosus, scleroderma, Sjögren's syndrome, myositis, psoriasis, fibromyalgia, alopecia areata, and sarcoidosis. [11] In the SLICC system, the criteria have been distributed over 11 clinical and six immunological criteria. In order to classify a patient with SLE, at least four criteria with at least one clinical and one immunological criterion must be present. Also, biopsy-proven nephritis compatible with SLE in the presence of ANAs or anti-dsDNA antibodies will also classify the patient as having SLE.

Lupus nephritis

The renal biopsy plays an important role in the management of patients with SLE. Renal manifestations may be the first sign of SLE and the renal biopsy may then aid in diagnosing the patient. Furthermore, in both these newly diagnosed patients and patients already diagnosed with SLE, the renal biopsy is instrumental in determining the type and extent of LN, as this cannot be accurately assessed on the basis of clinical manifestations.

Electron microscopy

Instrumental in the pathology of LN are immune deposits. These immune deposits can be visualized with immunofluorescence techniques (discussed below) and electron microscopy (EM). On EM, immune deposits are electron dense and can be present in the mesangium, subendothelially, intramembranous and subepithelially. Often in LN, immune deposits are found at more than one of these locations (Figure 1, panel A, C and D). The size and frequency of these deposits are extremely variable, ranging from sparse and small to abundant and large. Immune deposits are not restricted to the glomerulus and can be present along tubular basement membranes and in vessels. Another feature that may be seen by EM is the presence of tubuloreticular inclusions, which are mostly found in endothelial cells (Figure 1,

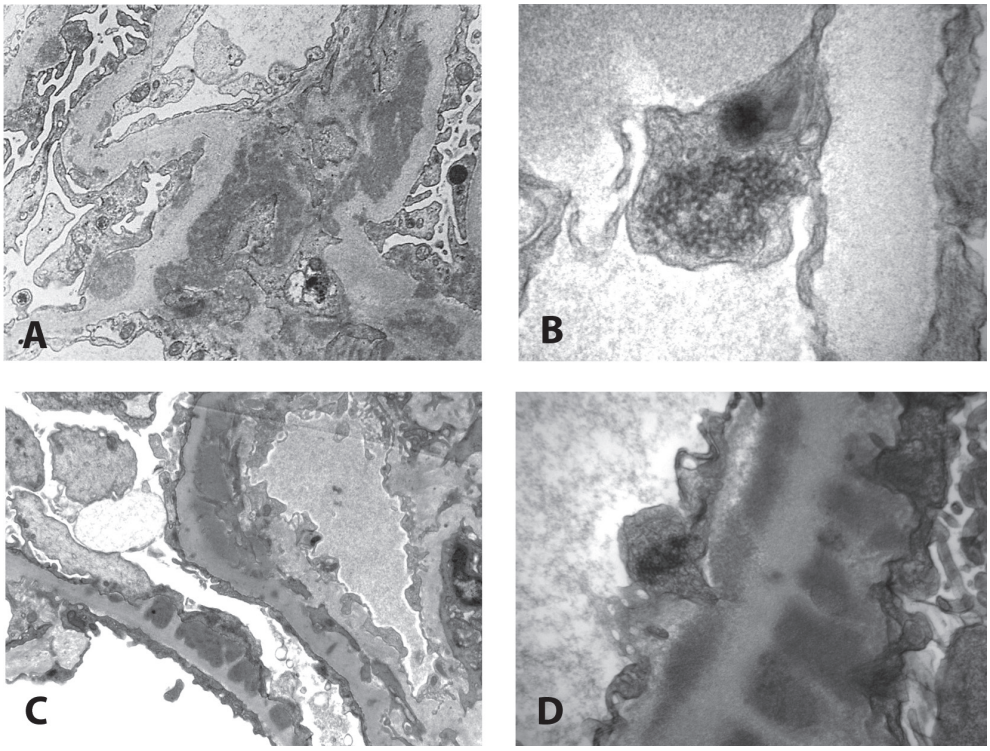


Figure 1. Examples of lesions in lupus nephritis, as seen by electron microscopy
(A) Extensive mesangial electron dense deposits. (B) Tubuloreticular inclusion in an endothelial cell. (C) and (D) Electron dense deposits at both subendothelial and subepithelial locations. This is accompanied by foot process effacement and microvillous transformation.

panel B). Although they are often present, they are not specific for SLE and may be seen in patients with HIV, other collagen-vascular diseases, renal allografts[12] and even a few healthy individuals. Finally, particularly in patients with subepithelial deposits, changes to the podocyte foot processes can be observed. These changes include foot process effacement, condensation of the cytoskeletal microfilaments and microvillous transformation.

Histology

LN has many different histological features which correspond to the location of the immune deposits seen on EM. These deposits can be visualized by EM, and by immunofluorescence techniques. In contrast to EM, immunofluorescence allows for the determination of the type of immune deposit (IgA, IgG, IgM antibodies) and of the presence of components of the complement system (C1q, C3). Often IgA, IgG, IgM, C1q and C3 are all present, which is

commonly referred to as a “full house” pattern. This pattern is highly suggestive of LN, but not completely specific as other renal diseases may occasionally show a full house pattern.[13] For assessing a biopsy with light microscopy, multiple special stains are used. Apart from the regular haematoxylin and eosin staining (H&E), special stains are used such as the Jones methenamine silver stain, the periodic acid-Schiff (PAS) stain and trichrome stain. The patterns of injury in LN can be divided into three groups. These patterns are not mutually exclusive and can occur together.

Mesangial pattern

In this pattern there is hypercellularity of the mesangium and accumulation of matrix due to the mesangial presence of immune complexes (Figure 2, panel A).

Endothelial pattern

A wide variety of lesions may be seen within this pattern. The most common feature is endocapillary hypercellularity, which causes a luminal reduction of the capillary loops (Figure 2, panel C). This endocapillary hypercellularity has two components which may vary in its contribution: endothelial cell swelling and leukocyte influx. Another feature is fibrinoid necrosis.[14] This is often accompanied by extracapillary hypercellularity, so called crescents, because there is destruction of the capillary walls causing the capillary contents to leak into Bowman’s space. This elicits an inflammatory response and proliferation of visceral and parietal epithelial cells. Crescents may also occur without fibrinoid necrosis (Figure 2, panel D). Wire loops, the light microscopical counterpart of large amounts of subendothelial immune complex deposits, can be a focal or diffuse phenomenon (Figure 2, panel B). Furthermore, a membranoproliferative pattern may occur showing cellular interposition of mesangial cells along capillary walls and duplication of the glomerular basement membrane. Finally, karyorrhexis and hyaline thrombi may be observed.

Epithelial pattern

When immune complexes accumulate on the subepithelial side of the glomerular basement membrane, new glomerular basement membrane is formed around these deposits. Because the glomerular basement membrane is black on silver stain, this newly formed basement membrane can be seen as black spikes along the outer aspect of the capillary walls (Figure 2, panels E and F). If this new glomerular basement membrane has not yet been formed, light microscopy may be normal with deposits visible only by immunofluorescence and electron microscopy.

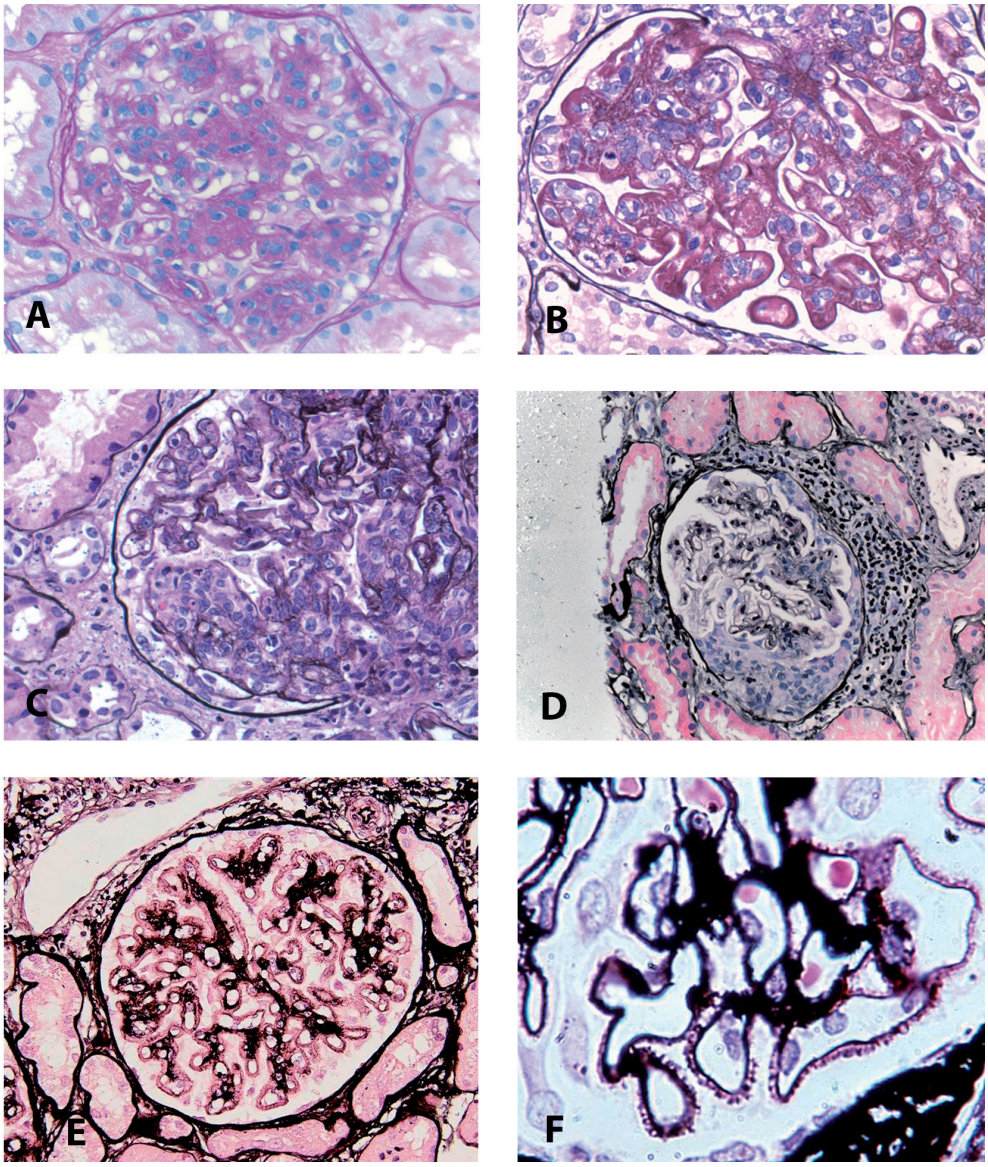


Figure 2. Examples of lesions in lupus nephritis, as seen by light microscopy
(A) Mostly mesangial hypercellularity and expansion with focal endocapillary hypercellularity (PAS stain). (B) Diffuse wire loops with mild endocapillary hypercellularity (silver stain). (C) Endocapillary hypercellularity with endothelial cell swelling and influx of inflammatory cells (silver stain). (D) Cellular crescent (silver stain). (E) and (F) Spikes along the outer aspect of the capillary walls (silver stain).

Table 1. Abbreviated ISN/RPS classification system of lupus nephritis (adapted from Weening, JASN/ Kidney International, 2004[15 16])

Class	Description
I	Minimal mesangial lupus nephritis
II	Mesangial proliferative lupus nephritis
III	Focal lupus nephritis[a]
IV	Diffuse segmental (IV-S) or diffuse global (IV-G) lupus nephritis[a]
V	Membranous lupus nephritis[b]
VI	Advanced sclerosing lupus nephritis

[a] Indicate proportion of glomeruli with active lesions, chronic lesions, fibrinoid necrosis and (cellular) crescents. [b] Class V may occur in combination with class III or IV, in which case both will be diagnosed. Indicate and grade (mild, moderate, severe) tubular atrophy, interstitial inflammation and fibrosis, arteriosclerosis or other vascular lesions.

Classification

When a diagnosis of LN has been made, the biopsy findings need to be classified according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) classification system from 2004.[15 16] It is imperative that a biopsy is classified correctly as the class will guide further treatment of the patient. Since its introduction, the classification system has undergone several revisions and now consists of 6 classes (Table 1).

In class I and II LN changes are restricted to the mesangium. Both classes show deposits by immunofluorescence, but only in class II is there mesangial hypercellularity or mesangial matrix expansion by light microscopy. In class II LN there may be a few isolated subendothelial or subepithelial deposits present by immunofluorescence (or EM) but not by light microscopy. Class III and IV LN show light microscopic abnormalities as described above under the endothelial pattern of injury. The distinction between class III and IV LN lies in the percentage of glomeruli involved with <50% being classified as class III and ≥50% as class IV. In both of these classes it should be indicated if there are either only active lesions (A), both active and chronic lesions (A/C), or only chronic lesions (C), although the latter is a rare event. Class IV LN is further subdivided into class IV-S and IV-G depending on whether the majority of involved glomeruli have segmental (IV-S) or global (IV-G) involvement, where segmental is defined as less than half of the glomerular tuft and global as more than half of the tuft. In class V there are subepithelial deposits by immunofluorescence (and EM), and possibly also by light microscopy. In class V LN any degree of mesangial hypercellularity may occur. When class III/class IV lesions coexist with class V lesions, the classification should consist of a combination of two classes, but only if the membranous component involves

more than 50% of the tuft in more than 50% of glomeruli. Finally, class VI LN is designated when ≥90% of glomeruli show global glomerulosclerosis, but only if there is clinical or pathological evidence that the sclerosis is attributable to LN. Furthermore, there should be no evidence of active nephritis. In addition to the class the pathology report should include details on tubulointerstitial and vascular lesions.

Treatment

After a diagnosis of SLE has been made, a treatment strategy can be devised. The treatment strategy depends on which organs are involved and to what extent. In the kidney, the class of LN plays a central role in the choice of treatment.

SLE

Treatment of SLE, without major organ involvement, consists of glucocorticoids, antimalarials (hydroxychloroquine), non-steroid anti-inflammatory drugs and, in severe, refractory cases, immunosuppressive agents. SLE patients may be at increased risk for several co-morbidities, including treatment-related morbidity. These include (urinary-tract) infections, hypertension, dyslipidaemia, diabetes mellitus, atherosclerosis, coronary heart disease, osteoporosis, avascular bone necrosis and certain types of cancer. Although there is no evidence that screening for co-morbidities will improve outcome, a high index of suspicion and diligent follow-up is recommended. Apart from treatment of these co-morbidities, when appropriate, preventive strategies may be considered such as low-dose aspirin in adult patients receiving glucocorticoids, in patients with anti-phospholipid antibodies, and in patients with at least one traditional risk factor for atherosclerotic disease.[17]

Lupus nephritis

Class III and class IV LN, and under certain circumstances class V LN, require aggressive treatment. Corticosteroids were the first available treatment for LN, and have since been an integral part of treatment. Treatment is comprised of two phases; induction and maintenance. The aim of the first is to achieve a meaningful renal response. The goal of maintenance is to consolidate the renal response and prevent renal flares. In the eighties and nineties of the previous century, NIH (National Institute of Health) studies demonstrated the added benefit of cyclophosphamide in the induction phase.[18-20] Cyclophosphamide is an alkylating agent thereby interfering in DNA replication. Later, mycophenolate mofetil (MMF) was shown to be equally effective.[21]

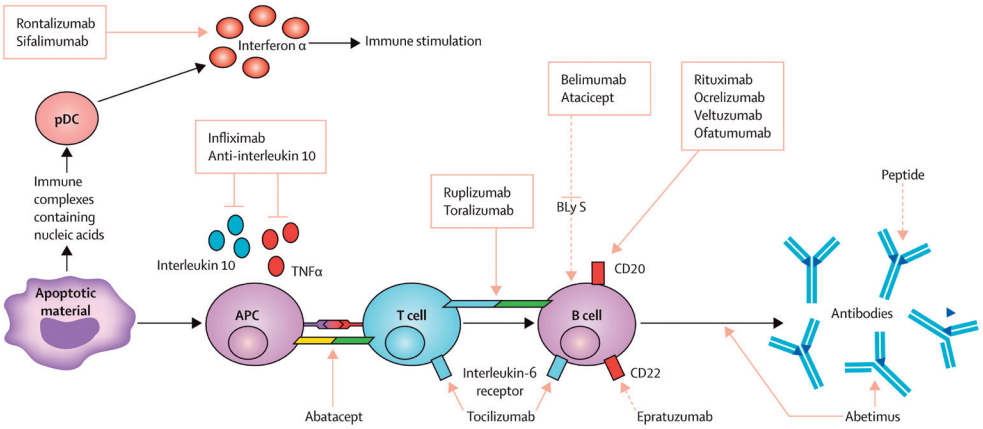


Figure 3. Targeted biological agents available and in present or previous clinical trials of systemic lupus erythematosus (reprinted from Murphy, Lancet, 2013[22], with permission from Elsevier)
The biological agents available all interact with the immune system. Some by immune stimulation through interferon a, others by targeting cytokines affecting antigen presenting cells. T cells interactions with antigen presenting cells and with B cells are also targeted, as well as the B cells and produces antibodies. Only belimumab (anti-BLyS) has so far been proven effective in SLE.
pDC, plasmacytoid dendritic cell; BLyS, B-lymphocyte stimulator; TNFα, tumor necrosis factor α; APC, antigen-presenting cell.

In the maintenance phase, treatment consists of either azathioprine, or MMF. MMF and azathioprine both inhibit purine synthesis, albeit by a different mechanism. Purine synthesis is important in the proliferation of B and T cells. In both the induction and maintenance phase, corticosteroids remain the backbone of treatment, although a phase 3 open-label multicenter investigator-led clinical trial (RITUXILUP, NCT01773616) is currently investigating a treatment strategy without steroids.

Most of the above mentioned drugs have serious possible side effects necessitating the search for effective drugs with less (severe) side effects. As more becomes known about the pathways involved in the pathogenesis of SLE and LN (discussed later), more targeted approaches are being developed (Figure 3).[22 23] For LN these drugs have not yet been proven effective. For the treatment of systemic disease belimumab, an anti-BLyS (B-lymphocyte stimulator) antibody, has been proven to be effective and is now registered for treatment of the disease.[24 25]

Pathogenesis

As discussed above, SLE may have many faces clinically. This complicates research into the pathogenesis of this complex and multifactorial disease even further. Although there are

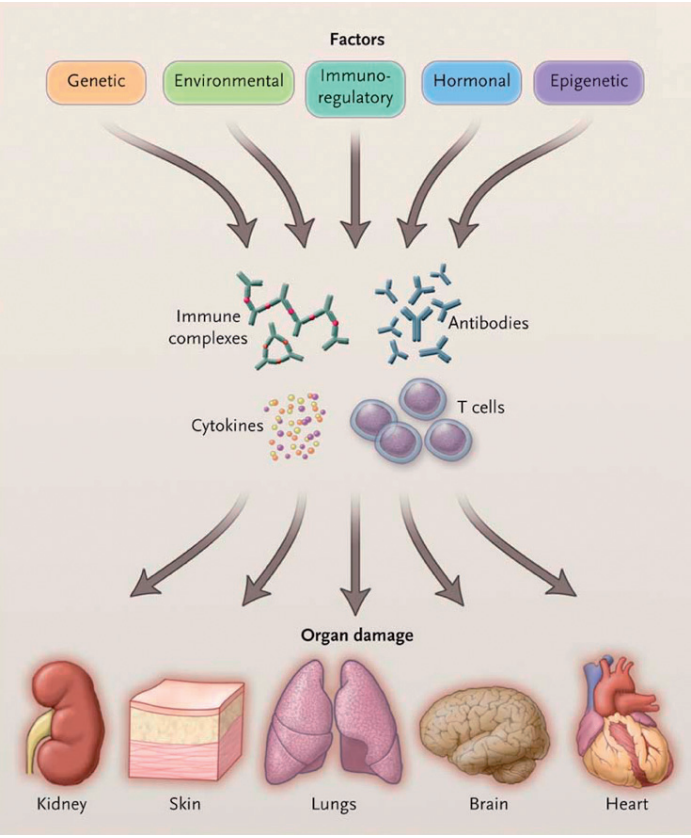


Figure 4. Factors involved in the pathogenesis of systemic lupus erythematosus (reproduced with permission from Tsokos, NEJM, 2011[39], Copyright Massachusetts Medical Society)

still many unknowns concerning the pathogenesis of the disease, substantial progress has been made in the last decades.

SLE

A multifactorial disease

Many factors contribute to the development of SLE, including (epi)genetic, environmental, hormonal and immunoregulatory factors (Figure 4).[26]

Genetic susceptibility to SLE is inherited as a complex trait, but the genetic contribution is thought to be significant in the etiology of SLE. Disease concordance in SLE is higher in monozygotic (25-50%) than in dizygotic twins (2%) and there is a high sibling risk ratio (λs) of 20-29.[26-28] Although some single disease-causing mutations have been described, such as

in DNASE1, these are only the cause of disease in rare cases.[29] Initial studies used linkage analysis in multiplex families to identify candidate genes. More recently, multiple genome wide association studies were performed in European-derived and Asian populations identifying multiple SLE susceptibility alleles.[30-38] Meta-analyses and large replication studies have expanded these further. Although some of these loci are located in coding sequences, many reside in non-coding regions. The HLA-region holds a prominent position within these susceptibility loci. The non-HLA SLE-associated genes play a role in multiple biological pathways: dendritic cell function and IFN signaling, T and B cell function and signaling, immune complex processing and innate immunity, transcriptional regulation, and cell cycle, apoptosis and cellular metabolism.[39] Despite all the advances made in the last decade, only a small proportion of the heritability is explained by these susceptibility loci.

Environmental factors also seem to play a role in pathogenesis. Epigenetic changes such as DNA hypomethylation have been attributed to medications known to cause SLE. Furthermore, epidemiologic studies have implicated smoking and exposure to ultraviolet light as risk factors. Finally, there is evidence to suggest that viruses may trigger SLE.[39]

The strong female predominance in SLE has led to research into hormonal influences. Although they contribute to the pathogenesis of SLE, the mechanisms involved are unknown. Data from animal studies suggests that the X chromosome may also contribute independently from hormones. Furthermore, CD40 is among the genes known to contribute to SLE and this gene is located on the X chromosome.[39]

Multiple abnormalities have been demonstrated in antigen presenting cells, and T and B cell signaling and function in SLE (for review, see Konya *et al*.,[40] Bird *et al.*[41] and Orme *et al.*[42]). Neutrophils have received much attention, particularly since the discovery of NETosis (formation of neutrophil extracellular traps, or NETs). NETosis has been described as a novel mechanism of cell death in which the neutrophils extrude their chromatin to trap and inactivate pathogens. NET formation appears to be enhanced in SLE leading to an increased exposure to autoantigens and increased production of IFN-α by plasmacytoid dendritic cells (for review, see Smith *et al.*[43]).

Autoantibodies against nuclear antigens are the hallmark of SLE. These antibodies are produced by autoreactive B cells (plasma cells) and may, along with immune complexes, autoreactive or inflammatory T cells and inflammatory cytokines, initiate and amplify organ damage. The factors described above are probably all involved in the production of these antibodies, but the question remains: why are these mainly directed towards nuclear antigens? The source of chromatin, the main auto-antigen in SLE, is most likely apoptotic

and/or necrotic cells, including NETs. Apoptosis, necrosis and NETosis explain how normally inaccessible autoantigens can be released and subsequently become exposed to the immune system. The autoantigens can be modified during apoptosis, possibly facilitating the breach of tolerance. In addition, impaired removal may lead to the accumulation of apoptotic cells and debris. There is convincing evidence for clearance defects op apoptotic cells and debris in SLE (for review, see Rekvig *et al.*[44]). Possibly, antibodies are not (only) generated in response to self-DNA and chromatin, but to DNA/chromatin from chimeric cells, as chimeric cells have been implicated in the pathogenesis of SLE. Before discussing the role of chimeric cells in SLE, first the definition, sources and techniques for detection of the chimeric cells are considered.

Microchimerism

Definition

The term 'chimerism' originates from Greek mythology. It refers to the Chimaera (Figure 5), which is a monster composed of parts of more than one animal. Homer's description in the Iliad is the earliest surviving literary reference: "…. an invincible inhuman monster, but divine in origin. Its front part was a lion, its rear a snake's tail, and in between a goat. She breathed deadly rage in searing fire."[45]

Figure 5. The "Chimera of Arezzo", as displayed at the Museo Archeologico Nazionale, Florence, Italy

In medicine, the term 'chimaera' is related to the term 'mosaicism', because in human cytogenetics both connote subjects with cells of two or more chromosomally different kinds. However, a chimaera "... is an organism whose cells derive from two or more distinct zygote lineages...", whereas a mosaic "... is formed of the cells of a single zygote lineage."[46] *Micro*chimerism (Mc) refers to the presence in an individual of a *small number* of genetically distinct cells of any type, originating from a different zygote.

Sources of microchimerism

Transplantation (solid organs[47] or bone marrow[48]), blood transfusions[49] and pregnancies[50] are possible sources of (micro)chimerism, the latter being the most common. During pregnancy, fetal cells can enter the maternal circulation leading to fetal Mc (FMc) in the mother. When maternal cells cross the placental barrier to the fetus, this can lead to maternal Mc (MMc). Pregnancies of all terms, including both miscarriages and pregnancies resulting in (live) birth, may lead to Mc.[51-53] Also, undetected pregnancies have the potential to cause FMc, making research into the relationship between pregnancy and long-term FMc difficult. Several studies investigated the kinetics of Mc during and after pregnancy in healthy individuals.[53-57] It was demonstrated that Mc tended to increase with gestational age and disappeared in the months postpartum. During pregnancy, not only can fetal cells circulate in the mother, fetal cell-free DNA can be detected in the maternal circulation.[53] The quick disappearance of this fetal cell-free DNA after delivery made it the perfect candidate for the prenatal detection of genetic defects in the fetus. Prenatal diagnostics are now being employed to detect trisomy 13, 18 and 21.[58] Both FMc[59] and MMc[60] have been detected in peripheral blood multiple decades after birth in healthy individuals.

During blood transfusion, genetically distinct cells are introduced into the host. Initial studies, however, were not able to demonstrate donor leukocyte survival beyond 6 days.[61 62] Interestingly, in a study characterizing the survival kinetics of donor subsets after elective surgery, it was accidently found that the 'control group' of women with blood transfusions after traumatic injury did have multilineage persistence of male donor leukocytes for 6 months to 1.5 years after blood transfusion. In the patients with elective surgery, the donor leukocytes were cleared within 14 days after transfusion.[49] Follow-up studies have confirmed these results.[63 64] Studies in other populations, such as in an HIV-infected population as a paradigm of an immunosuppressive state,[65] and sickle cell anaemia[66] reflecting chronic transfusion risk, did not show a significant increase in Mc or durability of Mc. Importantly, women receiving peripartum transfusions for maternal hemorrhage did not show durable Mc.[67]

Solid organ transplantation itself is a form of chimerism because an organ from a different individual (zygote) is transplanted into the patient. Furthermore, these patients also have circulating donor Mc in their peripheral blood.[68] So far, it is unclear if the presence or level of Mc in peripheral blood in recipients of solid organ transplantation has an effect on tolerance induction and graft function.[68-70]

Detection of microchimerism

The earliest detection of Mc employed karyotyping in metaphase figures in lymphocyte cultures from peripheral blood samples.[50] Later, many studies used *in situ* hybridisation for the Y chromosome.[55 71] This was followed by PCR techniques, first the non-quantitative nested PCR,[59] and later the quantitative PCR (qPCR).[72] Still, mostly the detection of the Y chromosome was used in women to measure Mc, although HLA genotype disparities were also sometimes used.[60] A drawback of detecting Mc using the Y chromosome is that only male Mc can be detected. This limits research to women and to FMc. It also means that in an individual all male cells are indiscriminately analyzed together and other sources of Mc (maternal, female children, female siblings) are missed. Using HLA disparities allows for studying MMc and for the detection of Mc in men. However, the presence of Mc is possibly linked to HLA disparities, making this method less desirable. In 2002, Alizadeh *et al.*[73] described a method using insertion-deletion polymorphisms (indels) for the detection of chimeric cells. Additional indels were described by Jimenez-Velasco *et al.*,[74] as well as a number of null alleles. In the latter study a sensitivity of 10^{-5} was reached, which is equal to the sensitivity reached with the detection of the Y-chromosome.[72] Maas *et al.*[75] developed an assay using SNPs, but this assay was less sensitive. Also, it was potentially less specific than the usage of indels or null alleles to detect Mc because it makes use of only a one base pair difference. Combining sets of indels and null alleles may reach a high informativity in differentiating different sources of Mc.

The number of chimeric cells detected in various circumstances is usually very small, ranging from 1 to up to 400 cells per 10^6. Therefore, both sensitivity and specificity are of the utmost importance. A sensitivity of at least 10^{-5} is necessary to study Mc. Differences in sensitivity and specificity of the techniques used in the field make comparison of the different studies difficult. This high sensitivity also requires a very clean work flow: while preparing samples all possible contaminants should be avoided. Also, all experiments require multiple negative controls.

Microchimerism in pregnancy

Schmorl *et al.* first described fetal Mc. He found syncytial aggregates in lungs of women who died of pre-eclampsia.[76] Although women with pre-eclampsia have more syncytial aggregates in their lungs, these placenta-derived syncytial aggregates have also been shown in women with normal pregnancies.[77] Also, fetal cells were detected in peripheral blood and various organs during pregnancy.[53 78] It is, however, unclear what role these chimeric cells play in normal pregnancies. Are they an epiphenomenon or do they play a central role in the immunology of pregnancy? Conversely, it is known that maternal chimeric cells can have an effect on the immune system of the child; Mold *et al.* showed that maternal alloantigens promote the development of tolerogenic fetal regulatory T cells in utero.[79]

Microchimerism in SLE

SLE mainly affects women and has a peak incidence in the reproductive years.[80] In mice, injection of parental lymphocytes in their offspring leads to a graft-versus-host response and a lupus-like disease in selected parent-to-F1 combinations.[81 82] Together, these data suggest that pregnancy-acquired Mc may be of pathogenic significance in the development of SLE. Studies investigating Mc in SLE have shown that women with SLE have a significantly higher prevalence of fetal Y chromosome-positive chimeric cells in tissue than healthy controls.[83-85] In two studies, SLE patients were shown to have male FMc in peripheral blood more frequently than controls.[86 87] However, other studies showed no differences between patients and controls.[88 89] Kanold *et al.* studied MMc in peripheral blood and did not find a difference between patients and controls.[90] However, their sensitivity of detecting chimeric cells was relatively low.

Kremer Hovinga *et al.*[91] formulated hypotheses regarding the role of Mc in SLE: *i*) Mc induces a graft-versus-host reaction; *ii*) Mc induces a host-versus-graft reaction, either directly or via cross-reactivity due to molecular mimicry; or *iii*) chimeric cells repair injured tissue. The first hypothesis is supported by the data from animal studies described above. From human studies the evidence is very circumstantial. The host-versus-graft hypothesis has more support from studies in humans, albeit also circumstantial. Anti-paternal antibodies have been demonstrated in mothers and have been shown to correlate to the presence of primed anti-paternal cytotoxic T lymphocytes.[92] Also, it was demonstrated in SLE patients that patients with LN had higher levels of Mc than patients without SLE, although overall disease activity was not correlated with Mc.[89] The presence of chimeric progenitor cells in

SLE patients lends support to the third hypothesis.[59] Also in animal studies chimeric cells were shown to have stem cells phenotypes[93] and chimeric cells have been demonstrated in bone marrow and rib sections of women with sons.[94] Furthermore, Mc was increased in animal models after injury.[95 96] Finally, in kidney biopsies of women with LN, the chimeric cells were shown to have multiple differentiated phenotypes, such as an endothelial cell.[83]

Lupus nephritis

The deposition of immune complexes in the kidney is the cause of the renal damage in LN. Depending on the location of the immune deposits, *i.e.* mesangial, subendothelial or subepithelial, different mechanisms leading to renal damage are triggered. These mechanisms involve activation of the *i*) classical complement pathway; *ii*) Fc, Toll-like and complement receptor activation; *iii*) local expression of cytokines, chemokines and adhesion molecules; *iv*) recruitment of leukocytes with pro-inflammatory effector functions; *v*) programmed death of renal parenchymal cells and reparative hyperproliferation; and *vi*) insufficient regeneration and scarring.[97] Macrophages and dendritic cells may play a role in the initiation as well as the progression of LN.[98 99]

It is, however, a question why immune complexes deposit in the kidney in the first place and what factors influence the location of these immune deposits. Traditionally, it was believed that immune complex deposition is a passive process. Now, several studies provide arguments against this notion. Yung *et al.* demonstrated binding of anti-dsDNA antibodies to mesangial annexin II. They also showed that this binding correlated with disease activity, and that annexin II colocalized with IgG and C3 deposits in human and murine LN.[100] In murine experimental LN Krishnan *et al.* demonstrated that only anti-DNA antibodies with glomerular basement membrane binding capacity were able to activate complement and induce proteinuria.[101] These studies suggest that cross-reactivities of anti-DNA antibodies may be responsible for initiating LN. In contrast, Mjelle *et al.* provide evidence that antibodies bind to nucleosomal antigens which in turn bind to components of mesangial matrix and the glomerular basement membrane.[102] Finally, failure to dismantle NETs has been shown to be correlated with kidney involvement in lupus, suggesting that neutrophils undergoing NETosis in the glomerulus may provide an additional source of nuclear antigens.[103]

Thesis outline

Diagnosing LN is the topic of the first two chapters. In **chapter 2** an investigation of the interobserver agreement in the recognition of class III and class IV LN nephritis is described. In **chapter 3** possible changes to be made to the current classification system of LN in order to further improve its usefulness and reproducibility are discussed.

In **chapter 4** of this thesis the comparison of six treatment guidelines of LN is presented, determining common ground in the treatment of LN between the guidelines and highlighting differences. These differences are areas where further research into the optimal treatment strategy is warranted.

The last three chapters of this thesis will focus on the pathogenesis of SLE, starting with the role of Mc. In the work described in **chapter 5** we investigated if Mc is more prevalent in peripheral blood of women with SLE than in controls. In the work described in **chapter 6** we aimed to determine if kinetics of Mc during and after pregnancy may be responsible for the difference we observed between SLE patients and controls. In order to gain further insight into the pathogenesis of LN, we compared patients with sporadic LN to patients with familial LN focusing on genetic, clinical, and histopathological aspects, as described in **chapter 7**.

Finally, in **chapter 8** we summarize and discuss the results of the research presented in the aforementioned chapters.

References

1. D'Cruz D. Systemic lupus erythematosus. *Lancet* 2007;369(9561):587-96.

2. Danchenko N, Satia JA, Anthony MS. Epidemiology of systemic lupus erythematosus: a comparison of worldwide disease burden. *Lupus* 2006;15(5):308-18.

3. Tucker LB, Menon S, Schaller JG*, et al.* Adult- and childhood-onset systemic lupus erythematosus: a comparison of onset, clinical features, serology, and outcome. *Br J Rheumatol* 1995;34(9):866-72.

4. Tucker LB, Uribe AG, Fernandez M*, et al.* Adolescent onset of lupus results in more aggressive disease and worse outcomes: results of a nested matched case-control study within LUMINA, a multiethnic US cohort (LUMINA LVII). *Lupus* 2008;17(4):314-22.

5. Seligman VA, Lum RF, Olson JL*, et al.* Demographic differences in the development of lupus nephritis: a retrospective analysis. *Am J Med* 2002;112(9):726-9.

6. Somers EC, Marder W, Cagnoli P*, et al.* Population-based incidence and prevalence of systemic lupus erythematosus: the Michigan Lupus Epidemiology and Surveillance program. *Arthritis Rheumatol* 2014;66(2):369-78.

7. Lim SS, Bayakly AR, Helmick CG*, et al.* The incidence and prevalence of systemic lupus erythematosus, 2002-2004: The Georgia Lupus Registry. *Arthritis Rheumatol* 2014;66(2):357-68.

8. Cohen AS, Reynolds WE, Franklin EC*, et al.* Preliminary criteria for the classification of systemic lupus erythematosus. *Bull Rheum Dis* 1971;21:643-48.

9. Tan EM, Cohen AS, Fries JF*, et al.* The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982;25(11):1271-7.

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

11. Petri M, Orbai AM, Alarcon GS*, et al.* Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus. *Arthritis Rheum* 2012;64(8):2677-86.

12. Willicombe M, Moss J, Moran L*, et al.* Tubuloreticular Inclusions in Renal Allografts Associate with Viral Infections and Donor-Specific Antibodies. *J Am Soc Nephrol* 2016;27(7):2188-95.

13. Wen Y-K, Chen M-L. Clinicopathological study of originally non-lupus "full-house" nephropathy. *Ren Fail* 2010;32:1025-30.

14. Bajema IM, Bruijn JA. What stuff is this! A historical perspective on fibrinoid necrosis. *J Pathol* 2000;191(3):235-8.

15. Weening JJ, D'Agati VD, Schwartz MM*, et al.* The classification of glomerulonephritis in systemic lupus erythematosus revisited. *J Am Soc Nephrol* 2004;15(2):241-50.

16. Weening JJ, D'Agati VD, Schwartz MM*, et al.* The classification of glomerulonephritis in systemic lupus erythematosus revisited. *Kidney Int* 2004;65(2):521-30.

17. Bertsias G, Ioannidis JP, Boletis J*, 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.

18. Austin HA, 3rd, Klippel JH, Balow JE*, et al.* Therapy of lupus nephritis. Controlled trial of prednisone and cytotoxic drugs. *N Engl J Med* 1986;314(10):614-9.

19. Boumpas DT, Austin HA, Balow JE*, et al.* Controlled trial of pulse methylprednisolone versus two regimens of pulse cyclophosphamide in severe lupus nephritis. *Lancet* 1992;340(8822):741-45.

20. Gourley MF, Austin HA, 3rd, Scott D*, et al.* Methylprednisolone and cyclophosphamide, alone or in combination, in patients with lupus nephritis. A randomized, controlled trial. *Ann Intern Med* 1996;125(7):549-57.

21. Appel GB, Contreras G, Dooley MA*, et al.* Mycophenolate mofetil versus cyclophosphamide for induction treatment of lupus nephritis. *J Am Soc Nephrol* 2009;20(5):1103-12.

22. Murphy G, Lisnevskaia L, Isenberg D. Systemic lupus erythematosus and other autoimmune rheumatic diseases: challenges to treatment. *Lancet* 2013;382(9894):809-18.

23. Jordan N, D'Cruz D. Key issues in the management of patients with systemic lupus erythematosus: latest developments and clinical implications. *Ther Adv Musculoskelet Dis* 2015;7(6):234-46.

24. Navarra SV, Guzman RM, Gallacher AE*, et al.* Efficacy and safety of belimumab in patients with active systemic lupus erythematosus: a randomised, placebo-controlled, phase 3 trial. *Lancet* 2011;377(9767):721-31.

25. Furie R, Petri M, Zamani O*, et al.* A phase III, randomized, placebo-controlled study of belimumab, a monoclonal antibody that inhibits B lymphocyte stimulator, in patients with systemic lupus erythematosus. *Arthritis Rheum* 2011;63(12):3918-30.

26. Block SR, Winfield JB, Lockshin MD*, et al.* Studies of twins with systemic lupus erythematosus. A review of the literature and presentation of 12 additional sets. *Am J Med* 1975;59(4):533-52.

27. Deapen D, Escalante A, Weinrib L*, et al.* A revised estimate of twin concordance in systemic lupus erythematosus. *Arthritis Rheum* 1992;35(3):311-8.

28. Alarcon-Segovia D, Alarcon-Riquelme ME, Cardiel MH*, et al.* Familial aggregation of systemic lupus erythematosus, rheumatoid arthritis, and other autoimmune diseases in 1,177 lupus patients from the GLADEL cohort. *Arthritis Rheum* 2005;52(4):1138-47.

29. Yasutomo K, Horiuchi T, Kagami S*, et al.* Mutation of DNASE1 in people with systemic lupus erythematosus. *Nat Genet* 2001;28(4):313-4.

30. Han JW, Zheng HF, Cui Y*, et al.* Genome-wide association study in a Chinese Han population identifies nine new susceptibility loci for systemic lupus erythematosus. *Nat Genet* 2009;41(11):1234-7.

31. Yang W, Shen N, Ye DQ*, et al.* Genome-wide association study in Asian populations identifies variants in ETS1 and WDFY4 associated with systemic lupus erythematosus. *PLoS Genet* 2010;6(2):e1000841.

32. Armstrong DL, Zidovetzki R, Alarcon-Riquelme ME*, et al.* GWAS identifies novel SLE susceptibility genes and explains the association of the HLA region. *Genes Immun* 2014;15(6):347-54.

33. Graham RR, Cotsapas C, Davies L*, et al.* Genetic variants near TNFAIP3 on 6q23 are associated with systemic lupus erythematosus. *Nat Genet* 2008;40(9):1059-61.

34. Harley JB, Alarcon-Riquelme ME, Criswell LA*, et al.* Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PXK, KIAA1542 and other loci. *Nat Genet* 2008;40(2):204-10.

35. Hom G, Graham RR, Modrek B*, et al.* Association of systemic lupus erythematosus with C8orf13-BLK and ITGAM-ITGAX. *N Engl J Med* 2008;358(9):900-9.

36. Okada Y, Shimane K, Kochi Y*, et al.* A genome-wide association study identified AFF1 as a susceptibility locus for systemic lupus eyrthematosus in Japanese. *PLoS Genet* 2012;8(1):e1002455.

37. Lee HS, Kim T, Bang SY*, et al.* Ethnic specificity of lupus-associated loci identified in a genome-wide association study in Korean women. *Ann Rheum Dis* 2014;73(6):1240-5.

38. Kozyrev SV, Abelson AK, Wojcik J*, et al.* Functional variants in the B-cell gene BANK1 are associated with systemic lupus erythematosus. *Nat Genet* 2008;40(2):211-6.

39. Tsokos GC. Systemic lupus erythematosus. *N Engl J Med* 2011;365:2110-21.

40. Konya C, Paz Z, Tsokos GC. The role of T cells in systemic lupus erythematosus: an update. *Curr Opin Rheumatol* 2014;26(5):493-501.

41. Bird AK, Meednu N, Anolik JH. New insights into B cell biology in systemic lupus erythematosus and Sjogren's syndrome. *Curr Opin Rheumatol* 2015;27(5):461-7.

42. Orme J, Mohan C. Macrophages and neutrophils in SLE-An online molecular catalog. *Autoimmun Rev* 2012;11(5):365-72.

43. Smith CK, Kaplan MJ. The role of neutrophils in the pathogenesis of systemic lupus erythematosus. *Curr Opin Rheumatol* 2015;27(5):448-53.

44. Rekvig OP, Van der Vlag J. The pathogenesis and diagnosis of systemic lupus erythematosus: still not resolved. *Semin Immunopathol* 2014;36(3):301-11.

45. Johnston I. The Iliad by Homer. Arlington, VA, USA: Richer Resources Publications, 2006.

46. Ford CE. Mosaics and chimaeras. *Br Med Bull* 1969;25(1):104-9.

47. Lagaaij EL, Cramer-Knijnenburg GF, van Kemenade FJ*, et al.* Endothelial cell chimerism after renal transplantation and vascular rejection. *Lancet* 2001;357(9249):33-37.

48. Korbling M, Katz RL, Khanna A*, et al.* Hepatocytes and epithelial cells of donor origin in recipients of peripheral-blood stem cells. *N Engl J Med* 2002;346(10):738-46.

49. Lee TH, Paglieroni T, Ohto H*, et al.* Survival of donor leukocyte subpopulations in immunocompetent transfusion recipients: frequent long-term microchimerism in severe trauma patients. *Blood* 1999;93(9):3127-39.

50. Walknowska J, Conte FA, Grumbach MM. Practical and theoretical implications of fetal-maternal lymphocyte transfer. *Lancet* 1969;293(7606):1119-22.

51. Sato T, Fujimori K, Sato A*, et al.* Microchimerism after induced or spontaneous abortion. *Obstet Gynecol* 2008;112(3):593-97.

52. Peterson SE, Nelson JL, Guthrie KA*, et al.* Prospective assessment of fetal-maternal cell transfer in miscarriage and pregnancy termination. *Hum Reprod* 2012;27(9):2607-12.

53. Ariga H, Ohto H, Busch MP*, et al.* Kinetics of fetal cellular and cell-free DNA in the maternal circulation during and after pregnancy: implications for noninvasive prenatal diagnosis. *Transfusion* 2001;41(12):1524-30.

54. Hamada H, Arinami T, Hamaguchi H*, et al.* Fetal nucleated cells in maternal peripheral blood after delivery. *Am J Obstet Gynecol* 1994;170(4):1188-93.

55. Hamada H, Arinami T, Kubo T*, et al.* Fetal nucleated cells in maternal peripheral blood: frequency and relationship to gestational age. *Hum Genet* 1993;91(5):427-32.

56. Adams Waldorf K. Dynamic Changes in Fetal Microchimerism in Maternal Peripheral Blood Mononuclear Cells, CD4+ and CD8+ cells in Normal Pregnancy. *Placenta* 2010;31(7):589-94.

57. Hsieh TT, Pao CC, Hor JJ*, et al.* Presence of fetal cells in maternal circulation after delivery. *Hum Genet* 1993;92(2):204-05.

58. Mersy E, Smits LJ, van Winden LA*, et al.* Noninvasive detection of fetal trisomy 21: systematic review and report of quality and outcomes of diagnostic accuracy studies performed between 1997 and 2012. *Hum Reprod Update* 2013;19(4):318-29.

59. Bianchi DW, Zickwolf GK, Weil GJ*, et al.* Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. *Proc Natl Acad Sci U S A* 1996;93(2):705-08.

60. Maloney S, Smith A, Furst DE*, et al.* Microchimerism of maternal origin persists into adult life. *J Clin Invest* 1999;104(1):41-47.

61. Schechter GP, Whang-Peng J, McFarland W. Circulation of donor lymphocytes after blood transfusion in man. *Blood* 1977;49(4):651-6.

62. Adams PT, Davenport RD, Reardon DA*, et al.* Detection of circulating donor white blood cells in patients receiving multiple transfusions. *Blood* 1992;80(2):551-5.

63. Dunne JR, Lee TH, Burns C*, et al.* Transfusion-associated microchimerism in combat casualties. *J Trauma* 2008;64(2 Suppl):S92-7; discussion S97-8.

64. Utter GH, Owings JT, Lee TH*, et al.* Blood transfusion is associated with donor leukocyte microchimerism in trauma patients. *J Trauma* 2004;57(4):702-7.

65. Kruskall MS, Lee TH, Assmann SF*, et al.* Survival of transfused donor white blood cells in HIV-infected recipients. *Blood* 2001;98(2):272-9.

66. Reed W, Lee TH, Vichinsky EP*, et al.* Sample suitability for the detection of minor white cell populations (microchimerism) by polymerase chain reaction. *Transfusion* 1998;38(11-12):1041-5.

67. Bloch EM, Busch MP, Lee TH*, et al.* Microchimerism in the transfused obstetric population. *Vox Sang* 2014;107(4):428-30.

68. Elwood ET, Larsen CP, Maurer DH*, et al.* Microchimerism and rejection in clinical transplantation. *Lancet* 1997;349(9062):1358-60.

69. Dutta P, Burlingham WJ. Microchimerism: tolerance vs. sensitization. *Curr Opin Organ Transplant* 2011;16(4):359-65.

70. Curcio M, Cantarovich D, Barbuti S*, et al.* Association of donor-specific microchimerism with graft dysfunction in kidney transplant patients. *Transpl Immunol* 2012;26(2-3):151-5.

71. Koopmans M, Kremer Hovinga I, Baelde HJ*, et al.* Chimerism in kidneys, livers and hearts of normal women: implications for transplantation studies. *Am J Transplant* 2005;5(6):1495-502.

72. Pujal JM, Gallardo D. PCR-based methodology for molecular microchimerism detection and quantification. *Exp Biol Med* 2008;233(9):1161-70.

73. Alizadeh M, Bernard M, Danic B*, et al.* Quantitative assessment of hematopoietic chimerism after bone marrow transplantation by real-time quantitative polymerase chain reaction. *Blood* 2002;99:4618-25.

74. Jimenez-Velasco A, Barrios M, Roman-Gomez J*, et al.* Reliable quantification of hematopoietic chimerism after allogeneic transplantation for acute leukemia using amplification by real-time PCR of null alleles and insertion/deletion polymorphisms. *Leukemia* 2005;19(3):336-43.

75. Maas F, Schaap N, Kolen S*, et al.* Quantification of donor and recipient hemopoietic cells by real-time PCR of single nucleotide polymorphisms. *Leukemia* 2003;17(3):621-29.

76. Schmorl G. *Pathologisch-anatomische Untersuchungen ueber Puerperal Eklampsie*. Leipzig: Vogel, 1896.

77. Buurma AJ, Penning ME, Prins F*, et al.* Preeclampsia is associated with the presence of transcriptionally active placental fragments in the maternal lung. *Hypertension* 2013;62(3):608-13.

78. Rijnink EC, Penning ME, Wolterbeek R*, et al.* Tissue microchimerism is increased during pregnancy: a human autopsy study. *Mol Hum Reprod* 2015;21(11):857-64.

79. Mold JE, Michaelsson J, Burt TD*, et al.* Maternal alloantigens promote the development of tolerogenic fetal regulatory T cells in utero. *Science* 2008;322(5907):1562-5.

80. Lisnevskaia L, Murphy G, Isenberg D. Systemic lupus erythematosus. *Lancet* 2014;384(9957):1878-88.

81. Via CS, Shearer GM. T-cell interactions in autoimmunity: insights from a murine model of graft-versus-host disease. *Immunol Today* 1988;9(7-8):207-13.

82. Gleichmann E, Van Elven EH, Van der Veen JP. A systemic lupus erythematosus (SLE)-like disease in mice induced by abnormal T-B cell cooperation. Preferential formation of autoantibodies characteristic of SLE. *Eur J Immunol* 1982;12(2):152-9.

83. Kremer Hovinga I, Koopmans M, Baelde HJ*, et al.* Chimerism occurs twice as often in lupus nephritis as in normal kidneys. *Arthritis Rheum* 2006;54(9):2944-50.

84. Kremer Hovinga I, Koopmans M, Baelde HJ*, et al.* Tissue chimerism in systemic lupus erythematosus is related to injury. *Ann Rheum Dis* 2007;66(12):1568-73.

85. Florim GM, Caldas HC, de Melo JC*, et al.* Fetal microchimerism in kidney biopsies of lupus nephritis patients may be associated with a beneficial effect. *Arthritis Res Ther* 2015;17(1):101.

86. Abbud Filho M, Pavarino-Bertelli EC, Alvarenga MP*, et al.* Systemic lupus erythematosus and microchimerism in autoimmunity. *Transplant Proc* 2002;34:2951-52.

87. Kekow M, Barleben M, Drynda S*, et al.* Long-term persistence and effects of fetal microchimerisms on disease onset and status in a cohort of women with rheumatoid arthritis and systemic lupus erythematosus. *BMC Musculoskelet Disord* 2013;14:325.

88. Gannage M, Amoura Z, Lantz O*, et al.* Feto-maternal microchimerism in connective tissue diseases. *Eur J Immunol* 2002;32(12):3405-13.

89. Mosca M, Curcio M, Lapi S*, et al.* Correlations of Y chromosome microchimerism with disease activity in patients with SLE: analysis of preliminary data. *Ann Rheum Dis* 2003;62(7):651-4.

90. Kanold AMJ, Svenungsson E, Gunnarsson I*, et al.* A Research Study of the Association between Maternal Microchimerism and Systemic Lupus Erythematosus in Adults: A Comparison between Patients and Healthy Controls Based on Single-Nucleotide Polymorphism Using Quantitative Real-Time PCR. *PLoS One* 2013;8(9):e74534.

91. Kremer Hovinga I, Koopmans M, de Heer E*, et al.* Chimerism in systemic lupus erythematosus--three hypotheses. *Rheumatology (Oxford)* 2007;46(2):200-08.

92. van Kampen CA, Versteeg-vd Voort Maarschalk MF, Langerak-Langerak J*, et al.* Kinetics of the pregnancy-induced humoral and cellular immune response against the paternal HLA class I antigens of the child. *Hum Immunol* 2002;63(6):452-8.

93. Seppanen E, Fisk NM, Khosrotehrani K. Pregnancy-acquired fetal progenitor cells. *J Reprod Immunol* 2013;97(1):27-35.

94. O'Donoghue K, Chan J, de la Fuente J*, et al.* Microchimerism in female bone marrow and bone decades after fetal mesenchymal stem-cell trafficking in pregnancy. *Lancet* 2004;364(9429):179-82.

95. Nassar D, Droitcourt C, Mathieu-d'Argent E*, et al.* Fetal progenitor cells naturally transferred through pregnancy participate in inflammation and angiogenesis during wound healing. *FASEB J* 2012;26(1):149-57.

96. Khosrotehrani K, Reyes RR, Johnson KL*, et al.* Fetal cells participate over time in the response to specific types of murine maternal hepatic injury. *Hum Reprod* 2007;22(3):654-61.

97. Lorenz G, Desai J, Anders HJ. Lupus nephritis: update on mechanisms of systemic autoimmunity and kidney immunopathology. *Curr Opin Nephrol Hypertens* 2014;23(3):211-7.

98. Rogers NM, Ferenbach DA, Isenberg JS*, et al.* Dendritic cells and macrophages in the kidney: a spectrum of good and evil. *Nat Rev Nephrol* 2014;10(11):625-43.

99. Davidson A, Bethunaickan R, Berthier C*, et al.* Molecular studies of lupus nephritis kidneys. *Immunol Res* 2015;63(1-3):187-96.

100. Yung S, Cheung KF, Zhang Q*, et al.* Anti-dsDNA antibodies bind to mesangial annexin II in lupus nephritis. *J Am Soc Nephrol* 2010;21(11):1912-27.

101. Krishnan MR, Wang C, Marion TN. Anti-DNA autoantibodies initiate experimental lupus nephritis by binding directly to the glomerular basement membrane in mice. *Kidney Int* 2012;82(2):184-92.

102. Mjelle JE, Rekvig OP, Van Der Vlag J*, et al.* Nephritogenic antibodies bind in glomeruli through interaction with exposed chromatin fragments and not with renal cross-reactive antigens. *Autoimmunity* 2011;44(5):373-83.

103. Hakkim A, Furnrohr BG, Amann K*, et al.* Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *Proc Natl Acad Sci U S A* 2010;107(21):9813-8.

2

Interobserver Agreement on Histopathological Lesions in Class III or IV Lupus Nephritis

Suzanne Wilhelmus, H. Terence Cook, Laure-Hélène Noël,
Franco Ferrario, Ron Wolterbeek, Jan A. Bruijn, and Ingeborg M. Bajema

Clinical Journal of the American Society of Nephrology 2015; 10: 47-53

Abstract

Background and objectives

To treat lupus nephritis effectively, proper identification of the histological class is essential. Although the classification system for lupus nephritis is nearly 40 years old, remarkably few studies have investigated interobserver agreement. Interobserver agreement among nephropathologists was studied, particularly with respect to the recognition of class III/IV lupus nephritis lesions, and possible causes of disagreement were determined.

Design, setting, participants and measurements

A link to a survey containing pictures of 30 glomeruli was provided to all 360 members of the Renal Pathology Society; 34 responses were received from 12 countries (a response rate of 9.4%). The nephropathologist was asked whether glomerular lesions were present that would categorize the biopsy as class III/IV. If so, additional parameters were scored. To determine the interobserver agreement among the participants, kappa or intraclass correlation values were calculated. The ICC or kappa value was also calculated for two separate levels of experience (specifically, nephropathologists who were new to the field or moderately experienced [less experienced] and nephropathologists who were highly experienced).

Results

Intraclass correlation for the presence of a class III/IV lesion was 0.39 (poor). The kappa/intraclass correlation values for the additional parameters were as follows: active, chronic, or both: 0.36; segmental versus global: 0.39; endocapillary proliferation: 0.46; influx of inflammatory cells: 0.32; swelling of endothelial cells: 0.46; extracapillary proliferation: 0.57; type of crescent: 0.46; and wire loops: 0.35. The highly experienced nephropathologists had significantly less interobserver variability compared to the less-experienced nephropathologists (P=0.004).

Conclusions

There is generally poor agreement in terms of recognizing class III/IV lesions. Because experience clearly increases interobserver agreement, this agreement may be improved by training nephropathologists. These results also underscore the importance of a central review by experienced nephropathologists in clinical trials.

Introduction

One of the most severe manifestations of systemic lupus erythematosus is lupus nephritis, a major cause of high morbidity and mortality, because of either the disease itself or to the adverse effects associated with immunosuppressive therapy.[1 2] In treating lupus nephritis, the histologic class plays a central role in guiding treatment decisions.[3-5] The current version of the classification system was proposed in 2003 by the International Society of Nephrology and the Renal Pathology Society (ISN/RPS)[6] and has been adopted by renal pathologists worldwide. After the introduction of this revised classification system, several studies were performed comparing the revised classification system with the 1995 World Health Organization (WHO) classification system. Each of these studies[7-9] found higher interobserver agreement using the revised system, and this was attributed to clearer definitions and fewer subclasses.

The most important decision with respect to treating lupus nephritis is whether the biopsy can be classified as either class III or IV rather than class I, II or V. On the basis of current guidelines for treating lupus nephritis,[3-5] a diagnosis of either class III or class IV is an indication for initiating immunosuppressive therapy. Furthermore, in the most recent version of the classification system, allowance has been made to include lesions that are not strictly proliferative—for example, the sole presence of wire loops—in classes III and IV. Because obtaining an accurate diagnosis is essential for determining the subsequent treatment, it is imperative that renal pathologists reach consensus regarding what constitutes a lesion that would place the biopsy in class III or IV.

While performing a central review of lupus nephritis trials, we noted that, even in a selected group of highly experienced nephropathologists, there were differences of opinion with respect to what constitutes a lesion that would categorize the biopsy as class III or IV. On the basis of this experience, we sought to measure the (global) interobserver agreement regarding the recognition of classes III and IV lesions specifically. In addition, we also attempted to determine the sources of possible disagreement among nephropathologists.

Methods

Case selection and survey

Three trained nephropathologists carefully selected 30 glomeruli from several randomly chosen biopsies from classes III and class IV lupus nephritis cases, thus obtaining a representative sample of the various lesions that occur in lupus nephritis (except membranous

lupus nephritis). All of the biopsies selected had high staining quality and suitable section thickness and were selected from the archives at Leiden University Medical Center. All biopsies 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). High-quality pictures of these glomeruli were included in the questionnaire as a PDF file. One half of the pictures were images of silver-stained glomeruli, and one half were images of periodic acid-Schiff-stained glomeruli, because these stains are commonly used to assess glomerular pathology. The entire membership (approximately 360 regular members) of the Renal Pathology Society was provided with a link to the questionnaire, with the exception of the pathologists who selected the glomeruli. We received a total of 34 responses from the following countries: Australia (2), Canada (3), India (3), Italy (1), Japan (1), the Netherlands (3), Poland (1), Romania (1), Spain (1), Thailand (2), the United Kingdom (2), and the United States (14). For each picture of a glomerulus, the participants were asked to decide whether the glomerulus contained a lesion that would classify the biopsy as either class III or IV. If the answer was no, the participants were then asked whether other lesions were present, after which they could move on to the next picture; if the answer was yes (*i.e.*, the biopsy could be classified as class III/IV), they were asked to score additional parameters, which are shown in Figure 1. The participants were also encouraged to provide comments regarding each glomerulus. Lastly, the respondents were asked to indicate their level of experience as a nephropathologist (*i.e.*, new to the

Does this glomerulus contain a lesion which would put the biopsy in class III or IV?			☐ Yes	☐ No
If so, please also indicate				
Active and/or chronic	☐ Active	☐ Chronic	☐ Active/chronic	
Segmental or global	☐ Segmental	☐ Global		
Endocapillary proliferation	☐ Present	☐ Absent		
Influx of inflammatory cells	☐ Present	☐ Absent		
Swelling of endothelial cells	☐ Present	☐ Absent		
Extracapillary proliferation	☐ Present	☐ Absent		
If present	☐ Cellular	☐ Fibrocellular	☐ Fibrous	
Wire loops	☐ Present	☐ Absent		
Fibrinoid necrosis	☐ Present	☐ Absent		
Karyorrhexis	☐ Present	☐ Absent		
Other lesions?	☐ Yes	☐ No	If yes, please describe	
Comments				

Figure 1. Scoring form sent to the membership of the Renal Pathology Society
Respondents were asked to score 30 images containing one glomerulus each

field, moderately experienced, or highly experienced). In addition to this self-assessment, the participating pathologists were asked how many years they had practiced as a renal pathologist, how many native biopsies they see each year, what percentage of these biopsies was diagnosed as lupus nephritis, and how many lupus nephritis cases they evaluated in the context of research. The answers to these questions were then combined into a single value that was used to estimate the total number of lupus biopsies evaluated by each pathologist prior to participation in this study.

Statistical procedures

For each outcome parameter, the kappa value[10] or intraclass correlation (ICC)[11] value was calculated in order to measure the degree of interobserver agreement (0 = no agreement, 1 = perfect agreement). To calculate these values, we compared the answers given by the participants, rather than comparing the answers with a gold standard. ICCs were calculated using a mixed model to estimate the variance components of the ICC. If there were more than two nominal (non-ordinal) categories an unweighted kappa value was calculated (in such cases, the ICC would be less appropriate, because ICC values imply quadratic weights for differences in agreement). The ICC or kappa value was also calculated for two separate levels of experience (specifically, nephropathologists who were new to the field/moderately experienced [less-experienced] and nephropathologists who were highly experienced) and different continents. We used a sign test (exact variant) to test the null hypothesis that within the set of parameters, the direction of the difference between less-experienced and highly experienced pathologists would be random. A kappa or ICC of <0.4, 0.4-0.6, 0.6-0.8, or >0.8 was considered to reflect poor, moderate, good or excellent agreement, respectively.[8] For the analysis, if no lesion was present that would categorize the biopsy as either class III or class IV, the other parameters were also considered absent, unless otherwise specified by the responding pathologist. A separate analysis was performed on the presence of endocapillary proliferation with respect to the presence of swelling of endothelial cells and influx of inflammatory cells. For this analysis the total number of observations (calculated by multiplying the number of participants by number of glomeruli) for endocapillary proliferation was used (*i.e.* the total number of times the question about endocapillary proliferation was answered with either absent or present). The answers to the (sub-) questions on swelling of endothelial cells and influx of inflammatory cells were related

to that number of observations (in percentages). In addition, we provided the number of pathologists who gave a specific combination of answers at least once.

We used the independent-samples Mann-Whitney *U* test to compare the total number of biopsies with lupus nephritis evaluated by less-experienced pathologists (*i.e.*, new to the field and moderately experienced pathologists) with the total number of biopsies with lupus nephritis evaluated by more experienced pathologists (highly experienced). This non-parametric test was used, because the data were not distributed normally. A *P*-value <0.05 was considered statistically significant. All analyses were performed using SPSS 20.0 (IBM, Armonk, NY).

Results

The ICC for the presence of a lesion that would classify the biopsy as class III or IV was 0.39, which is relatively poor. The kappa/ICC values for the additional parameters were as follows: active, chronic or both: 0.36; segmental versus global: 0.39; endocapillary proliferation: 0.46; influx of inflammatory cells: 0.32; swelling of endothelial cells: 0.46; extracapillary proliferation: 0.57; type of crescent: 0.46; and wire loops: 0.35. Fibrinoid necrosis and karyorrhexis were excluded from the analysis, because these two parameters lacked sufficient variance in our cohort to calculate a reliable kappa or ICC value. The highly experienced nephropathologists (n=19) had higher interobserver agreement for all parameters compared to the less-experienced nephropathologists (n=15; five were new to the field, and 10 were moderately experienced; *P*=0.004) (Table 1). Before their participation in this study, the pathologists who considered themselves to be highly experienced had evaluated significantly more lupus nephritis biopsies (median=525 biopsies per pathologist) than less-experienced pathologists who considered themselves to be either new to the field or moderately experienced (median=128 biopsies per pathologist; *P*=0.002 versus highly experienced pathologists) (Supplemental Figure 1).

In view of the regional variations in the prevalence of lupus (nephritis)[12 13], we examined whether interobserver agreement differs between continents. We found that the between-continent differences were not consistent for all parameters and seemed to primarily reflect the relative proportion of highly experienced nephropathologists in each continent (Supplemental Table 1).

Our study had a relatively low response rate (9.4%). To test for a possible response bias based on each participant's country of origin, we compared the distribution of participants

Table 1. Intraclass correlation and kappa-values for all parameters

Parameter	Statistical Method	Overall[a]	Level of Experience	
			New or Moderate[a] (n=15)	High[a] (n=19)
Class III/IV	Intraclass correlation	0.39	0.33	0.43
Active and/or chronic	Kappa	0.36	0.27	0.38
Segmental or global	Kappa	0.39	0.27	0.46
Endocapillary proliferation	Intraclass correlation	0.46	0.42	0.50
Influx of inflammatory cells	Intraclass correlation	0.32	0.19	0.45
Swelling of endothelial cells	Intraclass correlation	0.46	0.40	0.52
Extracapillary proliferation	Intraclass correlation	0.57	0.42	0.71
Type of crescent	Kappa	0.46	0.40	0.55
Wire loops	Intraclass correlation	0.35	0.27	0.41

[a] >0.8: excellent; 0.6–0.8: good; 0.4–0.6: moderate; <0.4: poor

Table 2. Inflammatory cell influx and swelling of endothelial cells in endocapillary proliferation, as scored by the participants

Endocapillary proliferation	Swelling of Endothelial Cells	Inflammatory Cell Influx	Observations n (%)	Pathologists[b] n
Present (observations; *n*=535[a])	-	-	10 (2)	5
	+	-	115 (21)	24
	-	+	48 (9)	17
	+	+	362 (68)	34
Absent (observations; *n*=65[a])	-	-	41 (63)	18
	+	-	14 (22)	4
	-	+	3 (5)	2
	+	+	7 (10)	4

[a] The total number of observations for endocapillary proliferation (calculated by multiplying the number of participants by the number of glomeruli) if answered with either absent or present

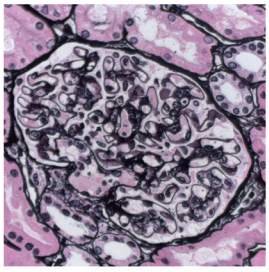
[b] Number of participating pathologists (total n=34) who gave this combination of answers at least one time

throughout the six continents with the distribution of RPS members in general and found similar distributions (Supplemental Table 2).

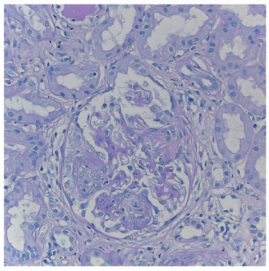
We next analyzed the role, as perceived by the participants, of swelling of endothelial cells and the influx of inflammatory cells in endocapillary proliferation. This analysis revealed that, in 23% of the times a glomerulus was considered to have endocapillary proliferation, it did not include the presence of inflammatory cell influx. In 15% of the times that a glomerulus was designated by the participant as lacking endocapillary proliferation, inflammatory cell

influx was marked as being present but apparently not considered to be representative of endocapillary proliferation. In an additional 22% of the times a glomerulus was designated as lacking endocapillary proliferation, swelling of endothelial cells was noted by the participant (Table 2).

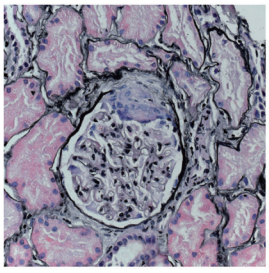
Figure 2 shows three example images of glomeruli. For each glomerulus, a selection of the scoring results is shown, including a selection of comments made by the responding participants.



Class III/IV	41% YES	59% NO	
Of this 41%:			
Segmental or global	50% SEGMENTAL	50% GLOBAL	
Endocapillary proliferation	86% PRESENT	14% ABSENT	
Inflammatory cell influx	92% PRESENT	8% ABSENT	
Wire loops	29% PRESENT	71% ABSENT	
Comments	"THERE ARE CIRCULATING CELLS IN CAPILLARIES: WOULD NOT CALL IT ENDOCAPILLARY PROLIFERATION"		



Class III/IV	100% YES	0% NO	
Active and/or chronic	35% ACTIVE	65% ACTIVE/CHRONIC	
Segmental or global	58% SEGMENTAL	42% GLOBAL	
Extracapillary proliferation	91% PRESENT	9% ABSENT	
Crescent type[a]	58% CELLULAR	42% FIBROCELLULAR	
Comments	"SEGMENTAL SCAR" "EXTRACAPILLARY PROLIFERATION BUT NOT YET A CRESCENT"		



Class III/IV	82% YES	18% NO	
Of this 82%:			
Active and/or chronic	48% ACTIVE	16% CHRONIC	36% ACTIVE/CHRONIC
Segmental or global	86% SEGMENTAL	14% GLOBAL	
Endocapillary proliferation	14% PRESENT	86% ABSENT	
Extracapillary proliferation	100% PRESENT	0% ABSENT	
Comments	"THERE IS A FIBROCELLULAR CRESCENT BUT NOT MORE THAN 25%. SILLY CLASSIFICATION SYSTEM!"		

Figure 2. Examples of glomeruli with poor agreement between observers and a selection of the scoring results

Left panel shows pictures of three glomeruli (two silver-stained and one periodic acid-Schiff-stained), and right panel shows the summary responses from the 34 respondents for each glomerulus. alf extracapillary proliferation is present.

Discussion

The aim of our study was to measure global interobserver agreement regarding the identification of class III/IV lesions in lupus nephritis and determine possible causes of disagreement. We found that agreement among 34 pathologists from 12 different countries was poor, leaving considerable room for improvement. Interestingly, the responding nephropathologists with more experience had higher agreement than less experienced pathologists. However, even within the subgroup of highly experienced nephropathologists, the agreement was only moderate at best.

Although the histologic classification system for lupus nephritis is nearly 40 years old, remarkably few studies have been performed to investigate interobserver agreement using this classification system. The most recent studies of interobserver agreement[7-9] have been done in the aftermath of the introduction of the revised ISN/RPS classification system in 2003. In contrast to these earlier studies, we decided to focus on interobserver agreement on the lesions of class III/IV – instead of the entire classification, including classes I, II, V and VI – because the recognition of these lesions has the highest clinical relevance. However, the results from our study can be compared to previous studies regarding the reproducibility of the activity and chronicity indices in lupus nephritis. Two studies from the 1990s examining interobserver and intraobserver agreement showed a low reproducibility of the (components of the) indices. Schwartz *et al.* [14] attributed this finding primarily to differences in interpreting the components of the indices among the four participating expert pathologists. Wernick *et al.* [15] attributed the low reproducibility to a lack of experience of the pathologists in their study's nonacademic setting. Wernick *et al.* also noted that the most experienced pathologist in their study had the highest intraobserver reliability; this effect of experience is consistent with our results. Moreover, as in our study, the components of the indices relating to endocapillary proliferation were less reproducible than the components relating to extracapillary proliferation.

To measure interobserver agreement regarding class III/IV lesions in lupus nephritis, we chose to use pictures of individual glomeruli rather than biopsy slides. The main advantage of this approach is that it minimizes the sources of variability that are inherent to looking at whole biopsies (*i.e.*, each pathologist looking at a different glomerulus). Our approach also allowed us to focus specifically on the reproducibility of class III/IV lesion identification and examine possible causes of variability in higher detail. However, because of the nature of our study, a drawback is that there is a bias the selection of the glomeruli used ; however, we made every effort to include a representative sample of the various lesions that occur in

lupus nephritis. Also, we noted that there may have been a response bias, because only 9.4% of all RPS members responded. However, the participants in this study seemed to reflect the RPS membership reasonably well with respect to the participants' distribution among the different continents and with respect to the wide range of experience levels among our participants. Finally, our results cannot be directly extrapolated to the reproducibility of the entire ISN/RPS classification system for lupus nephritis, because classifying an image of a glomerulus differs from classifying renal biopsy specimens that contain multiple glomeruli cut at multiple levels and stained with several different stains. Because we did not study whole biopsies, we can only speculate about the effect of our results on the agreement in clinical practice. On one hand, the agreement with respect to particular features of the biopsy could be worse, because instead of having only one glomerulus on to agree, there would be many glomeruli. Using the Banff classification system of renal transplant pathology, Furness *et al.* [16] reported that reproducibility increased when pictures were assessed rather than an entire biopsy. On the other hand, agreement with respect to class assignment may be higher: when one—or a few—glomeruli contain a clear lesion, the effect of other, less clear lesions would be diminished. Nevertheless, this does not detract from the value of this study in revealing that there is considerable room for improvement in the identification of lesions belonging to class III/IV lupus nephritis.

Several factors could have caused interobserver variation, including a lack of objectivity, technical variability, the participating pathologist's experience, ambiguous definitions of lesions, and nonadherence to the classification methodology. In this study, a lack of objectivity and technical variability likely played only a minor role. Objectivity was ensured by blinding the participants to the clinical data. Technical variability was minimized by using sections that were cut and stained in the same laboratory and by distributing the same pictures of glomeruli to all participants. However, the participating nephropathologists' experience may have played an important role, because more experienced nephropathologists have higher interobserver agreement than less experienced nephropathologists. Finally, the role of ambiguous lesion definitions and non-adherence to classification methodology are discussed below.

To characterize a lesion as belonging to class III/IV, one of the challenges is to decide whether or not endocapillary proliferation is present; in this respect, ICC was only 0.46. When studying how the influx of inflammatory cells and endothelial cell swelling were scored in this context, we found that, in 23% of times a glomerulus was perceived to have endocapillary proliferation, influx of inflammatory cells was marked as absent and that, in

9% of these times, the designation of endocapillary proliferation was based solely on the influx of inflammatory cells. Moreover, in cases where participants considered endocapillary proliferation to be absent, they still marked influx of inflammatory cells to be present in 15% and endothelial cell swelling to be present in 22% of cases. Thus, it seems to be unclear how to interpret the definition of endocapillary proliferation. In the ISN/RPS 2003 classification system,[6] endocapillary proliferation is defined two times. The first definition is "endocapillary hypercellularity due to increased numbers of mesangial cells, endothelial cells, AND infiltrating monocytes, AND causing narrowing of the glomerular capillary lumina", and the second definition is "endocapillary hypercellularity with OR without leukocyte infiltration AND with substantial lumen reduction". These two definitions have in common that a lumen reduction is required for endocapillary proliferation. It is, however, unclear how narrowed the lumina should be, which might explain why, in 37% of the observations with perceived absence of endocapillary proliferation, influx of inflammatory cells and/or endothelial cell swelling was marked as present. However, these two definitions differ with respect to the composition of the endocapillary hypercellularity. In contrast to the first definition, the second definition states that an influx of inflammatory cells is not necessarily a part of endocapillary proliferation. It is also not clear if the influx of inflammatory cells alone is sufficient. This ambiguity seems to be reflected in the abovementioned scoring of endocapillary proliferation by the participants. Finally, the question remains if it is even possible to reliably distinguish between the different components of endocapillary proliferation.

The agreement on the distribution of lesions within the glomeruli (*i.e.*, segmental or global) was poor (kappa value=0.39). Segmental lesions are currently defined as "involving less than half of the glomerular tuft". However, this definition does not tell the pathologist how to account for extracapillary proliferation (which occurs outside the tuft). Furthermore, wire loops that are not obviously global are difficult to incorporate in this context. Moreover, the relevance of distinguishing between segmental (S) and global (G) lesions has been a subject of debate. For example, Haring *et al.*[17] performed a meta-analysis and found no difference in clinical outcome between patients with class IV-S and patients with class IV-G lesions. However, other groups suggest that a biological difference exists between IV-S and IV-G and argue that the distinction should remain in the classification system.[18] If this distinction remains, more explicit definitions should be devised in order to make the distinction both reliable and reproducible.

Finally, extracapillary proliferation and the designation of lesions as active or chronic (or

both) caused confusion among the respondents. First, although the classification system states that extracapillary proliferation should occupy at least one quarter of the glomerular capsular circumference to qualify as extracapillary proliferation, only a few of the responding pathologists used this criterion in their scoring (even in the highly experienced pathologists group). Second, although fibrocellular crescents are designated as active lesions, many respondents seem to interpret them as chronic or active/chronic lesions. Third, although double contours are not listed as chronic lesions in the classification system, some of the respondents apparently perceived them as such**.**

Although the introduction of the 2003 ISN/RPS classification system significantly improved interobserver agreement relative to the 1995 WHO system, our results indicate there is still considerable room for improvement in the identification of lesions (in individual glomeruli) belonging to class III/IV lupus nephritis. Improving interobserver agreement—particularly with respect to the presence of class III/IV lesions—has high clinical relevance, because correctly identifying the histologic class plays an essential role in deciding whether to initiate immunosuppressive therapy when treating patients with lupus nephritis. The observation that highly experienced pathologists have higher agreement than less experienced pathologists suggests that agreement can be improved—at least in part—by educating nephropathologists. Moreover, as discussed above, agreement might be improved by revising and clarifying some of the definitions in the current classification system. Finally, our results underscore the need for a central review of biopsies in clinical trials by a minimum of two experienced nephropathologists.

Disclosures

All the authors declared no competing interest.

Acknowledgments

We would like to thank the Renal Pathology Society, in particular H.K. Singh, and all of the participating members for their contribution to this study. Furthermore, we would like to thank M. Mengel for reviewing the manuscript. The results presented in this paper have been presented in abstract form at the Annual American Society of Nephrology Kidney Week, Atlanta, Georgia, November 5-10, 2013.

References

1. Bernatsky S, Boivin JF, Joseph L, et al. Mortality in systemic lupus erythematosus. *Arthritis Rheum* 2006;54(8):2550-7.

2. Bono L, Cameron JS, Hicks JA. The very long-term prognosis and complications of lupus nephritis and its treatment. *Q J Med* 1999;92(4):211-18.

3. Bertsias GK, Tektonidou M, Amoura Z, et al. Joint European League Against Rheumatism and European Renal Association–European Dialysis and Transplant Association (EULAR/ERA-EDTA) recommendations for the management of adult and paediatric lupus nephritis. *Ann Rheum Dis* 2012;71(11):1771-82.

4. Hahn BH, McMahon MA, Wilkinson A, et al. American College of Rheumatology guidelines for screening, treatment, and management of lupus nephritis. *Arthritis Care Res* 2012;64(6):797-808.

5. Kidney Disease: Improving Global Outcomes (KDIGO) Glomerulonephritis Work Group. KDIGO Clinical Practice Guideline for Glomerulonephritis. *Kidney Int Suppl* 2012;2:139-274.

6. Weening JJ, D'Agati VD, Schwartz MM, et al. The classification of glomerulonephritis in systemic lupus erythematosus revisited. *J Am Soc Nephrol* 2004;15(2):241-50.

7. Furness PN, Taub N. Interobserver reproducibility and application of the ISN/RPS classification of lupus nephritis-a UK-wide study. *Am J Surg Pathol* 2006;30:1030-5.

8. Grootscholten C, Bajema IM, Florquin S, et al. Interobserver agreement of scoring of histopathological characteristics and classification of lupus nephritis. *Nephrol Dial Transplant* 2008;23:223-30.

9. Yokoyama H, Wada T, Hara A, et al. The outcome and a new ISN/RPS 2003 classification of lupus nephritis in Japanese. *Kidney Int* 2004;66(6):2382-8.

10. Silcocks PB. Measuring repeatability and validity of histological diagnosis--a brief review with some practical examples. *J Clin Pathol* 1983;36(11):1269-75.

11. Fleiss JL, Cohen J. Equivalence of weighted kappa and intraclass correlation coefficient as measures of reliability. *Educ Psychol Meas* 1973;33(3):613-19.

12. Pons-Estel GJ, Alarcon GS, Scofield L, et al. Understanding the epidemiology and progression of systemic lupus erythematosus. *Semin Arthritis Rheum* 2010;39(4):257-68.

13. Feldman CH, Hiraki LT, Liu J, et al. Epidemiology and sociodemographics of systemic lupus erythematosus and lupus nephritis among US adults with Medicaid coverage, 2000-2004. *Arthritis Rheum* 2013;65(3):753-63.

14. Schwartz MM, Lan SP, Bernstein J, et al. Irreproducibility of the activity and chronicity indices limits their utility in the management of lupus nephritis. *Am J Kidney Dis* 1993;21(4):374-77.

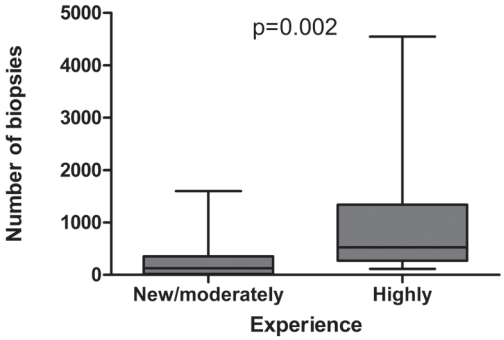
15. Wernick RM, Smith DL, Houghton DC, et al. Reliability of histologic scoring for lupus nephritis: a community-based evaluation. *Ann Intern Med* 1993;119(8):805-11.

16. Furness PN, Taub N, Assmann KJ, et al. International variation in histologic grading is large, and persistent feedback does not improve reproducibility. *Am J Surg Pathol* 2003;27(6):805-10.

17. Haring CM, Rietveld A, van den Brand JA, et al. Segmental and global subclasses of class IV lupus nephritis have similar renal outcomes. *J Am Soc Nephrol* 2012;23(1):149-54.

18. Hill GS, Delahousse M, Nochy D, et al. Class IV-S versus class IV-G lupus nephritis: clinical and morphologic differences suggesting different pathogenesis. *Kidney Int* 2005;68(5):2288-97.

Supplement



Supplemental Figure 1. The total number of biopsies with lupus nephritis that were evaluated by pathologists prior to participation in this study, grouped by experience

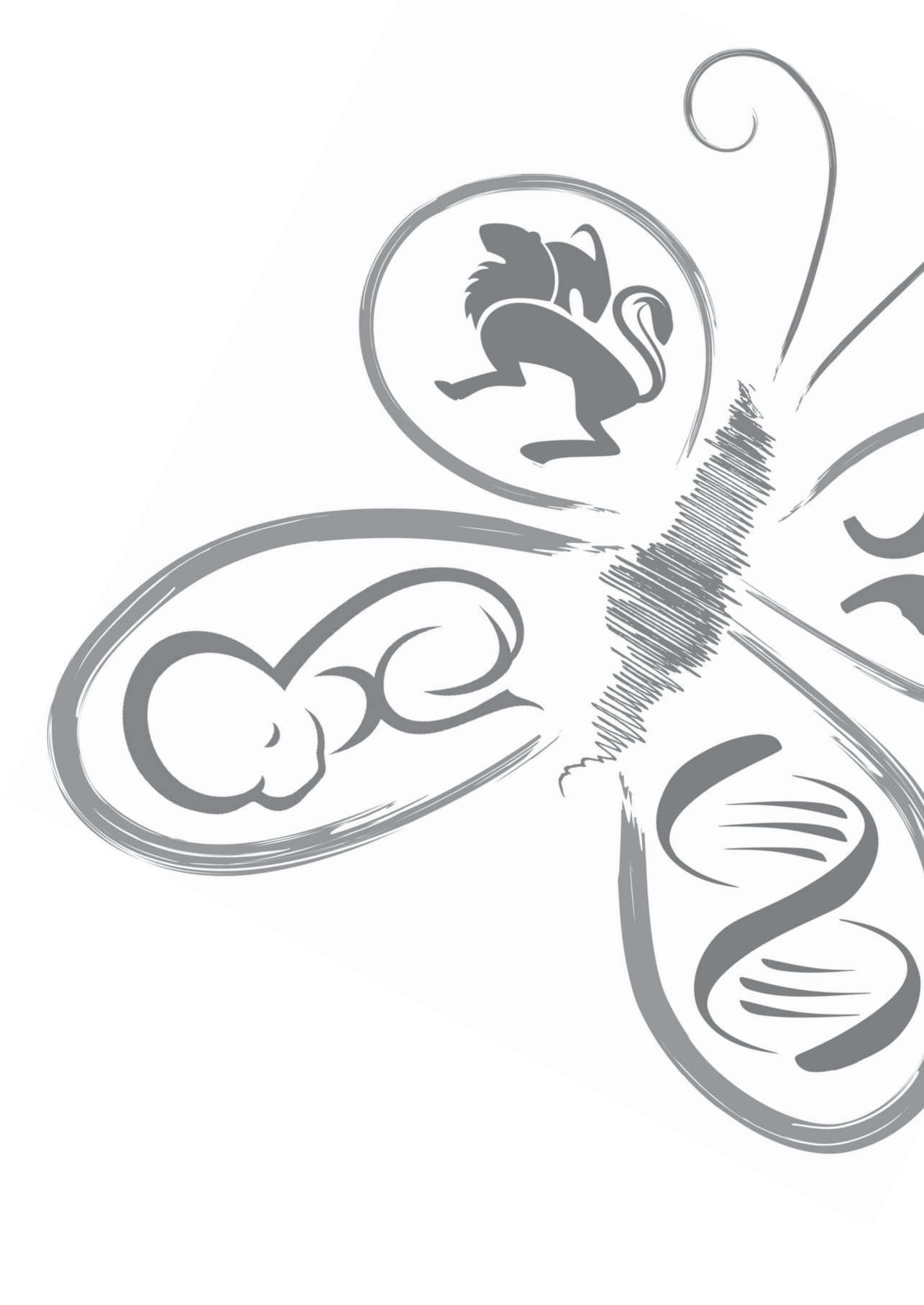
Supplemental Table 1. Kappa and ICC values for all parameters, analyzed per continent

Parameter	Statistical method	Overall[a]	Continent			
			Europe[b] (n=9)	Asia[c] (n=7)	Australia[d] (n=2)	North America[e] (n=16)
Class III/IV	ICC	0.39	0.32	0.36	0.61	0.42
Active and/or chronic	Kappa	0.36	0.34	0.25	0.51	0.36
Segmental or global	Kappa	0.37	0.33	0.28	0.49	0.38
Endocapillary proliferation	ICC	0.46	0.45	0.41	0.73	0.47
Influx of inflammatory cells	ICC	0.32	0.43	0.20	0.12	0.31
Swelling of endothelial cells	ICC	0.46	0.51	0.37	0.84	0.46
Extracapillary proliferation	ICC	0.57	0.51	0.38	0.68	0.68
Type of crescent	Kappa	0.46	0.46	0.34	0.61	0.60
Wire loops	ICC	0.35	0.39	0.33	0.57	0.34

[a] >0.8: excellent; 0.6–0.8: good; 0.4–0.6: moderate; <0.4: poor
[b] Europe: 2 new, 2 moderately experienced, and 5 highly experienced nephropathologists.
[c] Asia: 6 moderately experienced and 1 highly experienced nephropathologist.
[d] Australia: 1 moderately experienced and 1 highly experienced nephropathologist. Comparison with other continents was not due to the small number of pathologists from this continent.
[e] North America: 3 new, 1 moderately experienced, and 12 highly experienced nephropathologists.
ICC, intraclass correlation

Supplemental Table 2. Distribution of the study participants and the entire RPS membership by continent

	Our study (%)	RPS membership (%)
Europe	26.5	15.2
Asia	20.6	16.3
Australia	5.9	1.6
North America	47.1	62.6
South America	0.0	3.5
Africa	0.0	0.8



3

The Revisited Classification of Glomerulonephritis in Systemic Lupus Erythematosus at 10 years: Time to Re-evaluate Histopathologic Lesions

Suzanne Wilhelmus, Charles E. Alpers, H. Terence Cook, Franco Ferrario, Agnes B. Fogo, Mark Haas, Kensuke Joh, Laure-Hélène Noël, Surya V. Seshan, Jan A. Bruijn, and Ingeborg M. Bajma

Journal of the American Society of Nephrology 2015; 26: 2938-2946

Abstract

Over 10 years have passed since the latest revision of the histopathologic classification of lupus nephritis. This revision was a significant improvement compared to the previous version, mainly because of clearer and more concise definitions and the elimination of mixed subclasses. Despite these improvements, there are still some difficulties in the classification for lupus nephritis, many of which are in the definitions provided. In this review, we focus on the difficulties surrounding the evaluation of classes III and IV lesions, particularly the definitions of endocapillary and extracapillary proliferation, the use of the terms endocapillary proliferation and hypercellularity, the clinical relevance of segmental and global subdivisions in class IV, and the value of distinguishing lesions that indicate activity and chronicity. Vascular and tubulointerstitial lesions are also discussed. Furthermore, we give an overview of the history of the classification to provide background on the origin and development of the definitions in lupus nephritis. The issues raised in this review, as well as the suggestions for improvements may assist with a revision of the lupus nephritis classification in the near future.

Introduction

Over 10 years have elapsed since the latest revision of the classification of glomerulonephritis (GN) in SLE, which also known as the International Society of Nephrology/ Renal Pathology Society (ISN/RPS) lupus nephritis (LN) classification.[1 2] This revision is generally considered an improvement to the previous classification. The improvement was mainly attributed to clearer and more precise definitions of classes and lesions and elimination of the mixed subclasses of membranous LN and class III/IV lesions, which led to a relatively high reproducibility compared with previous versions.[3 4] Nevertheless, from experience in a group of nephropathologists who specialized in LN, it became apparent that there are still many difficulties in the current version of the classification, mostly originating from uncertainties and inconsistencies in the definitions of histologic parameters. In a recent study focussing on class III and IV lesions, considerable interobserver variation among nephropathologists in evaluating these lesions was shown.[5] Taking the opportunity to further improve the classification may add to its usefulness in clinical practice and to better interobserver agreement among nephropathologists. Therefore, the purpose of this paper is to provide a critical reading of the latest version of the classification,[1 2] list points to be considered for clarification, and offer suggestions for improvements, which may be used to guide a revision of the classification in the near future.

Biopsy requirements

Reporting of the number of glomeruli in a biopsy confers a level of certainty with regard to the accuracy of the assigned class.[6] In the ISN/RPS classification paper,[1 2] a minimum of 10 glomeruli is advised for the classification of LN, but it is uncertain what to do with incomplete glomeruli on the edge of the biopsy or small tangential sections of glomeruli. For research purposes, such as in the Oxford IgA nephropathy (IgAN) classification,[7] a glomerulus is required to have at least three mesangial areas to be included in the number of glomeruli scorable for mesangial hypercellularity.

In the ISN/RPS classification paper[1 2] it is recommended to cut the biopsy at multiple levels. Although useful in clinical practice, it is a complicating factor in classifying LN, because there are no guidelines on how to establish the final decision on class after this exercise. It is cumbersome and not always possible to track each glomerulus through different levels. Furthermore, it is currently unclear if a glomerular lesion should be designated as segmental or global when this differs between multiple levels of the same glomerulus. The segmental or global involvement of a glomerulus already has been shown to have low interobserver

agreement when one the basis of one glomerulus at one level.[5] All of these considerations are of particular importance in LN, because they can make the difference between class III and IV or between classes IV-segmental (IV-S) and IV-global (IV-G), the latter distinction being especially complex and controversial. We conclude that more specific guidelines are needed on how to deal with multiple levels and incomplete glomeruli in classifying LN.

Classes I and II

The lack of quantitative guidelines, which ideally would define cutoff values, is a common problem in many definitions, despite the improvements already made in the ISN/RPS classification. In class I, glomeruli show deposits by immunofluorescence and electron microscopy, whereas they should appear normal by light microscopy. Class II is defined as mesangial proliferative LN. This class is characterized by any degree of mesangial hypercellularity, where the hypercellularity is defined as three or more mesangial cells per mesangial area in a 3-micron-thick section. The origin of this cutoff is unclear. The only previously described cutoff stems from the 1974/1975 World Health Organization (WHO) classification,[8] in which mesangial hypercellularity is defined as more than three cells per mesangial area away from the vascular pole (Box 1). The latter is equivalent to the Oxford IgAN classification, where mesangial hypercellularity is defined as four or more mesangial cells per area[7] rather than three or more cells. In LN, although three or more cells in a mesangial area is a clear-cut guideline, there is, unfortunately, limited information on the extent of mesangial proliferative lesions necessary to classify a biopsy as class II. The definition that any degree of mesangial proliferation would suffice for class II implies that one glomerulus, independent of the total number of glomeruli, with one mesangial area containing three cells would be enough to classify the biopsy as class II. It is questionable if this is what was meant. Ultimately, it may be questioned if the amount of mesangial proliferation defining either class I or II has any clinical relevance. This was at least not what was intended in the current division in class I and II. Apart from mesangial cell proliferation, mesangial matrix expansion is also used to define class II (table 3 in the ISN/RPS classification paper[1,2]). However, no definition of mesangial matrix expansion is given.

It is not entirely clear how many subepithelial and subendothelial deposits are allowed in class II. It is stated that “a few isolated subepithelial or subendothelial deposits may be visible by immunofluorescence or electron microscopy, but not by light microscopy”.[1,2] Quantifying what is meant by a few isolated subepithelial or subendothelial deposits would be helpful to make the diagnosis of class II LN more straightforward and most importantly, to clearly

Box 1: A history of lupus nephritis with a focus on terminology

1955

Preceding the first attempt towards a classification of LN in 1964, Pirani and Pollak in collaboration with Muehrcke, Kark, and Steck reported in detail on the individual histologic lesions in LN in 1955.[38] Muehrcke *et al*. [38] reported that the earliest detectable histologic lesions consisted of minute foci of hypercellularity at the periphery of the glomerular tufts as a result of endothelial cell proliferation. This was called local glomerulitis. Local was used, because initially, the lesion consisted of one or two patches of proliferating endothelial cells near the periphery of the tuft. The term proliferation in this article is always used in conjunction with the endothelium. It uniquely referred to endothelial proliferation, although this was never actually proven.

1964

The Natural History of Renal Manifestations of SLE was reported on in 1964[39] (a reprint of this article together with the original authors' comments appeared in 1997 in JASN[40]). Histologic findings in 176 renal biopsy and necropsy specimens were grouped according to the following categories: (1) no histologic evidence of renal involvement, (2) lupus glomerulitis, (3) active lupus GN, and (4) membranous lupus GN.

Among the histologic findings considered to reflect the presence of activity was cellular proliferation in glomeruli. The description of lesions found in the four classes in the 1964 article[39] are at the basis of the classification of LN as we know it today. It is interesting that, in these early beginnings, confusion on how to define the separate components of the glomerular changes already became apparent. Lupus glomerulitis was distinguished from active lupus GN. Most likely, this distinction hinged on whether the interstitium was involved in the inflammation, but there were also glomerular lesions that were more characteristic of one versus the other. Local necrosis, obliteration, karyorrhexis, and fibrinoid changes are specifically mentioned as part of lupus glomerulitis. For active lupus GN, areas with glomerular hypercellularity and on occasion, the occurrence of a few polymorphonuclear leukocytes were mentioned.

1970

Baldwin *et al.*[41] described clinical histopathologic correlates of patients with focal proliferative LN, diffuse proliferative LN, or membranous LN. It was observed that in focal LN, for the most part, only small portions of glomeruli were affected, whereas in diffuse LN, usually larger

portions of each glomerulus were involved. Cutoff points in terms of percentages were not given. The difference in morphologic appearance, severity, and clinical course was suggested to point towards different pathogenic mechanisms.

1974/1975

The WHO classification for LN resulted from deliberation at international conferences in Buffalo, New York and Geneva, Switzerland in 1974 and 1975, respectively. An official WHO classification was never published in the peer-reviewed literature; however, the first journal article that referred to the classification was by Appel *et al.*[8] in 1978. This WHO system included a purely mesangial form of LN as well as focal, diffuse, and membranous forms. The pathologic definition of the purely mesangial form of LN was already quite complex: "segmental or global, focal or diffuse hypercellularity confined to the mesangium – more than three cells per mesangial area away from the vascular pole in two to four micron sections and/or increased matrix with widening of the mesangial stalk".[8] Zimmerman *et al.*[42] had independently described the mesangial proliferative variant in 1975, and Baldwin *et al.* [43] added this variant to their classification in 1977.

In the publication by Appel *et al.*,[8] 56 patients with LN were entered into a clinicopathologic analysis using the WHO classification from 1974/1975, and Roman numerals were, for the first time, used to identify the different classes. In this 1978 publication, descriptions of the five classes were enriched by immunofluorescence and electron microscopy data.[8] In fact, in the discussion, Appel *et al.*[8] concluded that the location of immune complex deposits as defined by immunofluorescence studies and the host response that these immune complexes stimulate form the basis of the histologic classification of LN. Although classes III and IV were regarded as two forms of LN reflecting different stages of the same process, it was also mentioned that class IV may have a membranoproliferative variant.

1982

Eight years after the introduction of the first WHO scheme, it was modified by a consensus conference held during the International Study of Kidney Diseases in Children Meeting in Paris in 1980.[44] Instead of the 50% cutoff to differentiate between classes III and IV, which was introduced in the 1974/1975 version, class III was defined as focal segmental GN, and class IV was defined as diffuse GN. Because of the lack of a definitive explanation of the distinction between classes III and IV, there was substantial controversy over the importance of segmental

inflammatory lesions versus % of glomeruli involved in distinguishing between classes III and IV. Classes III and IV were subdivided into three and four subclasses, respectively. Also, class V was subdivided for the possible combinations with class II, III, or IV LN. Finally, class VI (advanced sclerosing GN) was introduced but not specifically defined. This classification was considered too complicated by many pathologists, causing them to continue using the unofficial version published by Appel *et al.*.[8]

1995

Additional modifications were made and published in the second edition of the book on the classification of glomerular diseases by Churg *et al*.[45] These modifications consisted of the elimination of classes Vc and Vd to describe combined membranous and class III or IV LN. Also, the original 50% cutoff in class III versus class IV was mentioned again, but it was stated that "... this division is not clear-cut. Rather there is a continuum of changes, and the clinical behavior usually parallels the proportion of involved glomeruli. It might be better, therefore, to include all cases of proliferative LN in class IV and to specify the degree of involvement as mild, moderate, or severe. The class III designation should be reserved for cases with focal segmental necrotizing lesions".[45]

2004

Another classification was proposed by the ISN/RPS Consensus Conference on the Classification of Lupus Glomerulonephritis. This system resembled the WHO system but has more detailed definitions and clearer distinctions among the classes.[1 2] Notably, in the overview of the classes, classes III and IV are not called proliferative, because pure chronic sclerosing lesions were also included. This is in accord with the WHO classification, which also did not use proliferative in the diagnostic terms for class III and IV[44 45]; however many published accounts of the classification inserted the term proliferative inappropriately, and the term has been widely used in practice. This probably stems from the use of proliferative in the unofficial reference to the WHO classification in the article by Appel *et al.*,[8] which used terminology in use by Baldwin[41] and Pirani.[39] In the current classification, the term proliferative is still used in the description of the classes III/IV-A(/C).LN, for the most part, only small portions of glomeruli were affected, whereas in diffuse LN, usually larger portions of each glomerulus were involved. Cutoff points in terms of percentages were not given. The difference in morphologic appearance, severity, and clinical course was suggested to point towards different pathogenic mechanisms.

distinguish it from class III. However, it would be challenging to establish an evidence-based quantitative standard for this using information currently available.

Class III and IV

Endocapillary proliferation

Endocapillary proliferation, a key feature of active classes III and IV LN, is defined as: "endocapillary hypercellularity due to an increased number of mesangial cells, endothelial cells, and infiltrating monocytes, and causing narrowing of the glomerular capillary lumina" (table 5 in the ISN/RPS classification paper[1 2]). The increased number of mesangial cells in this definition could be confusing, because it is stated in table 3 of the ISN/RPS classification paper[1 2] that classes III and IV are characterized by GN with or without mesangial alterations. What is also not clear from the definition is whether all or only some of the mentioned criteria should be present. The wording suggests that all items should be present. In our experience, many nephropathologists would call lesions, such as those depicted in Figure 1, A and B, endocapillary proliferation, although some of the mentioned criteria are lacking. Of interest is the mention of the monocyte as the inflammatory cell characteristic of endocapillary proliferation (table 5 in the ISN/RPS classification paper[1 2]), whereas in table 6 of the same paper,[1 2] under the descriptions of active lesions, the looser term leukocyte infiltration is used. Substantial luminal reduction is also part of the definition, but how substantial remains unclear. These issues together have probably contributed to the high interobserver variation in recognizing these lesions, which was shown in a recent study.[5]

Another important source of interobserver variation in LN seems to lie in the confusion around the terms proliferation and hypercellularity. In *Dorland's Illustrated Medical Dictionary,*[9] proliferation is defined as "the reproduction or multiplication of similar forms ... see also hyperplasia and hypertrophy", and hypercellularity is defined as "a state characterized by an abnormal increase in the number of cells present, …". It is likely that part of what we consider endocapillary proliferation in LN is not actually "reproduction or multiplication of similar forms", although many instances do represent "a state characterized by an abnormal increase in the number of cells".[9] Often, we do not exactly know which cell types are responsible for what we call endocapillary proliferation or hypercellularity. In our opinion, the lesions characteristic of classes III and IV LN should be clearly redefined, because both there is the large interobserver variation with respect to these lesions[5] and the same terminology is beginning to cause similar problems in other areas of nephropathology, such

as IgAN (I. Bajema, M. Haas, and T. Cook, personal communications). An option would be to avoid the term proliferation altogether, which would have the added benefit of avoiding confusion around the term mesangial proliferative LN for class II.

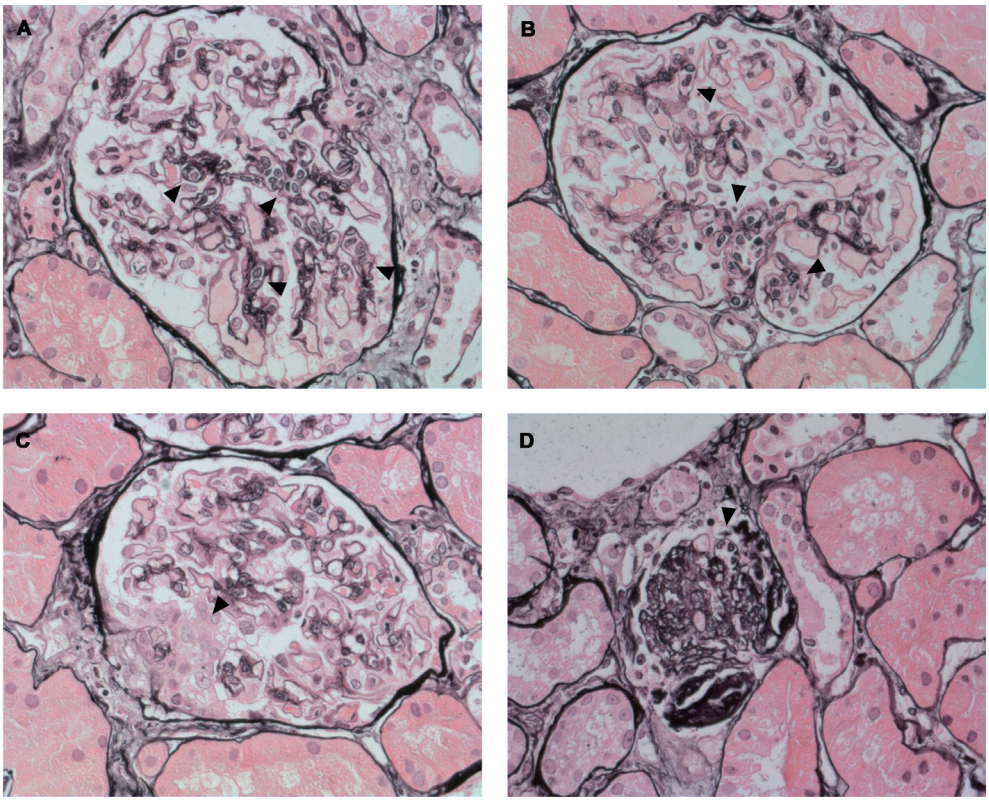


Figure 1. Examples of problematic lesions in LN
(A and B) Is this endocapillary proliferation according to the definition? Arrowheads point to areas that could signify endocapillary proliferation, because there is reduction of the capillary lumen most likely caused by influx of inflammatory cells and/or endothelial swelling. (C) Is this extracapillary proliferation (arrowhead)? According to the classification, it does not qualify, because it spans <25% of the capsular circumference. (D) A globally sclerosed glomerulus located not far from the capsule and adjacent to another globally sclerosed glomerulus (not shown). Is this global sclerosis caused by LN or to another cause? The arrowhead points to two inflammatory cells in the capillary lumen. Silver methenamine stain. Original magnification, X 400.

Extracapillary proliferation

The evaluation of extracapillary proliferation is another challenging issue in classes III and IV LN. The definition of extracapillary proliferation or a cellular crescent given in table 5

in the ISN/RPS classification paper[1 2] is “extracapillary cell proliferation of more than two cell layers occupying one fourth or more of the glomerular capsular circumference”. This definition only holds for a cellular crescent; fibrocellular and fibrous crescents lack a definition. Fibrocellular and fibrous crescents are only mentioned in table 6 of the ISN/RPS classification paper,[1 2] which states that both cellular and fibrocellular crescents are regarded as active lesions and that fibrous crescents are regarded as chronic lesions. The “one fourth or more of the glomerular capsular circumference” is an addendum that many nephropathologists probably disregard, because it would entail that a lesion, such as depicted in Figure 1C, would not be considered to represent extracapillary proliferation. How extracapillary proliferation contributes to determining whether a biopsy falls into either the IV-S or IV-G subcategories is a complicated issue. The segmental or global character of class IV lesions is defined by the extent of the lesions within the glomerular tuft, which consists of glomerular capillaries and mesangial cells,[10] and does not include Bowman’s space and Bowman’s capsule. By definition, therefore, extracapillary proliferation can never contribute to the segmental or global nature of a class IV lesion. If we want to include extracapillary proliferation when assessing the segmental or global nature of the lesion, the area should be redefined in which both endocapillary and extracapillary lesions can occur to establish whether we are dealing with segmentally or globally affected glomeruli. Finally, the term extracapillary proliferation holds some of the same objections as the term endocapillary proliferation. Therefore, one could consider using the term extracapillary hypercellularity rather than extracapillary proliferation.

Segmental and global subdivision

There is a belief among many nephropathologists and nephrologists that a subclass of LN characterized by segmental lesions with fibrinoid necrosis resembling those typically seen in ANCA-associated vasculitis would be clinically relevant. In the latest version of the classification, the segmental and global subdivision within class IV was introduced. This subdivision was based on data from a study by Najafi *et al.*[11] suggesting that this would lead to a subclass of segmental lesions, which comprised the more vasculitic-like lesions in LN, possibly with poor outcome. In a recent meta-analysis by Haring *et al.,*[12] it was demonstrated that there is little clinical significance in relation to outcome of segmental and global LN, as defined in the ISN/RPS classification.[1 2] However, before a final decision is made regarding potential elimination of the IV-S and IV-G subcategories, it should be considered how the definitions of segmental and global were applied in different studies. Most notably, Najafi *et al.*[11] defined their segmental lesions differently from the definition given in the ISN/RPS

classification,[1 2] including lesions involving >50% but not the entire glomerular tuft. In a later study of Schwartz *et al.*[13] these latter lesions, which they termed class IV-Q, were found to have a worse prognosis than segmental lesions involving <50% of the tuft or lesions involving the entire tuft. An older study by Schwartz *et al.*[14] from 1987 did not show a difference in prognosis between cases with segmental (subtotal) involvement of the tuft in more than 50% of glomeruli and patients with a diffuse pattern in >80% of glomeruli. We conclude that it is, thus far, unclear what subdivision (if any) within classes III and IV would be of clinical or prognostic relevance. Most importantly, the premise of the vasculitic-like lesion, which is at the basis of a possible subclass, in time was replaced by the notion of a segmental lesion. It seems evident that, whereas most vasculitic-like lesions will be segmental, many other segmental lesions exist that are not vasculitic-like. Because of the many different definitions that were used in different studies, it seems that only by starting from scratch with new data will it become possible to investigate this issue for future purposes.

Activity and chronicity

In table 6 in the ISN/RPS classification paper[1 2] a summary is given of markers of activity and chronicity of LN to be included in the report. Presumably, these also serve as guidelines towards the usage of the active (A), chronic (C), and A/C subclasses, which are important for making treatment decisions. Although this is incorporated in classes III and IV by the addition of A, C, or A/C, this denotation does not provide any information on the extent of the activity or chronicity. Therefore, it is recommended in the ISN/RPS classification paper[1 2] to report the proportion of glomeruli affected by active and chronic lesions in the diagnostic line. Also the proportion of glomeruli with fibrinoid necrosis and crescents should be reported. Furthermore, it is stated that the activity and chronicity indices by Austin *et al*. [15] can be used. However, the added benefit of these indices is unclear. So far, they have not unequivocally been shown to be of prognostic value when added to clinical information and the histologic class.[15-19] Moreover, they do not show good reproducibility.[20 21] There are some lesions for which the A or C status is debatable (*e.g.,* the membranoproliferative pattern, of which it is stated that this pattern "…is particularly common in the chronic phase of lupus nephritis",[1 2] although no literature reference is provided). Another issue is global glomerulosclerosis. If considered to be the consequence of LN, global sclerosis is cause to designate the biopsy as having a chronic component, but it can be very difficult and often impossible to determine whether global glomerulosclerosis is the result of LN or another cause (Figure 1D). This is also the case for other chronic glomerular lesions, particularly segmental sclerosis, which may result from podocyte injury (*e.g.*, in class V lesions as

discussed below) or postinflammatory scarring. Nevertheless, such lesions may lead to a classification of LN class III C or IV C, with the potential for clinical confusion. To make the distinction between nonspecific glomerulosclerosis and chronic lupus lesions, it may be helpful to look at the location of the glomerulus in question within the biopsy (subcapsular or not), other signs of ischemia, signs of previous active lesions (for example, a convincing fibrous/fibrocellular crescent), or a fragmented-appearing scarred tuft.

Class V

The definition of class V LN seems quite straightforward. The major difficulty is in chronic lesions. It is mentioned that, as class V evolves to chronicity, the development of segmental or global glomerulosclerosis is typical. However, if segmental or global glomerulosclerosis is regarded as sequelae of class III/IV lesions, the biopsy should be designated as class III/IV C + V. Similar to the discussion raised above, it may be challenging to reliably distinguish between segmental or global sclerosis caused by class III/IV or V LN. Review of previous biopsies, if available, for any active class III/IV lesions and subendothelial deposits may be helpful in making this distinction.

Class VI

The definition of class VI LN is relatively straightforward, being on the basis of >90% of globally sclerotic glomeruli without any active glomerular lesions. The primary reason for including this cutoff was to end the arbitrary use of class VI in the WHO system, with some pathologists using class VI for >50%, others >75% or 80% global sclerosis. However, >90% global sclerosis is a rare event, and one may ponder about its clinical usefulness. This class could be combined with biopsies otherwise classified as pure chronic class III or IV as a new chronic LN class VI, in which the extent of sclerosis has to be specified. This has practical implications, in that none of the pure chronic lesions are likely to benefit from immunosuppressive treatment, although the management of the individual patient may vary depending on the percentage of sclerosed glomeruli and clinical presentation.

Glomerular lesions not included in the classification

Apart from the typical histopathologic glomerular lesions on which the classification is based, a number of other glomerular lesions may be encountered. Although these lesions are not part of the classification, they do require the attention of the pathologist and should be reported in the diagnostic line. These lesions include (lupus) podocytopathy, collapsing

glomerulopathy, and thrombotic microangiopathy (TMA). The latter can occur within the context of antiphospholipid syndrome nephropathy, which has been shown to be present in 10%-32% of biopsies with LN.[22-24] However, TMA is not specific for antiphospholipid syndrome nephropathy and can also been seen in, for example, malignant hypertension. In some patients with SLE and a nephrotic syndrome, diffuse foot process effacement without capillary wall deposits can be found by electron microscopy. This finding can be either coincidental idiopathic minimal change disease or more likely, some form of lupus podocytopathy, possibly mediated through T cell activation in SLE.[25-27] Finally, collapsing glomerulopathy can sometimes be encountered in patients with SLE either with or without concomitant LN. Whether this represents coincidental idiopathic collapsing glomerulopathy or should be seen in the context of a lupus podocytopathy remains to be determined. An argument in favor of the latter is that, in the largest patient series reported, 16 of 19 patients had active extrarenal lupus symptoms at time of biopsy.[28]

Vascular lesions

In the current classification, little attention is given to vascular lesions in lupus, although they do seem to have clinical significance. The most common lesion is the presence of isolated immune complex deposits. Furthermore, TMA, lupus vasculopathy, and arterioarteriolosclerosis can occur, while vasculitis is uncommon[29]; Banfi *et al.* [30] showed decreased renal survival if one of the latter four lesions was present. Although it was thought that isolated vascular immune complex deposits did not affect outcome, in a recent study by Wu *et al.,* [31] a worse renal outcome was shown. The current classification does recommend reporting vascular lesions, such as vascular deposits, thrombi, vasculitis, or sclerosis, in the diagnostic line and grading them as mild, moderate, or severe. Specific criteria for this grading are not provided. For intimal sclerosis, it can be considered to use the cutoff values set in the Banff classification of renal transplant biopsies.[32] It has been suggested that the inclusion of a detailed description of renal vascular lesions in the ISN/RPS classification of LN may strengthen the predictive value for renal outcome.[31]

Tubulointerstitial lesions

Tubulointerstitial lesions are correlated with glomerular lesions, but they have also been shown to be prognostic of renal outcome in LN independent of glomerular lesions.[33] Therefore, tubular atrophy, interstitial inflammation, and fibrosis have to be reported in the diagnostic line and graded as mild, moderate, or severe. No cutoff values for this grading

system are provided. It is also unclear if all three parameters should be graded separately or can be combined into one grade for tubulointerstitial damage, because interstitial fibrosis and tubular atrophy have been shown to correlate with tubulointerstitial inflammation in LN.[34] Interestingly, the possible significance of tubulitis in LN has not yet been studied extensively. The reported interobserver agreement for visual assessment of tubular atrophy and interstitial fibrosis using routine stains applied in nephropathology is quite variable.[7 35 36] The reproducible approach reported in the Oxford IgAN classification,[37] in which tubular atrophy and interstitial fibrosis are combined into one grading system, therefore seems most practical.

Concluding remarks

We have given a close reading of the latest version of the LN classification, pointing out problematic issues in the definitions of histopathologic lesions used to classify LN. Solving these problematic issues is not an easy task. Importantly, one has to realize that, in making workable definitions, there is a delicate balance between maximum precision and Gestalt interpretation. Strict definitions may be most useful for research studies and relatively inexperienced nephropathologists, whereas Gestalt interpretation may sometimes serve clinical practice better, because it allows for a more liberal interpretation by experienced pathologists, which may sometimes overrule the strict boundaries of the definitions. The latest version of the classification reflects the compromises that have been made in the very long history of this classification, in which many experts over the years have tried to capture the complex nature of LN. For details on the historic development of the terminology, we refer to Box 1. The lupus classification is one of the few nephropathologic classifications that is closely linked to therapeutic interventions, making it clinically very relevant. Therefore, it is of the utmost importance to clearly define the histopathologic lesions, which form the basis of the classification, to obtain good interobserver agreement among nephropathologists worldwide. In addition, future iterations of the classification may incorporate certain immunologic and/or molecular markers if they are shown to improve diagnostic accuracy and/or clinical correlation beyond histology alone. Points of consideration for further improvement of the classification are listed in Table 1.

Disclosures

None.

Table 1. Concerns and suggestions for improvement for the future revision of the LN classification

Topic and Concerns	Suggestions for Improvement
Biopsy requirements	
No details on how to deal with small or incomplete glomeruli	Following the Oxford IgAN classification: include glomeruli with at least three mesangial fields in the number of scorable glomeruli
No guidelines on how to establish the final decision on classification after evaluation of multiple levels	No clear-cut suggestions from the literature; needs to be further discussed
Class II	
Mesangial proliferation	
It is unclear if the current cutoff for mesangial hypercellularity (three mesangial cells per mesangial area) implies that hypercellularity in one mesangial area suffices for a diagnosis of class II	Redefine cutoff; consider guidelines as used in the Oxford IgAN classification for mesangial hypercellularity and mesangial matrix expansion
Any degree of mesangial matrix expansion (not further defined) is mentioned to be part of mesangial proliferative LN (class II), but it is unclear if and how this is part of the definition	Alternatively, an evidence-based approach in LN specifically can be considered
Subendothelial or subepithelial immune complexes	
By EM or IF, scattered immune complexes are not defined	Define what scattered immune complexes are by EM or IF
Class III/IV	
Endocapillary proliferation	
Definitions are unclear and inconsistent. How many criteria are needed? Leukocyte influx only? Which inflammatory cell type? How much lumen reduction? Specific involvement of endothelial cells?	The term proliferation may not be appropriate to designate these lesions; consider avoidance of this term, redefine the characteristics of class III/IV lesions, consider separate definition for MPGN lesions, disentangling the mesangial and capillary wall changes of MPGN
Extracapillary proliferation	Redefine definition
Extracapillary cell proliferation involving less than 25% of the capsular circumference is not considered extracapillary proliferation	
Unclear how to incorporate these lesions in the segmental and global subdivision, because this subdivision is based on lesions within the tuft	Redefine or remove segmental and global subdivision
Lack of definitions for fibrocellular and fibrous crescents	Provide definitions for these lesions
Extracapillary proliferation does not merely consist of proliferating cells	Consider replacing the term proliferation with hypercellularity
Segmental and global lesions	
The validity of the subdivision can be questioned	Option 1: maintain subdivision but clarify definitions
It is unclear what to do if exactly 50% of involved glomeruli have segmental lesions and 50% have global lesions; both of the definitions provided (IV-S if >50% segmental and IV-G if >50% global [in the text] or IV-S if ≥50% segmental and IV-G if ≥50% global [table 3 in refs. 1 and 2]) lack this provision	Option 2: eliminate S and G subdivision and define an alternative subdivision that may achieve the goal of identifying the more vasculitic-like lesions in LN

Table 1. Continued.

Topic and Concerns	Suggestions for Improvement
Activity and chronicity	
It is unclear if a membranoproliferative pattern of injury is to be considered as active, chronic, or both	Decide whether double contours are indicative of activity or chronicity
Difficult to reliably distinguish between global sclerosis caused by LN or other causes	Redefine the role of global sclerosis in the classification
Class V	
Segmental scarring	
Difficult to reliably differentiate between segmental sclerosis as a consequence of class V LN and segmental sclerosis as a consequence of class III/IV LN	Redefine the role of segmental sclerosis in the classification
Other glomerular lesions	
Specific attention for other glomerular abnormalities occurring in the context of SLE is lacking	Consider including lesions, such as lupus podocytopathy, collapsing glomerulopathy, and TMA
Vascular lesions	
Vascular lesions should be graded into mild, moderate, or severe, but criteria for this grading are not provided	Define criteria for severity of vascular lesions (use *e.g.*, Banff guidelines)
Very little attention is given to vascular lesions occurring in the context of SLE	Consider including vascular lesions, such as immune complex deposition, lupus vasculopathy, TMA and vasculitis
Tubulointerstitial lesions	
There are no cutoff values provided for the reporting of severity of tubulointerstitial lesions; it is unclear if interstitial inflammation, tubular atrophy, and interstitial fibrosis need to be graded separately or if these can be combined into one or two parameters	Provide cutoff values: use, for example, Oxford IgAN classification guidelines

EM, electron microscopy; IF, immunofluorescence; MPGN, membranoproliferative GN.

References

1. Weening JJ, D'Agati VD, Schwartz MM*, et al.* The classification of glomerulonephritis in systemic lupus erythematosus revisited. *J Am Soc Nephrol* 2004;15(2):241-50.

2. Weening JJ, D'Agati VD, Schwartz MM*, et al.* The classification of glomerulonephritis in systemic lupus erythematosus revisited. *Kidney Int* 2004;65(2):521-30.

3. Yokoyama H, Wada T, Hara A*, et al.* The outcome and a new ISN/RPS 2003 classification of lupus nephritis in Japanese. *Kidney Int* 2004;66(6):2382-8.

4. Furness PN, Taub N. Interobserver reproducibility and application of the ISN/RPS classification of lupus nephritis-a UK-wide study. *Am J Surg Pathol* 2006;30:1030-5.

5. Wilhelmus S, Cook HT, Noel LH*, et al.* Interobserver agreement on histopathological lesions in class III or IV lupus nephritis. *Clin J Am Soc Nephrol* 2015;10(1):47-53.

6. Corwin HL, Schwartz MM, Lewis EJ. The importance of sample size in the interpretation of the renal biopsy. *Am J Nephrol* 1988;8(2):85-9.

7. Working Group of the International IgA Nephropathy Network and the Renal Pathology Society, Roberts IS, Cook HT*, et al.* The Oxford classification of IgA nephropathy: pathology definitions, correlations, and reproducibility. *Kidney Int* 2009;76(5):546-56.

8. Appel GB, Silva FG, Pirani CL*, et al.* Renal involvement in systemic lupus erythematosus (SLE). *Medicine (Baltimore)* 1978;57:371-410.

9. Dorland's illustrated medical dictionary. 31st ed: Elsevier Health Sciences, 2007.

10. Bonsib SM. Renal anatomy and histology. In: Jennette J, Olson J, Silva F, D'Agati V, eds. Heptinstall's pathology of the kidney. 7th ed. Philadelphia: Wolters Kluwer, 2015:1-66.

11. Najafi CC, Korbet SM, Lewis EJ*, et al.* Significance of histologic patterns of glomerular injury upon long-term prognosis in severe lupus glomerulonephritis. *Kidney Int* 2001;59(6):2156-63.

12. Haring CM, Rietveld A, van den Brand JA*, et al.* Segmental and global subclasses of class IV lupus nephritis have similar renal outcomes. *J Am Soc Nephrol* 2012;23(1):149-54.

13. Schwartz MM, Korbet SM, Lewis EJ. The prognosis and pathogenesis of severe lupus glomerulonephritis. *Nephrol Dial Transplant* 2008;23(4):1298-306.

14. Schwartz MM, Kawala KS, Corwin HL*, et al.* The prognosis of segmental glomerulonephritis in systemic lupus erythematosus. *Kidney Int* 1987;32(2):274-9.

15. Austin III HA, Muenz LR, Joyce KM*, et al.* Diffuse proliferative lupus nephritis: identification of specific pathologic features affecting renal outcome. *Kidney Int* 1984;25:689-95.

16. Magil AB, Puterman ML, Ballon HS*, et al.* Prognostic factors in diffuse proliferative lupus glomerulonephritis. *Kidney Int* 1988;34(4):511-7.

17. Esdaile JM, Levinton C, Federgreen W*, et al.* The clinical and renal biopsy predictors of long-term outcome in lupus nephritis: a study of 87 patients and review of the literature. *Q J Med* 1989;72(269):779-833.

18. Schwartz MM, Lan SP, Bernstein J*, et al.* Role of pathology indices in the management of severe lupus glomerulonephritis. *Kidney Int* 1992;42(3):743-48.

19. Austin III HA, Boumpas DT, Vaughan EM*, et al.* High-risk features of lupus nephritis: importance of race and clinical and histological factors in 166 patients. *Nephrol Dial Transplant* 1995;10(9):1620-28.

20. Wernick RM, Smith DL, Houghton DC*, et al.* Reliability of histologic scoring for lupus nephritis: a community-based evaluation. *Ann Intern Med* 1993;119(8):805-11.

21. Schwartz MM, Lan SP, Bernstein J*, et al.* Irreproducibility of the activity and chronicity indices limits their utility in the management of lupus nephritis. *Am J Kidney Dis* 1993;21(4):374-77.

22. Erre GL, Bosincu L, Faedda R*, et al.* Antiphospholipid syndrome nephropathy (APSN) in patients with lupus nephritis: a retrospective clinical and renal pathology study. *Rheumatol Int* 2014;34(4):535-41.

23. Daugas E, Nochy D, Huong DL*, et al.* Antiphospholipid syndrome nephropathy in systemic lupus erythematosus. *J Am Soc Nephrol* 2002;13(1):42-52.

24. Miranda JM, Jara LJ, Calleja C*, et al.* Clinical significance of antiphospholipid syndrome nephropathy (APSN) in patients with systemic lupus erythematosus (SLE). *Reumatol Clin* 2009;5(5):209-13.

25. Dube GK, Markowitz GS, Radhakrishnan J*, et al.* Minimal change disease in systemic lupus erythematosus. *Clin Nephrol* 2002;57(2):120-6.

26. Hertig A, Droz D, Lesavre P*, et al.* SLE and idiopathic nephrotic syndrome: coincidence or not? *Am J Kidney Dis* 2002;40(6):1179-84.

27. Kraft SW, Schwartz MM, Korbet SM*, et al.* Glomerular podocytopathy in patients with systemic lupus erythematosus. *J Am Soc Nephrol* 2005;16(1):175-9.

28. Salvatore SP, Barisoni LM, Herzenberg AM*, et al.* Collapsing glomerulopathy in 19 patients with systemic lupus erythematosus or lupus-like disease. *Clin J Am Soc Nephrol* 2012;7(6):914-25.

29. Appel GB, Pirani CL, D'Agati V. Renal vascular complications of systemic lupus erythematosus. *J Am Soc Nephrol* 1994;4(8):1499-515.

30. Banfi G, Bertani T, Boeri V*, et al.* Renal vascular lesions as a marker of poor prognosis in patients with lupus nephritis. Gruppo Italiano per lo Studio della Nefrite Lupica (GISNEL). *Am J Kidney Dis* 1991;18(2):240-8.

31. Wu LH, Yu F, Tan Y*, et al.* Inclusion of renal vascular lesions in the 2003 ISN/RPS system for classifying lupus nephritis improves renal outcome predictions. *Kidney Int* 2013;83(4):715-23.

32. Racusen LC, Solez K, Colvin RB*, et al.* The Banff 97 working classification of renal allograft pathology. *Kidney Int* 1999;55(2):713-23.

33. Yu F, Wu LH, Tan Y*, et al.* Tubulointerstitial lesions of patients with lupus nephritis classified by the 2003 International Society of Nephrology and Renal Pathology Society system. *Kidney Int* 2010;77(9):820-9.

34. Hsieh C, Chang A, Brandt D*, et al.* Predicting outcomes of lupus nephritis with tubulointerstitial inflammation and scarring. *Arthritis Care Res (Hoboken)* 2011;63(6):865-74.

35. Furness PN, Taub N, Assmann KJ*, et al.* International variation in histologic grading is large, and persistent feedback does not improve reproducibility. *Am J Surg Pathol* 2003;27(6):805-10.

36. Farris AB, Adams CD, Brousaides N*, et al.* Morphometric and visual evaluation of fibrosis in renal biopsies. *J Am Soc Nephrol* 2011;22(1):176-86.

37. Working Group of the International IgA Nephropathy Network and the Renal Pathology Society, Cattran DC, Coppo R*, et al.* The Oxford classification of IgA nephropathy: rationale, clinicopathological correlations, and classification. *Kidney Int* 2009;76(5):534-45.

38. Muehrcke RC, Kark RM, Pirani CL*, et al.* Histological and clinical evolution of lupus nephritis. *Ann Rheum Dis* 1955;14:371-77.

39. Pollak VE, Pirani CL, Schwartz FD. The natural history of the renal lupus erythematosus manifestations. *J Lab Clin Med* 1964;63:537-50.

40. Pollak VE, Pirani CL, Schwartz FD. The natural history of the renal manifestations of systemic lupus erythematosus. *J Am Soc Nephrol* 1997;8:1189-98.

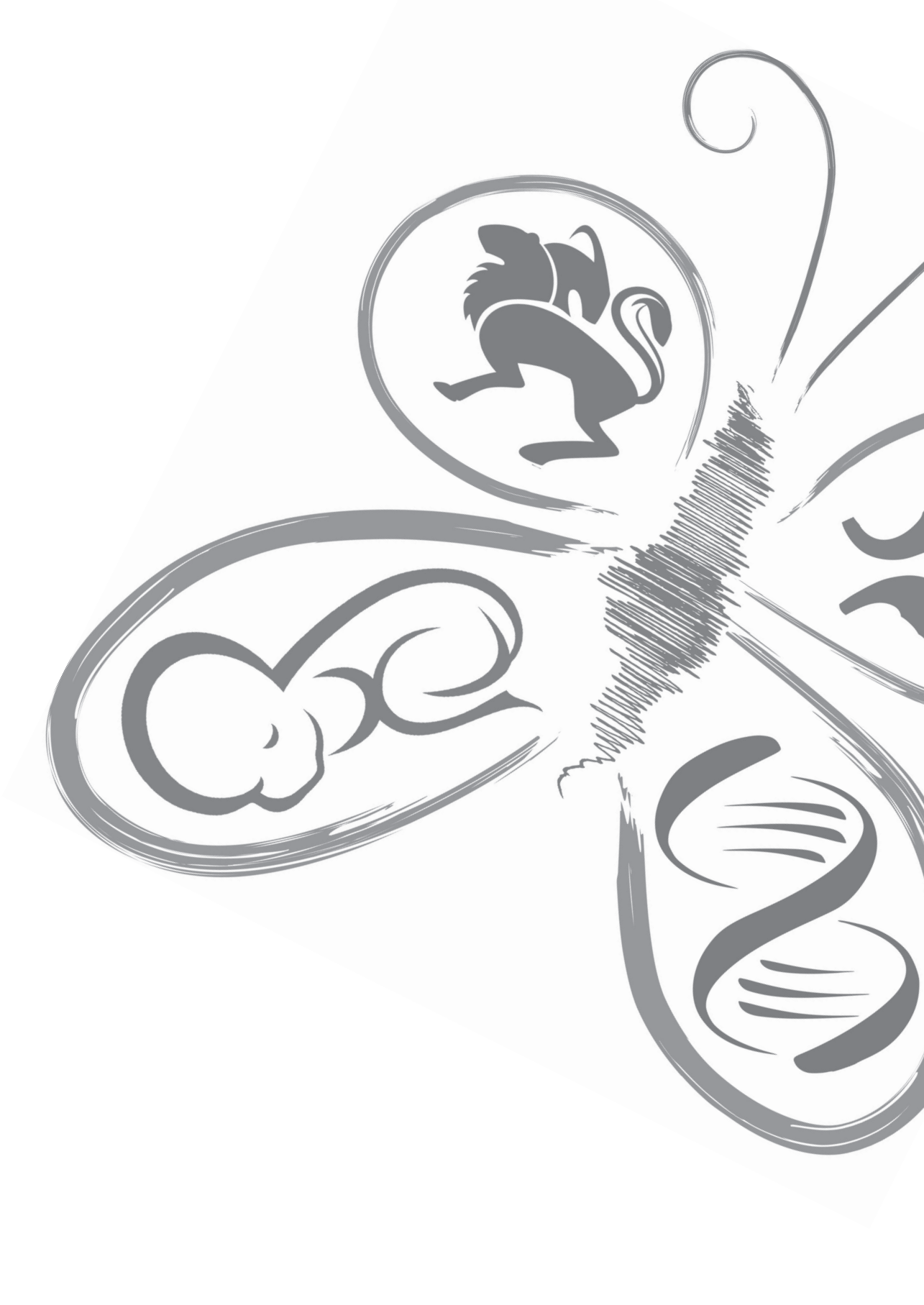
41. Baldwin DS, Lowenstein J, Rothfield NF*, et al.* The clinical course of the proliferative and membranous forms of lupus nephritis. *Ann Intern Med* 1970;73:929-42.

42. Zimmerman SW, Jenkins PG, Shelf WD*, et al.* Progression from minimal or focal to diffuse proliferative lupus nephritis. *Lab Invest* 1975;32(5):665-72.

43. Baldwin DS, Gluck MC, Lowenstein J*, et al.* Lupus nephritis. Clinical course as related to morphologic forms and their transitions. *Am J Med* 1977;62(1):12-30.

44. Churg J, Sobin L. Lupus nephritis. Renal disease: classification and atlas of glomerular diseases. Tokyo: Igaku-Shoin, 1982:127-31.

45. Churg J, Bernstein J, Glassock R. Lupus nephritis. Renal disease: classification and atlas of glomerular diseases. 2nd ed. New York: Igaku-Shoin, 1995:151-77.



4

Lupus Nephritis Management Guidelines Compared

Suzanne Wilhelmus, Ingeborg M. Bajema, George K. Bertsias, Dimitrios T. Boumpas, Caroline Gordon, Liz Lightstone, Vladimir Tesar, David R. Jayne

Nephrology Dialysis Transplantation 2016; 31: 904-913

Abstract

In the past years many (randomized) trials have been performed comparing the treatment strategies for lupus nephritis. In 2012 these data were incorporated in six different guidelines for treating lupus nephritis. These guidelines are European, American and internationally based, with one separate guideline for children. They offer information on different aspects of the management of lupus nephritis including induction and maintenance treatment of the different histologic classes, adjunctive treatment, monitoring of the patient, definitions of response and relapse, indications for (repeat) renal biopsy, and additional challenges such as the presence of vascular complications, the pregnant SLE patient, treatment in children and adolescents, and considerations about end-stage renal disease and transplantation. In this review we summarize the guidelines, determine the common ground between them, highlight the differences and discuss recent literature.

Introduction

Lupus nephritis (LN) is associated with poor survival[1 2] and considerable morbidity, particularly for patients who develop end-stage renal disease (ESRD) and require renal replacement therapy. The development of renal involvement within the course of disease ranges from ~20 to 60% of systemic lupus erythematosus (SLE) patients[3] with the highest risk of renal disease and renal failure in young black women.[4 5] Therapeutic possibilities have expanded from the solitary use of corticosteroids to the addition of a wide range of immunosuppressive drugs and other supportive treatment. Many trials have been conducted in the past 40 years leading to the publication of six guidelines in 2012 on the management of LN (Table 1).[6-11] These guidelines are American and European based, with separate guidelines from Spain and the Netherlands, with the addition of the KDIGO (Kidney Disease Improving Global Outcomes) guideline that is considered to be international. All guidelines were developed on the basis of extensive literature searches and (consensus)

Table 1. Guidelines that were compared

From	Date of publication	Geography	Population
EULAR/ERA-EDTA : European League Against Rheumatism and European Renal Association–European Dialysis and Transplant Association[6]	July 2012	Europe	Adults and children/adolescents
ACR: American College of Rheumatology[7]	June 2012	USA	Adults, particularly those receiving care in the USA Includes interventions available in the USA as of February 2012
KDIGO: Kidney Disease: Improving Global Outcomes Glomerulonephritis Work Group[8]	May 2012	International	Adults and children/adolescents
GEAS: Systemic auto-immune disease group of the Spanish Society of Internal Medicine and Spanish Society of Nephology[9]	March 2012	Spain	Not specified
DWP: Dutch Working Party on Systemic Lupus Erythematosus[10]	March 2012	The Netherlands	Not specified For proliferative LN only
CARRA: Childhood Arthritis and Rheumatology Research Alliance[11]	March 2012	North America	Children/adolescents For proliferative LN only Consensus treatment plan, not a guideline

LN, lupus nephritis; USA, United States of America

meetings. Furthermore, each guideline indicated the level of evidence or strength of a statement/recommendation, or both, for all topics (Supplemental Table 3). All guidelines were published in the same year and based on the same body of evidence and their main statements are congruent. However, there are also notable differences between them. The aim of this review is to compare the recent guidelines, outline a common view and highlight the differences, in particular in relation to indications for (repeat) renal biopsy, induction and maintenance treatment of the different classes, adjunctive treatment, monitoring of the patient, definitions of response and relapse, and additional circumstances such as the presence of vascular complications, the pregnant SLE patient, treatment in children and adolescents, and considerations about end-stage renal disease (ESRD) and transplantation (Tables 2 and 3, Supplemental Tables 1 and 2). We will also discuss recent literature and how to proceed further to increase the level of evidence based patient care.

Renal biopsy

All guidelines recommend a renal biopsy when there is a suspicion of renal involvement, because clinical and laboratory parameters cannot accurately predict the histologic class. Early diagnosis and treatment have been shown to improve outcomes.[12 13] The criteria for suspicion of renal involvement, however, differ. The common view is that an unexplained decrease in renal function, and proteinuria are indications for a renal biopsy. Also, an active urine sediment raises the level of suspicion of renal involvement and may be an additional argument for a renal biopsy. The GEAS (Spanish Society of Internal Medicine and Spanish Society of Nephrology) considers an active urine sediment alone a sufficient cause for biopsy. The required levels of proteinuria differ between the guidelines, but most use a urine protein-creatinine ratio of 50 mg/mmol (equivalent to ~0.5 g/24h) as a cutoff.

The biopsy is classified according to the system proposed by the International Society of Nephrology/ Renal Pathology Society (ISN/RPS) in 2003.[14] A minimum of 10 glomeruli is required in order to reasonably exclude focal disease and the biopsy should be examined by light microscopy, immunofluorescence and if possible, electron microscopy. Furthermore, data on activity and chronicity should be quantified (though activity and chronicity indices are not obligatory) and vascular and interstitial lesions described. The histologic class plays a fundamental role in the ensuing therapeutic decision process.

Although the evidence is sparse, in cases of worsening of disease, disease refractory to treatment or relapse, a repeat biopsy can be considered to determine activity and chronicity or detect other pathologies. Some also suggest taking a biopsy at the end of induction

Table 2. Guidelines compared; common views and differences

	Common view	Differences
Indication for renal biopsy	Inexplicable (persistent) decrease in renal function Reproducible proteinuria (required levels: different) Active sediment raises level of suspicion for LN and may be an additional argument for a renal biopsy	Proteinuria: – Most: isolated proteinuria ≥0.5 g/24 h – ACR: isolated proteinuria ≥1.0 g/24 h *or* ≥0.5 g/24 h and hematuria (5 RBCs/HPF) or cellular casts Active sediment: sufficient to warrant biopsy in GEAS, others consider a biopsy, sometimes when in combination with low levels of proteinuria
Biopsy evaluation	According to ISN/RPS 2003 classification system for LN Examine by light microscopy, immuno-fluorescence and if possible electron microscopy Quantify data on activity and chronicity and describe vascular and interstitial lesions	-
Indication for repeat biopsy	Consider in case of: – Worsening of disease or disease refractory to treatment – Relapse, in order to demonstrate change or progression in histological class, change in activity and chronicity (index) or other pathologies	-
Treatment class II	Treat proteinuria with RAAS (first)	ACR: no immunosuppressive treatment EULAR/ERA-EDTA: proteinuria >1 g/24 h, especially in the presence of glomerular hematuria; low to moderate doses oral glucocorticoids (0.25-0.5 mg/kg/day) alone or in combination with AZA (1-2 mg/kg/day), if necessary KDIGO: proteinuria <1 g/24 h: treat as dictated by extrarenal manifestations. Proteinuria >3 g/24 h: corticosteroids or CNI as described for MCD GEAS: significant proteinuria (>1-2 g/24 h) and/or deteriorated renal function that is not attributable to functional factors; steroids up to 0.5 mg/kg/day, possibly plus AZA or MMF for 6-12 months
Induction treatment class III/IV *without* crescents (and/or other adverse parameters)	Oral glucocorticoids with or without three iv pulses methylprednisolone (MP) at start induction + ivCYC or MMF	Dosage and preferences for different severities (see also next section) and ethnic groups: Glucocorticoids: – MP dose ranging from 250 to 1000 mg/day (or weight dependent in children) – MP not always recommended; dependent on combination with MMF or ivCYC, or on severity – Oral dose ranging from 0.5 to 1 mg/kg/day, sometimes depending on combination with MP, MMF or ivCYC

Table 2. Continued

	Common view	Differences
		– Tapering schedule: unclear MMF: – Ranging from 2 to 3 g total daily dose – Sometimes preferred over ivCYC in patients of African or Hispanic descent ivCYC: – Either high dose (NIH; 0.5-1 g/m^2 monthly for 6 months) or low dose (Eurolupus; 500 mg fortnightly for 3 months): low dose usually preserved for (European) Caucasians and sometimes only for relatively mild disease – In case of low dose ivCYC, combine pulses MP
Induction treatment class IV or IV/V *with* crescents (and/or other adverse parameters)	No consensus	KDIGO, DWP, CARRA: same as without crescents (and/or other adverse parameters) ACR: ivCYC or MMF + three iv pulses MP + oral glucocorticoids; MMF and oral glucocorticoids at highest doses (MMF 3 g total daily dose; oral glucocorticoids 1 mg/kg/day) EULAR/ERA-EDTA: high dose (see above) ivCYC can also be prescribed GEAS: three pulses MP (250-1000 mg/day) and include ivCYC in regimen
Induction treatment class V	If nephrotic range proteinuria (≥3 g/24 h): oral glucocorticoids (0.5 mg/kg/day) combined with other immunosuppressive medication (except in GEAS)	GEAS: also in patients with non-nephrotic range proteinuria; oral glucocorticoids up to 1 mg/kg/day (max 60 mg) combined with either ivCYC, MMF, AZA or CNIs Type of additional immunosuppressive medication: – EULAR/ERA-EDTA: preferably MMF (3 g total daily dose), alternatives; high dose ivCYC, CNIs or rituximab – ACR: MMF (2-3 g total daily dose) – KDIGO: ivCYC, CNIs, MMF or AZA
Treatment class VI	Suggestions from different guidelines: – Prepare for renal replacement therapy – Treat with immunosuppressives only as dictated by extrarenal disease – Maintain RAAS inhibition and monitor for complications	-
Maintenance treatment	Class III/IV: – AZA (1.5-2.5 mg/kg/day) or MMF (1-2 g/day) – Plus low dose oral glucocorticoids Class V: – As class III/IV – CNIs can be considered	Class III/IV: – EULAR/ERA-EDTA recommends MMF over AZA if there was a response to MMF during the induction phase – GEAS recommends MMF over AZA – Duration of treatment: at least 3 years (EULAR/ERA-EDTA) or at least 1 (KDIGO) or 2 (GEAS) years after (complete) remission

Table 2. Continued

	Common view	Differences
Adjunctive treatment	HCQ for all unless contraindicated; screening ophthalmologist for retinopathy at baseline and yearly after 5 years (recommended by most) RAAS inhibition for proteinuria and to control blood pressure (<130/80 mmHg) Treat hyperlipidemia with statins, target LDL <100 mg/dL or 2.6 mmol/L Other treatment suggestions supported by one or more guidelines: – Calcium and vitamin D supplements – Bisphosphonates depending on glucocorticoid dose, age and renal function – Low dose acetylsalicylic acid in patients with aPL – Consider anti-coagulant treatment in patients with nephrotic syndrome and albumin <20 g/L – Avoid vaccination with live or attenuated viruses during immune suppression – GnRH analogues in women over 35 years if cumulative CYC dose >10 g	Required level of proteinuria to start treatment with RAAS inhibition: ranging from 0.5 g/24h or uPCR >50 mg/mmol to 1.0 g/24 h, if specified at all
Treatment for refractory disease	Switch from ivCYC to MMF or vice versa with or without three accompanying iv pulses MP Alternative treatments: rituximab (as add-on or monotherapy), CNIs or intravenous immunoglobulins	-
Pregnancy	Continue HCQ Allowed: glucocorticoids (non-fluorinated), AZA, CNIs, methyldopa, labetalol or nifedipine *Not*-allowed: MMF, ivCYC, RAAS inhibitor Consider low dose acetylsalicylic acid to reduce risk of pre-eclampsia and fetal loss Monitor closely, preferably by a multi-disciplinary team Do not taper glucocorticoids or AZA during pregnancy or within 3 months thereafter (KDIGO)	Plan pregnancy when: – EULAR/ERA-EDTA: stable (uPCR <50 mg/mmol, GFR preferably over 50 mL/min) for 6 months – GEAS: (partial) remission for 6 months – KDIGO: preferably delay until complete remission – ACR: not specified
Vascular complications	No consensus	EULAR/ERA-EDTA: ASPN; consider HCQ and/or antiplatelet/anticoagulant treatment. In case of definite APS, start anticoagulant treatment ACR: treat TMA with plasma exchange therapy KDIGO/GEAS: ASPN; anticoagulant treatment (INR 2-3) KDIGO: treatment for TTP is plasma exchange as in patients without lupus

Table 2. Continued

	Common view	Differences
Monitoring	Determine at each visit: body weight, BP, sCr, proteinuria, urinary sediment, C3/C4, anti-dsDNA (and serum albumin and complete blood count) Schedule visits: – Active nephritis: approximately monthly, or more frequently – No active nephritis: every 3-6 months	ACR: – Some parameters can be determined at larger intervals than others; BP and urinalysis most often, anti-dsDNA least often – Separate schedule for pregnancy; in short, if active LN, once a month, and if LN in history but none currently, BP and urinalysis once a month and uPCR, sCr, C3/C4 and anti-dsDNA every 3 months
Management of ESRD	Renal replacement therapy: – Increased risk of infection in patients still on immunosuppressives (EULAR/ERA-EDTA) – Increased risk of vascular access thrombosis in patients with aPL (EULAR/ERA-EDTA) – If lupus is inactive offer peritoneal dialysis; if lupus is active offer hemodialysis (GEAS) Transplantation: – Determine aPL; associated with increased risk of vascular events in the transplant	Transplantation: – If lupus activity absent or low for 3-6 (EULAR/ERA-EDTA) or 6-12 (GEAS) months

uPCR 100 mg/mmol ≡ 1000 mg/g ≡ 1(g/g) ≈ 1 g/24 h.[55] ACR, American College of Rheumatology; aPL, antiphospholipid antibodies; APS, antiphospholipid syndrome; APSN, antiphospholipid-associated nephropathy; AZA, azathioprine; BP, blood pressure; CARRA, Childhood Arthritis and Rheumatology Research Alliance; CNI, calcineurin inhibitor; anti-dsDNA, antibodies to double stranded DNA; DWP, Dutch Working Party on Systemic Lupus Erythematosus; ESRD, end-stage renal disease; EULAR/ERA-EDTA, European League Against Rheumatism and European Renal Association–European Dialysis and Transplant Association; GEAS, Systemic auto-immune disease group of the Spanish Society of Internal Medicine and Spanish Society of Nephology; GFR, glomerular filtration rate; HCQ, hydroxychloroquine; HPF, high power field; ISN/RPS, International Society of Nephrology/Renal Pathology Society; ivCYC, intravenous cyclophosphamide; KDIGO, Kidney Disease: Improving Global Outcomes Glomerulonephritis Work Group; LN, lupus nephritis; MCD, minimal change disease; MMF, mycophenolate mofetil; MP, methylprednisolone; NIH, National Institute of Health; RAAS, renin-angiotensin-aldosterone system; RBC, red blood cell; sCr, serum creatinine; uPCR, urine protein-creatinine ratio.

treatment in order to determine the histologic response, as clinical parameters may underestimate (histologic) response.[15 16] However, this strategy has not been officially tested in a controlled study but repeat renal biopsy has been shown to have prognostic value.[17-20]

Treatment class II

There is little agreement among the guidelines on treatment of class II LN due to lack of evidence. Proteinuria should primarily be managed with renin-angiotensin-aldosterone

system (RAAS) inhibitors. The role of immunosuppression, however, is less clear. The ACR (American College of Rheumatology) guideline states that class II LN generally does not require immunosuppressive treatment. The EULAR/ERA-EDTA (European League Against Rheumatism and European Renal Association-European Dialysis and Transplant Association), however, recommends low to moderate doses of oral glucocorticoids (0.25-0.5 mg/kg/day) alone or in combination with azathioprine (AZA, 1-2 mg/kg/day), if necessary as a steroid sparing agent, in cases of proteinuria over 1 g/24 h, especially in the presence of glomerular hematuria. In the GEAS guideline steroids up to 0.5 mg/kg/day, if necessary with AZA or mycophenolate mofetil (MMF), for 6-12 months are suggested for class II nephritis with proteinuria (>1-2 g/24 h) and/or a deteriorated renal function that are not attributable to functional factors. The suggestions in the KDIGO guideline for the use of immunosuppressive therapy focuses on the presence/co-existence of podocytopathy [*i.e.*, minimal change disease (MCD)] in a subset of patients with class II LN, [21 22] and KDIGO suggests treating such patients with nephrotic range proteinuria (>3 g/24 h) with corticosteroids or calcineurin inhibitors (CNIs) as for MCD, but this presentation was not discussed in the ACR guidelines.

Induction and maintenance treatment class III/IV

Over the past decade several randomized controlled trials (RCTs) have been conducted for class III and IV LN, both in the induction and in the maintenance phase. Consequently, the guidelines are uniform in their recommendations for induction treatment: intravenous cyclophosphamide (ivCYC) or MMF (2-3 g total daily dose) in combination with oral glucocorticoids with or without three pulses of intravenous methylprednisolone (MP) at start of induction treatment. Although in general the use of both oral and intravenous glucocorticoids has been proven effective, evidence is scarce concerning dose and duration, and recommendations are mainly based on expert opinion. In the guidelines, the initial dose of oral glucocorticoids varies from 0.5 to 1.0 mg/kg/day. Only one small RCT compared high (1 mg/kg) and low (0.5 mg/kg) dose oral glucocorticoids (in a background of enteric coated mycophenolic acid). This study demonstrated an equal percentage (~20%) of complete responses at 24 weeks, although non-inferiority was not proven. It did, however, show a decrease in infections in favour of the low dose group.[23] Furthermore, advice for tapering of glucocorticoids is usually fairly general, except for the guideline from the Dutch Working Party on SLE (DWP), which devised a schedule for tapering (Supplemental Table 1). The use of pulse MP at induction is not always recommended and is reserved by some of the

guidelines for more severe cases. However, there is some indication that the use of pulse MP combined with medium dose oral glucocorticoids may be as effective as high dose oral glucocorticoids in inducing remission, but with less toxicity.[24] MMF and ivCYC have similar efficacy and adverse event rates when used with glucocorticoids for remission induction, but MMF avoids adverse effects on fertility. For ivCYC, both the low dose Eurolupus regimen (500 mg fortnightly for 3 months) and the higher dose NIH regimen (0.5-1 g/m^2 monthly for 6 months) can be used. However, the low dose is usually preferred for (European) Caucasians and sometimes only for milder cases because the original trials were mostly in this group of patients.[25 26] The ACCESS trial, communicated after publication of the guidelines, showed no benefit of abatacept as add-on to induction therapy. However, in a predominantly non-Caucasian study population comparable response rates to low dose ivCYC were observed to those previously reported, suggesting that low dose ivCYC may be as effective in non-Caucasians as in Caucasians,[27] although further evidence will be required. Finally, MMF is sometimes preferred over ivCYC in patients from African or Hispanic descent, based on a 'post-hoc' subgroup analysis of the ALMS trial.[28] Some of the guidelines advise more aggressive therapy in patients with crescents in the biopsy specimen, as detailed in Table 2. The EULAR/ERA-EDTA and KDIGO guidelines also state that patients should have active lesions (class III/IV_A or class III/$IV_{A/C}$) in order to be treated and should not have merely chronic lesions (class III/IV_C).

For severe LN, although not adequately defined, there is less evidence as these patients are often excluded from RCTs. However, a subgroup analysis of the ALMS trial in patients with a baseline estimated glomerular filtration rate (eGFR) <30 mL/min did not reveal a difference between ivCYC and MMF [29]. Unfortunately, numbers were small (32 in total) and there was no follow-up beyond the induction phase. Recently, Rovin *et al*. performed a systematic review using results extracted from clinical trials and drawn from expert opinion. Severe LN was arbitrarily defined by renal histology, resistance to therapy, or GFR at presentation. They showed that ivCYC and MMF are equally effective in inducing remission. For long-term follow-up (5 years), however, results from retrospective and observational studies suggest there may be a better preservation of renal function and fewer relapses with ivCYC.[30] Long-term follow-up data from RCTs, however, are lacking.

In the maintenance phase of treatment, MMF (1-2 g/day) or AZA (1.5-2.5 mg/kg/day) is recommended by all guidelines, supported by low dose oral glucocorticoids. The EULAR/ERA-EDTA recommends MMF over AZA if there was a response to MMF at induction based

on the combined results from the ALMS [31] and MAINTAIN trials.[32] The GEAS advises MMF over AZA, based on the results from the ALMS trial, although long-term effects of MMF are still lacking. Also, a recent meta-analysis of four trials (including MAINTAIN and ALMS) showed that there is no difference between MMF and AZA with respect to preventing relapse, progression to end-stage renal failure, death and doubling of serum creatinine.[33] Finally, with respect to duration of treatment, the guidelines differ: at least 3 years (EULAR/ERA-EDTA) or at least 1 (KDIGO) or 2 (GEAS) years after (complete) remission. Due to the length of completed studies, there is no advice on the optimal duration of therapy beyond 3 years.

Induction and maintenance treatment class V

Evidence in support of immunosuppressive therapy in patients with pure class V LN is less robust. Most of the guidelines suggest initiating immunosuppressive treatment if there is nephrotic range proteinuria (>3 g/24 h). If proteinuria is subnephrotic, management with RAAS inhibitors is recommended to reduce the levels of protein excretion. The GEAS, on the other hand, advises immunosuppression irrespective of the level of proteinuria. There is also no consensus on which immunosuppressive therapy to initiate, although there is agreement that glucocorticoids should be included in the regimen. The EULAR/ERA-EDTA and ACR guidelines prefer the addition of MMF over other immunosuppressives (ivCYC, CNIs, AZA or rituximab), in contrast to the GEAS and KDIGO that do not state a preference for any of the aforementioned possibilities. The preference for MMF is mainly based on a combined retrospective analysis of class V LN patients of two RCTs, demonstrating that MMF 2-3 g total daily dose plus daily prednisone for 6 months and ivCYC (0.5-1.0 mg/kg monthly) plus prednisone for 6 months resulted in similar improvement.[34] Unfortunately, due to the short follow-up of this study, the long-term efficacy remains unknown. Another RCT compared prednisone (40 mg/m^2 orally, tapered after 8 weeks to reach 10 mg/m^2 by 12 months) alone on alternate days with the addition of either ivCYC (500-1000 mg/m^2 every 2 months for six doses) or ciclosporin (5 mg/kg for 11 months). Results showed that the combination of prednisone with ivCYC or ciclosporin led to higher remission rates than prednisone alone, but relapse of nephrotic syndrome occurred significantly more often after completion of ciclosporin than after ivCYC.[35] As evidence is lacking on maintenance therapy in class V LN, it is suggested to treat according to maintenance regimens for class III/IV LN. The efficacy in idiopathic membranous glomerulopathy of tacrolimus, ciclosporin and rituximab also supports a therapeutic role for these agents in lupus membranous nephropathy.[36-38]

Monitoring

The guidelines differ in their approach but agree that patients with active nephritis should have a visit scheduled at least every month, particularly at induction, relapse and withdrawal of treatment. If there is no active nephritis every 3 to 6 months should suffice, although vigilance is required for prompt identification of disease relapse. At each visit body weight, blood pressure, serum creatinine (sCr), proteinuria, urinary sediment, complement levels, anti-dsDNA titres and according to some serum albumin and complete blood count, should be determined. The ACR states that some of the aforementioned can be determined at larger intervals than others (blood pressure and urinalysis frequent; anti-dsDNA less frequent) and drafted a separate monitoring schedule for pregnancy (Table 2 and Supplemental Table 1). Recommendations in this area are all based on expert opinion. Nevertheless, they can still serve as a guideline for the practicing physician. Also, a recommendation from the EULAR for monitoring patients with SLE was previously published.[39]

Adjunctive treatment/treatment for comorbidities

All guidelines recommend blood pressure control (target <130/80 mmHg), treatment of hyperlipidemia with statins (target LDL < 100 mg/dL or 2.6 mmol/L) and treatment of proteinuria with RAAS inhibition. The guidelines agree that all SLE patients should have a background of hydroxychloroquine (HCQ) unless contraindicated, since this is associated with less damage accrual.[40] There is a paucity of randomized evidence for the efficacy of HCQ on nephritis with only two retrospective studies supporting its use.[41 42] Patients receiving HCQ have a risk of developing retinopathy and should therefore be screened by the ophthalmologist at baseline and yearly after 5 years. Patients with severe renal or hepatic disease are at higher risk for developing retinopathy, due to less clearance of the drug. In those patients reducing the dose should be considered to avoid toxicity. Other recommendations made by one or more of the guidelines are listed in Table 2 and involve treatment for side effects of drugs, prevention of clotting events and osteoporosis. There are no clear recommendations from the guidelines on infective prophylaxis, such as for pneumocystis jirovecii pneumonia, or surveillance for other pathogens.

Definitions of response and relapse

When communicating about patients, either in trials or in clinical practice, it is essential that definitions for disease parameters such as partial and complete response and relapse or flare are the same. Previously, a very stringent European consensus statement was published on the terminology used in the management of lupus nephritis.[43] However, the choice

Table 3. Definitions of response to treatment and flares; common views and differences

	Common view	Differences
Complete response	Proteinuria: uPCR <50 mg/mmol or <500 mg/g, or <0.5 g/24 h (except CARRA) Plus (near) normal renal function	Proteinuria: uPCR <200 mg/g or age appropriate (CARRA) Renal function: ranging from normalization to baseline to baseline plus 25%. Or sCr <1.2 mg/dL (106 µmol/L) (GEAS) Plus inactive urinary sediment (<5 red blood cells/HPF, <5 leukocytes/HPF, no casts) (GEAS/CARRA) Plus serum albumin >30 g/dL (GEAS)
Partial response	≥50% decrease in proteinuria, to at least sub-nephrotic levels (except CARRA) Plus stabilization or improvement of sCr	GEAS: if proteinuria >3.5 g/24 h, partial response if proteinuria <3.5 g/24 h Renal function: – EULAR/ERA-EDTA: (near) normal – KDIGO/GEAS: stabilization (±25%) or improvement of sCr – DWP: sCr within 125% of baseline CARRA: different approach, for details, see Supplemental Table 2
Flare	Increase or recurrence of active urinary sediment (hematuria from <5 RBC/HPF to >15 RBC/HPF Or an increase in sCr (exact criteria different in the different guidelines, for details, see Supplemental Table 2) Or an increase in proteinuria: – If proteinuria <500 mg/g (complete response), an increase to ≥1000 mg/g is required – If proteinuria >500 mg/g (partial response), a doubling of uPCR to ≥2000 mg/g is required (EULAR/ERA-EDTA, KDIGO, GEAS) Depending on which of the above-mentioned criteria are met, the flare can be designated at either nephrotic or nephritic.	With respect to proteinuria the bar is raised in the DWP guideline and lowered in the CARRA guideline: – DWP: development of nephrotic syndrome if lowest proteinuria was <2 g/24 h, or proteinuria of ≥1.5 g/24 h in previously non-proteinuric patient – CARRA: after a complete response an increase to >500 mg/g is required and after a partial response a doubling of sCr to >1000 mg/g
Refractory disease	There is no consensus between guidelines and some guidelines state that a consensus was not reached	For details, see Supplemental Table 2

uPCR 100 mg/mmol ≡ 1000 mg/g ≡ 1(g/g) ≈ 1 g/24 h.55 ACR, American College of Rheumatology; CARRA, Childhood Arthritis and Rheumatology Research Alliance; DWP, Dutch Working Party on Systemic Lupus Erythematosus; EULAR/ERA-EDTA, European League Against Rheumatism and European Renal Association–European Dialysis and Transplant Association; GEAS, Systemic auto-immune disease group of the Spanish Society of Internal Medicine and Spanish Society of Nephrology; HPF, high power field; KDIGO, Kidney Disease: Improving Global Outcomes Glomerulonephritis Work Group; RBC, red blood cell; sCr, serum creatinine; uPCR, urine protein-creatinine ratio.

of primary endpoint in clinical trials can also substantially influence the ability to detect therapeutic benefit, as demonstrated by Wofsy *et al.*.[44] The common ground and differences for the definitions of complete and partial response, relapse or flare, and refractory disease are outlined in Table 3 and Supplemental Table 2.

Treatment for refractory disease

Although the definition for refractory disease is stated differently by the various guidelines and there is no clinical trial evidence for these approaches, there is agreement on the treatment. It is generally advised to switch from MMF to ivCYC or vice versa if induction treatment fails. Some guidelines also state that again three pulses of intravenous MP should be administered. If this approach fails, the guidelines recommend other options: rituximab, as add-on or monotherapy, CNIs (also as add-on or monotherapy) or intravenous immunoglobulins. Of these, the main focus in literature has been on the use of rituximab, although with the LUNAR trial of rituximab as add-on to a steroid-MMF combination failing to meet its endpoint, it has not yet been proven effective in an RCT. Putative explanations for this failure include the possible overtreatment of relatively mild disease, short follow-up and underpowered study for the detection of an effect mainly consisting of partial responses.[45] Recently, a summary of the literature on the use of rituximab in refractory LN was published,[46] which suggests that rituximab can induce a response in patients who did not achieve remission on standard therapy. Also, Jónsdóttir and colleagues recently showed in a group of 25 patients that add-on of rituximab to ivCYC and glucocorticoids resulted in both clinical and histologic improvements in the majority of patients.[47] A recent, non-randomized, prospective study found promising results for a steroid sparing induction regimen [48] consisting of two doses of rituximab (1 g) and MP (500 mg) on day 1 and 15, and maintenance with MMF without oral steroids. A phase 3 open label multicentre investigator led RCT (RITUXILUP, NCT01773616) will start in 2015 comparing this regimen with a 'standard' oral glucocorticoid/MMF regimen.

Although RCTs are lacking, there is a growing body of evidence that CNIs may be useful in refractory disease, but one should be aware of the nephrotoxic effects, especially in patients with decreased renal function. These nephrotoxic effects (reviewed by Naesens *et al*.[49]) seem to be less for tacrolimus than for ciclosporin. Although not studied in refractory disease, in a recent Chinese randomized trial the combination of MMF (1.0 g/day) with tacrolimus (4 mg/day) was proven superior to ivCYC (0.5-1 g/m^2 every 4 weeks for six doses) in achieving complete remission in patients with class IV, class V and class IV + V LN.[50] This

could be due to a faster anti-proteinuric effect of tacrolimus and longer follow-up data are needed to determine the comparable efficacy of the two regimens.

Pregnancy

Pregnancy should not be planned until remission is reached and maintained for 6 months (EULAR/ERA-EDTA and GEAS). HCQ should be continued as multiple studies (reviewed by Ruiz-Irastorza *et al*.[40]) have demonstrated its safety in pregnancy. RAAS inhibitors, MMF and cyclophosphamide are prohibited during pregnancy. As alternatives AZA, CNIs, methyldopa, labetalol or nifedipine can be prescribed, despite the classification of AZA (the same as MMF and ivCYC) as category D by the Food and Drug Administration ("positive evidence of human fetal risk based on adverse reaction data, potential benefits may warrant use of the drug in pregnant women despite the potential risk"). AZA is considered safe during pregnancy as there is no evidence that AZA increases the risk of congenital abnormalities (in contrast to MMF and CYC) and AZA cannot be metabolized to the active metabolite 6-mercaptopurine by the fetal liver.[51 52] Low dose oral glucocorticoids (non-fluorinated) are acceptable. It is advised by the KDIGO not to taper glucocorticoids or AZA during pregnancy or for 3 months thereafter. Furthermore, low dose acetylsalicylic acid should be considered to reduce the risk of pre-eclampsia. Finally, all patients should be monitored closely, preferably by a multidisciplinary team that is used to managing such patients and is aware of the need to distinguish between a flare and pre-eclampsia, which may also co-exist.

Vascular complications

Anti-phospholipid syndrome-associated nephropathy (APSN) is a vascular nephropathy that can occur in SLE patients and may be associated with the presence of anti-phospholipid (aPL) antibodies. The EULAR/ERA-EDTA guideline takes the use of HCQ and/or antiplatelet or anticoagulant treatment into consideration, while the KDIGO and GEAS merely suggest treatment with anticoagulants (INR 2-3). The ACR suggests treating thrombotic microangiopathy (TMA) primarily with plasma exchange. This area is further complicated by the inconsistent terminology used. TMA is a histologic lesion, which is part of the APSN spectrum, but also has a clinical counterpart with systemic manifestations such as the presence of schistocytes in peripheral blood. Thrombotic thrombocytopenia (TTP) is a clinical syndrome associated with TMA in the renal biopsy, recommended to be treated promptly with plasma exchange by KDIGO (and other guidelines for idiopathic TTP, as TTP especially in SLE has a high mortality). In summary, recommendations differ because of inconsistent

terminology and lack of evidence. Until this is solved, we recommend viewing TMA in the renal biopsy in the clinical context when determining treatment. If APSN is considered to be a small vessel manifestation of APS and laboratory criteria for the diagnosis of APS are met, it may be wise to treat it as such (with antiplatelet or anticoagulation therapy), at least until new evidence becomes available.

Management of ESRD and transplantation

The modality of dialysis should be determined by patient choice. However, the risk of infection in increased with the use of immunosuppressive drugs. Hence, the GEAS suggests peritoneal dialysis should only be offered to patients with inactive disease on minimal immunosuppression. Hemodialysis is suitable for patients with active disease/more immune suppression.

It is advised to determine the presence of aPL antibodies because this can increase the risk of vascular access thrombosis during dialysis and of vascular events in the transplant. Lupus activity should be absent or low for a period of 3-6 months (EULAR/ERA-EDTA) or 6-12 months (GEAS) to be eligible for transplantation. Although ESRD is often associated with remission of lupus activity, this is not universal and extra-renal lupus flares can still occur, patients should be managed accordingly.

Children and adolescents

The rate of developing LN during the course of disease is higher in children than in adults.[53] However, large trials comparing different treatment strategies in juvenile LN are lacking. The guidelines generally advise the same treatment strategies as for adults, except for the CARRA guideline, which is specifically aimed at children and adolescents. For dosages of the immunosuppressive drugs in children, we refer to this guideline. In 2012, the first results from an RCT, a subgroup analysis of the ALMS trial, were published.[54] This subgroup analysis included adolescents aged 12 to 18 years. Although the numbers were small (24 patients in the induction phase and 16 in the maintenance phase) and therefore not sufficient to yield statistically significant results, it was noted that in general there was similar efficacy in adolescents and adults. Due to the small numbers the effect of ethnicity could not be determined.

Conclusion

Although a substantial part of the management of LN is evidence-based, a significant part still rests on uncontrolled trials and expert opinion. Despite an increase in clinical trial activity during the last decade, there are areas where evidence is lacking, such as for the treatment of severe and refractory LN and of children. Furthermore, although the most important outcome is the long-term follow-up beyond 10 years due to the risk of end-stage renal failure at this time despite initial improvement in disease parameters, these data are scarce. Finally, it must be kept in mind that all guidelines are meant to assist physicians in the management of LN, but they can never replace the insight of the experienced clinician in reaching a therapeutic strategy tailored to the individual patient.

Funding

G.K.B. is supported by the FP7-2011-REGPOT-1 (TransPOT: 'Enhancing University of Crete Medical School Scientific Excellence and Translational Research Potential in Human Diseases'). V.T. is supported by the research project 455 PRVOUK-P25/LF1/2.

Conflict of interest statement

I.M.B. is a consultant for Roche; L.L.: Roche are providing drugs free of charge for the Rituxilup trial; honoraria /advisory boards/lecturing—GSK, Anthera Pharmaceuticals, MedIm- mune, Merck, Aspreva/Vifor Pharma, Biogen-Idec, UCB; C.G. is a consultant on clinical trial design and has received 465 honoraria for consultancy, participation in scientific advisory boards and lecturing for UCB, GSK and Bristol-Myers Squibb, and has received honoraria from Aspreva/Vifor Pharma, Med- Immune, Genentech, Roche and Merck Serono. D.J.: Roche/ Genentech is providing drugs for the RITAZAREM trial; 470 honoraria/advisory boards/lecturing—GSK, Medimmune, Merck, Biogen-Idec and UCB; V.T.: Lecturing for GSK and Roche.

References

1. Bernatsky S, Boivin JF, Joseph L*, et al.* Mortality in systemic lupus erythematosus. *Arthritis Rheum* 2006;54(8):2550-7.

2. Cervera R, Khamashta MA, Font J*, et al.* Morbidity and mortality in systemic lupus erythematosus during a 10-year period: a comparison of early and late manifestations in a cohort of 1,000 patients. *Medicine (Baltimore)* 2003;82(5):299-308.

3. Seligman VA, Lum RF, Olson JL*, et al.* Demographic differences in the development of lupus nephritis: a retrospective analysis. *Am J Med* 2002;112(9):726-9.

4. Somers EC, Marder W, Cagnoli P*, et al.* Population-based incidence and prevalence of systemic lupus erythematosus: the Michigan Lupus Epidemiology and Surveillance program. *Arthritis Rheumatol* 2014;66(2):369-78.

5. Lim SS, Bayakly AR, Helmick CG*, et al.* The incidence and prevalence of systemic lupus erythematosus, 2002-2004: The Georgia Lupus Registry. *Arthritis Rheumatol* 2014;66(2):357-68.

6. Bertsias GK, Tektonidou M, Amoura Z*, et al.* Joint European League Against Rheumatism and European Renal Association–European Dialysis and Transplant Association (EULAR/ERA-EDTA) recommendations for the management of adult and paediatric lupus nephritis. *Ann Rheum Dis* 2012;71(11):1771-82.

7. Hahn BH, McMahon MA, Wilkinson A*, et al.* American College of Rheumatology guidelines for screening, treatment, and management of lupus nephritis. *Arthritis Care Res* 2012;64(6):797-808.

8. Kidney Disease: Improving Global Outcomes (KDIGO) Glomerulonephritis Work Group. KDIGO Clinical Practice Guideline for Glomerulonephritis. *Kidney Int Suppl* 2012;2:139-274.

9. Ruiz Irastorza G, Espinosa G, Frutos MA*, et al.* Diagnosis and treatment of lupus nephritis. Consensus document from the systemic auto-immune disease group (GEAS) of the Spanish Society of Internal Medicine (SEMI) and Spanish Society of Nephrology (S.E.N.). *Nefrologia* 2012;32 Suppl 1:1-35.

10. van Tellingen A, Voskuyl AE, Vervloet MG*, et al.* Dutch guidelines for diagnosis and therapy of proliferative lupus nephritis. *Neth J Med* 2012;70(4):199-207.

11. Mina R, von Scheven E, Ardoin SP*, et al.* Consensus treatment plans for induction therapy of newly diagnosed proliferative lupus nephritis in juvenile systemic lupus erythematosus. *Arthritis Care Res (Hoboken)* 2012;64(3):375-83.

12. Wakasugi D, Gono T, Kawaguchi Y*, et al.* Frequency of class III and IV nephritis in systemic lupus erythematosus without clinical renal involvement: an analysis of predictive measures. *J Rheumatol* 2012;39(1):79-85.

13. Christopher-Stine L, Siedner M, Lin J*, et al.* Renal biopsy in lupus patients with low levels of proteinuria. *J Rheumatol* 2007;34(2):332-5.

14. Weening JJ, D'Agati VD, Schwartz MM*, et al.* The classification of glomerulonephritis in systemic lupus erythematosus revisited. *J Am Soc Nephrol* 2004;15(2):241-50.

15. Condon MB, Lightstone L, Cairns T*, et al.* Is rebiopsy required to identify complete remission in patients treated for lupus nephritis? *J Am Soc Nephrol* 2009;20:405A.

16. Gunnarsson I, Sundelin B, Jonsdottir T*, et al.* Histopathologic and clinical outcome of rituximab treatment in patients with cyclophosphamide-resistant proliferative lupus nephritis. *Arthritis Rheum* 2007;56(4):1263-72.

17. Grootscholten C, Bajema IM, Florquin S*, et al.* Treatment with cyclophosphamide delays the progression of chronic lesions more effectively than does treatment with azathioprine plus methylprednisolone in patients with proliferative lupus nephritis. *Arthritis Rheum* 2007;56(3):924-37.

18. Hill GS, Delahousse M, Nochy D, *et al.* Predictive power of the second renal biopsy in lupus nephritis: significance of macrophages. *Kidney Int* 2001;59:304-16.

19. Alsuwaida A, Husain S, Alghonaim M, *et al.* Strategy for second kidney biopsy in patients with lupus nephritis. *Nephrol Dial Transplant* 2012;27(4):1472-8.

20. Pagni F, Galimberti S, Goffredo P, *et al.* The value of repeat biopsy in the management of lupus nephritis: an international multicentre study in a large cohort of patients. *Nephrol Dial Transplant* 2013;28(12):3014-23.

21. Kraft SW, Schwartz MM, Korbet SM, *et al.* Glomerular podocytopathy in patients with systemic lupus erythematosus. *J Am Soc Nephrol* 2005;16(1):175-9.

22. Mok CC, Cheung TT, Lo WH. Minimal mesangial lupus nephritis: a systematic review. *Scand J Rheumatol* 2010;39(3):181-9.

23. Zeher M, Doria A, Lan J, *et al.* Efficacy and safety of enteric-coated mycophenolate sodium in combination with two glucocorticoid regimens for the treatment of active lupus nephritis. *Lupus* 2011;20(14):1484-93.

24. Ruiz-Irastorza G, Danza A, Perales I, *et al.* Prednisone in lupus nephritis: how much is enough? *Autoimmun Rev* 2014;13(2):206-14.

25. Houssiau FA, Vasconcelos C, D'Cruz D, *et al.* The 10-year follow-up data of the Euro-Lupus Nephritis Trial comparing low-dose and high-dose intravenous cyclophosphamide. *Ann Rheum Dis* 2010;69(1):61-4.

26. Houssiau FA, Vasconcelos C, D'Cruz D, *et al.* Immunosuppressive therapy in lupus nephritis: the Euro-Lupus Nephritis Trial, a randomized trial of low-dose versus high-dose intravenous cyclophosphamide. *Arthritis Rheum* 2002;46(8):2121-31.

27. The ACCESS Trial Group. Treatment of Lupus Nephritis With Abatacept: The Abatacept and Cyclophosphamide Combination Efficacy and Safety Study. *Arthritis Rheumatol* 2014;66(11):3096-104.

28. Isenberg D, Appel GB, Contreras G, *et al.* Influence of race/ethnicity on response to lupus nephritis treatment: the ALMS study. *Rheumatology (Oxford)* 2010;49(1):128-40.

29. Walsh M, Solomons N, Lisk L, *et al.* Mycophenolate mofetil or intravenous cyclophosphamide for lupus nephritis with poor kidney function: a subgroup analysis of the Aspreva Lupus Management Study. *Am J Kidney Dis* 2013;61(5):710-5.

30. Rovin BH, Parikh SV, Hebert LA, *et al.* Lupus nephritis: induction therapy in severe lupus nephritis - should MMF be considered the drug of choice? *Clin J Am Soc Nephrol* 2013;8(1):147-53.

31. Dooley MA, Jayne D, Ginzler EM, *et al.* Mycophenolate versus azathioprine as maintenance therapy for lupus nephritis. *N Engl J Med* 2011;365(20):1886-95.

32. Houssiau FA, D'Cruz D, Sangle S, *et al.* Azathioprine versus mycophenolate mofetil for long-term immunosuppression in lupus nephritis: results from the MAINTAIN Nephritis Trial. *Ann Rheum Dis* 2010;69(12):2083-9.

33. Feng L, Deng J, Huo DM, *et al.* Mycophenolate mofetil versus azathioprine as maintenance therapy for lupus nephritis: a meta-analysis. *Nephrology (Carlton)* 2013;18(2):104-10.

34. Radhakrishnan J, Moutzouris DA, Ginzler EM, *et al.* Mycophenolate mofetil and intravenous cyclophosphamide are similar as induction therapy for class V lupus nephritis. *Kidney Int* 2010;77(2):152-60.

35. Austin HA, 3rd, Illei GG, Braun MJ, *et al.* Randomized, controlled trial of prednisone, cyclophosphamide, and cyclosporine in lupus membranous nephropathy. *J Am Soc Nephrol* 2009;20(4):901-11.

36. Praga M, Barrio V, Juarez GF, *et al.* Tacrolimus monotherapy in membranous nephropathy: a randomized controlled trial. *Kidney Int* 2007;71(9):924-30.

37. Howman A, Chapman TL, Langdon MM, *et al.* Immunosuppression for progressive membranous nephropathy: a UK randomised controlled trial. *Lancet* 2013;381(9868):744-51.

38. Ruggenenti P, Cravedi P, Chianca A*, et al.* Rituximab in idiopathic membranous nephropathy. *J Am Soc Nephrol* 2012;23(8):1416-25.

39. Mosca M, Tani C, Aringer M*, 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;69(7):1269-74.

40. Ruiz-Irastorza G, Ramos-Casals M, Brito-Zeron P*, et al.* Clinical efficacy and side effects of antimalarials in systemic lupus erythematosus: a systematic review. *Ann Rheum Dis* 2010;69(1):20-8.

41. Barber CE, Geldenhuys L, Hanly JG. Sustained remission of lupus nephritis. *Lupus* 2006;15(2):94-101.

42. Kasitanon N, Fine DM, Haas M*, et al.* Hydroxychloroquine use predicts complete renal remission within 12 months among patients treated with mycophenolate mofetil therapy for membranous lupus nephritis. *Lupus* 2006;15(6):366-70.

43. Gordon C, Jayne D, Pusey C*, et al.* European consensus statement on the terminology used in the management of lupus glomerulonephritis. *Lupus* 2009;18(3):257-63.

44. Wofsy D, Hillson JL, Diamond B. Comparison of alternative primary outcome measures for use in lupus nephritis clinical trials. *Arthritis Rheum* 2013;65(6):1586-91.

45. Lightstone L. The landscape after LUNAR: rituximab's crater-filled path. *Arthritis Rheum* 2012;64(4):962-5.

46. Weidenbusch M, Rommele C, Schrottle A*, et al.* Beyond the LUNAR trial. Efficacy of rituximab in refractory lupus nephritis. *Nephrol Dial Transplant* 2013;28(1):106-11.

47. Jonsdottir T, Zickert A, Sundelin B*, et al.* Long-term follow-up in lupus nephritis patients treated with rituximab - clinical and histopathological response. *Rheumatology (Oxford)* 2013;52(5):847-55.

48. Condon MB, Ashby D, Pepper RJ*, et al.* Prospective observational single-centre cohort study to evaluate the effectiveness of treating lupus nephritis with rituximab and mycophenolate mofetil but no oral steroids. *Ann Rheum Dis* 2013;72(8):1280-6.

49. Naesens M, Kuypers DR, Sarwal M. Calcineurin inhibitor nephrotoxicity. *Clin J Am Soc Nephrol* 2009;4(2):481-508.

50. Liu Z, Zhang H, Liu Z*, et al.* Multitarget therapy for induction treatment of lupus nephritis: a randomized trial. *Ann Intern Med* 2015;162(1):18-26.

51. Ostensen M, Khamashta M, Lockshin M*, et al.* Anti-inflammatory and immunosuppressive drugs and reproduction. *Arthritis Res Ther* 2006;8(3):209.

52. Goldstein LH, Dolinsky G, Greenberg R*, et al.* Pregnancy outcome of women exposed to azathioprine during pregnancy. *Birth Defects Res A Clin Mol Teratol* 2007;79(10):696-701.

53. Livingston B, Bonner A, Pope J. Differences in clinical manifestations between childhood-onset lupus and adult-onset lupus: a meta-analysis. *Lupus* 2011;20(13):1345-55.

54. Sundel R, Solomons N, Lisk L. Efficacy of mycophenolate mofetil in adolescent patients with lupus nephritis: evidence from a two-phase, prospective randomized trial. *Lupus* 2012;21(13):1433-43.

55. Kidney Disease: Improving Global Outcomes (KDIGO) Blood Pressure Work Group. KDIGO Clinical Practice Guideline for the Management of Blood Pressure in Chronic Kidney Disease. *Kidney Int Suppl* 2012;2(5):337-414.

Supplemental Table 1. Guidelines compared; overview of all guidelines

	EULAR/ERA-EDTA	ACR	KDIGO	GEAS	DWP	CARRA
From	European League Against Rheumatism and European Renal Association–European Dialysis and Transplant Association	American College of Rheumatology	Kidney Disease: Improving Global Outcomes Glomerulonephritis Work Group	Systemic auto-immune disease group of the Spanish Society of Internal Medicine and Spanish Society of Nephology	Dutch Working Party on Systemic Lupus Erythematosus	Childhood Arthritis and Rheumatology Research Alliance
Indication for renal biopsy	• Reproducible proteinuria ≥0.5 g/24h, especially with glomerular hematuria and/or cellular casts (2C) • Consider: – Persisting isolated glomerular hematuria – Isolated leucocyturia (other causes excluded) – Unexplained renal insufficiency with normal urinary findings	• Increasing serum creatinine without compelling alternative causes (such as sepsis, hypovolemia, or medication) (C) • Confirmed proteinuria of ≥1g/24h (C) • Combinations of the following (confirmed in 2 tests in short period of time and in absence of alternative causes) (C) – Proteinuria ≥0.5 g/24h AND hematuria (≥5 RBCs/HPF) – Proteinuria ≥0.5 g/24h AND cellular casts	Not provided	• Confirmed proteinuria: ≥0.5 g/24h urine samples or protein/creatinine ratio in first morning samples ≥0.5, or a ratio ≥0.5 ratio calculated in 24h urine sample, or active urinary sediment (microhematuria/ leucocyturia/casts) • Inexplicable decrease in renal function (NG)	• >0.5 g/24h proteinuria, independent of presence of hematuria or elevated serum creatinine (C) • ≤0.5 g/24h proteinuria: – Normal creatinine and microscopic hematuria → consider biopsy – Elevated creatinine without microscopic hematuria → consider biopsy when either: ○ Persistent elevation of serum creatinine of >30% ○ Other causes of renal impairment are excluded ○ Positive antiphospholipid antibodies ○ Extrarenal involvement/presence of anti-dsDNA	Not provided

Supplemental Table 1. Continued

	EULAR/ERA-EDTA	ACR	KDIGO	GEAS	DWP	CARRA
					antibodies/hypo-complementemia (C)	
Biopsy evaluation	• At least 8 glomeruli • Score according to ISN/RPS (2C) with assessment of active and chronic glomerular (1A) and tubulo-interstitial changes (2B) and of vascular lesions associated with aPL/APS (3C) • Examine with HE, PAS, Ag, trichrome, IF and if possible EM	Not provided	Not provided	• Classify according to ISN/RPS (NG) • Optimal optical microscope and IF techniques and EM recommended (NG) • Quantified data on activity and chronicity and a description of vascular and interstitial lesions should be provided (NG)	Not provided	According to ISN/RPS classification system
Indication for repeat biopsy	In selected cases: – Worsening or refractory to treatment (failure to decrease proteinuria ≥50%, persistent proteinuria beyond 1 year and/or worsening of GFR) – At relapse, to demonstrate change or progression in histological class, change in activity	Not provided	Consider if: – No complete remission after 1 year (NG) – During relapse if there is suspicion that the histologic class has changed or there is uncertainty whether a rise in sCr or proteinuria represents	Only if findings can lead to a change in treatment or prognosis (NG): – Increase or reappearance of proteinuria, nephrotic syndrome, or active urinary sediment, especially if the first biopsy revealed a non-proliferative form – Increased sCr or inexplicable evolution towards renal failure – Refractory to immunosuppressives	Only if therapeutic consequences (C): – Persistence proteinuria after partial response (despite optimal supportive treatment): active or chronic disease or progression to FSGS – Failure to respond at 12 months, in order to differentiate between chronic and active	No consensus reached

Supplemental Table 1. Continued

	EULAR/ERA-EDTA	ACR	KDIGO	GEAS	DWP	CARRA
	and chronicity index, to provide prognostic information and to detect other pathologies (3C)		disease activity or chronicity (NG) – In patients with worsening sCr and/or proteinuria after completing one of the initial treatment regimens in order to distinguish active LN from scarring (NG)	– Uncertainty with regard to activity/chronicity of renal lesions (deciding upon treatment) – Suspicion of non-lupus related nephropathy		
Treatment class II	Proteinuria >1 g/24h despite RAAS inhibition, especially in the presence of glomerular hematuria; low to moderate doses oral glucocorticoids (0.25-0.5 mg/kg/day) alone or in combination with AZA (1-2 mg/kg/day)	No immunosuppressive treatment (C)	• Proteinuria <1 g/24h: treat as dictated by extrarenal manifestations (2D) • Proteinuria >3 g/24h: corticosteroids or CNI as described for MCD (2D)	Significant proteinuria (>1-2 g/24h despite renal protective treatment) and/or deteriorated renal function that is not attributable to functional factors; steroids up to 0.5 mg/kg/day, possibly plus AZA or MMF for 6-12 months (2D)	Not provided	Not provided
Induction treatment class III/IV *without* crescents (and/or other adverse parameters)	• For A or A/C classes (ISN/RPS 2003) • Regimens: – Glucocorticoids: 3 iv pulses MP; 500-750 mg/day (3C) + oral; 0.5	• Regimens: – Glucocorticoids: 3 iv pulses MP; 500-1000 mg/day + oral; 0.5-1 mg/kg/day and taper (C) – MMF (2-3 g total	• Regimens: – Glucocorticoids: 3 iv pulses MP (widely used for more severe disease, no dose provided) + oral; (1A) up to 1	• Regimens: – Glucocorticoids: 3 iv pulses MP (250-1000 mg/day) in presence of extracapillary proliferation or acute deterioration of renal function (2C) + oral; start	• Regimens: – MMF to 3 g total daily dose in 3 weeks + oral glucocorticoids; 1 mg/kg/day, max 60 mg (A) and taper: after 4 weeks 10 mg every 4 weeks to 20	• Regimens: – Glucocorticoids: 3 iv pulses MP (30 mg/kg/dose up to 1000 mg/dose)

Supplemental Table 1. Continued

	EULAR/ERA-EDTA	ACR	KDIGO	GEAS	DWP	CARRA
	mg/kg/day for 4 weeks, reducing to ≤10 mg/day by 4-6 months (C) – MMF (3 g total daily dose) for 6 months (seems preferable) (1A) or low dose ivCYC (1B) (in Caucasians) • African descent: MMF might be better but further confirmation needed • Mild cases: AZA (2 mg/kg/day) can be considered (2B)	daily dose; Asian 2 g considered) or ivCYC (white European high or low dose (B); rest high dose) • African Americans and Hispanics: favor MMF • MMF over ivCYC if child bearing concerns • Keep up for 6 months unless worsening at 3 months	mg/kg/day and taper according to clinical response over 6-12 months – MMF (1B) or ivCYC (1B); low dose ivCYC effective in Caucasians with not too severe disease, unclear if also case for other ethnicities and severe disease • MMF equivalent to high dose ivCYC in short term, not clear for long-term • If worsening LN (rising sCr, worsening proteinuria) in first 3 months → change to alternative initial (induction) therapy or repeat kidney biopsy (2D) • Race: further information required	up to 1 mg/kg/day (max 60 mg), 0.5 mg/kg/day can be used with concomitant pulses of MP (2C) and if possible taper to 5 mg/day – MMF 2-2.5 g/day (1B) or ivCYC; either monthly 750 mg/m^2 for 6 months (NIH), or fortnightly 500 mg for 3 months (Eurolupus) with 3 MP pulses (750 mg/day), followed by oral prednisone 0.5 mg/kg/day (1B)	mg, followed by 5 mg every 4 weeks to 10 mg – Low dose ivCYC + 3 iv pulses MP + oral glucocorticoids 0.5-1 mg/kg/day (A) and taper: after 4 weeks every 2 weeks with 2.5 mg to 5-7.5 mg at 30 months • Race: MMF may be better in Blacks	and/or oral glucocorticoids depending on which of the 3 scheme's (primarily oral, primarily iv or mixed oral/iv) is chosen – ivCYC; 6 monthly doses, initial dose 500 mg/m^2, subsequent doses higher but not more than 1500 mg (C) (most often used in practice), or MMF; 600 mg/m^2/day twice daily with a max of 1500 mg twice a day (C)

Supplemental Table 1. Continued

	EULAR/ERA-EDTA	ACR	KDIGO	GEAS	DWP	CARRA
Induction treatment class IV or IV/V *with* crescents (and/or other adverse parameters)	With adverse prognostic profile (acute deterioration renal function, substantial cellular crescents and/or fibrinoid necrosis): – Same regimen (MMF (2B); low dose ivCYC (4C)) – CYC can also be prescribed monthly iv at higher doses (0.75-1 g/m^2) for 6 months (1A) or orally (2-2.5 mg/kg/day) for 3 months (3B)	Either CYC or MMF(3 g total daily dose, instead of 2-3 g) (C) + 3 pulses MP and oral glucocorticoids at 1 mg/kg/day, instead of 0.5-1 mg/kg/day	Not different from without crescents (and/or other adverse parameters)	• 3 pulses MP (250-1000 mg/day) in presence of extracapillary proliferation or acute deterioration of renal function (2C) • Include ivCYC if severe decrease in renal function (sCr >3 mg/dL) or cellular crescents or fibrinoid necrosis (2C)	Not different from without crescents (and/or other adverse parameters)	Not different from without crescents (and/or other adverse parameters)
Induction treatment class V	• If nephrotic range proteinuria (≥3 g/24h): prednisone (0.5 mg/kg/day) and MMF 3 g total daily dose for 6 months(2B) • Alternatives: high dose ivCYC (2A), CNIs (ciclosporin (2A); tacrolimus (3B)) or rituximab (4C)	If nephrotic range proteinuria (≥3 g/24h): prednisone (0.5 mg/kg/day) and MMF 2-3 g total daily dose (A)	• If normal kidney function, non-nephrotic range proteinuria → no immunosuppressives unless dictated by extrarenal disease (2D) • Persistent nephrotic range proteinuria: corticosteroids plus immunosuppres	• Oral steroids up to 1 mg/kg/day (max 60 mg) initially and taper • Plus one of: – ivCYC (1B), dose as in class III/IV – CNIs (ciclosporin, dose 2-5 mg/kg/day (1B); tacrolimus, dose 0.15-0.2 mg/kg/day (2C)) – MMF (1B), dose as III/IV – AZA (1C), dose 1.5-2 mg/kg/day	Not provided	Not provided

Supplemental Table 1. Continued

	EULAR/ERA-EDTA	ACR	KDIGO	GEAS	DWP	CARRA
			sive (ivCYC (2C), CNI (2C), MMF (2D) or AZA (2D))			
Treatment class VI	Not provided	Prepare for renal replacement therapy	Treat with corticosteroids and immunosuppressives only as dictated by extrarenal disease (2D)	• Maintain RAAS inhibition and monitor for complications (2C) • Slowly decrease immune suppression until it can be discontinued (unless dictated by extrarenal disease) (1B)	Not provided	Not provided
Maintenance treatment	• Class III/IV: – AZA (2 mg/kg/day) or MMF (2 g/kg/day) (1A) for at least 3 years (3C) – If response to MMF at induction, stay on MMF (C) – Plus low dose oral glucocorticoids (5-7.5 mg/day) • Pure class V: – As class III/IV – CNIs can be considered (4C)	• Class III/IV: – AZA or MMF (A) – Plus low dose oral glucocorticoids	• Class III/IV: – AZA (1.5-2.5 mg/kg/day) or MMF (1-2g/day) (1B) – Plus low dose oral glucocorticoids (≤10 mg/day prednisone equivalent) – CNIs if intolerant to MMF or AZA (2D) – After complete remission, continue maintenance for at least 1 year (2D) – No complete remission after 1 year → consider	• Class III/IV: – MMF (1.5-2 g/day) (over AZA (1.5-2 mg/kg/day)) (2A) – Plus low dose oral glucocorticoids – Duration of treatment: at least 2 years after remission has been reached (2C) • Pure Class V: – Low dose steroids and MMF, CNIs or AZA (2B) – Dosage and duration as in class III and IV	• Class III/IV: – MMF over AZA (A) – Plus low dose oral glucocorticoids	Not provided

Supplemental Table 1. Continued

	EULAR/ERA-EDTA	ACR	KDIGO	GEAS	DWP	CARRA
			repeat biopsy (NG) – If during tapering renal function deteriorates and/or proteinuria worsens, increase to previous level (2D)			
Adjunctive treatment	• HCQ for all (3C); screening ophthalmologist for retinopathy (baseline and yearly after 5 years) • RAAS inhibition for patients with proteinuria (uPCR >50 mg/mmol) or hypertension (target <130/80) (2B) • Statins (target LDL <100 mg/dL = 2.58 mmol/L)) (C) • Acetylsalicylic acid in patient with aPL (C), calcium and vitamin D supplementation (C), and	• Background HCQ unless contraindicated (C) • Proteinuria ≥0.5 g/24h → RAAS inhibition (A) • Control hypertension, target ≤130/80 (A) • LDL >100 mg/dL → statins (C)	• HCQ for all unless contraindicated (2C); screening ophthalmologist for retinopathy (baseline and yearly after 5 years) • Leuprolide/testosterone should be offered to protect fertility • In general in glomerular disease: – Blood pressure control – Treatment of hyperlipidemia – RAAS inhibition in managing	• HCQ for all unless contraindicated (1B); screening ophthalmologist for retinopathy (baseline and yearly thereafter) (1C) • RAAS inhibition in patients with hypertension and/or proteinuria (1B) • Weight loss against proteinuria (1C) if obese • Reduce cardiovascular risks (1B) (lifestyle, BP <130/80, statins) • Calcium and vitamin D (1A) for patients on oral glucocorticoids; bisphosphonates if older than 50 years (1A) • Drugs for gastric protection if history of gastrointestinal	• Background HCQ (B); screening by ophthalmologist for retinopathy (baseline and yearly after 5 years) • Proteinuria ≥1 g/24h → RAAS inhibition (A) • Hypertension control, target <130/80 (A; if proteinuria >1 g/24h) • Treatment hyperlipidemia (C); target 2.6 mmol/L • Calcium and vitamin D (osteoporosis) for patients on oral glucocorticoids; bisphosphonates if >15 mg oral glucocorticoids daily or if >70 years old and 7.5-15 mg oral	Not provided

Supplemental Table 1. Continued

	EULAR/ERA-EDTA	ACR	KDIGO	GEAS	DWP	CARRA
	immunization with non-live vaccines (C) may reduce treatment or disease related comorbidities and should be considered • Consider anti-coagulant treatment in nephrotic syndrome with albumin <20 g/L, especially if also aPL (C)		proteinuria – Manage hypercoagulability	hemorrhage or peptic ulcer disease, or with combination of corticosteroids and NSAIDs (1B) • Avoid vaccines containing live or attenuated viruses during immune suppression (1B) • GnRH analogues in women over 35 y if cumulative CYC dose >10 g (1C)	glucocorticoids daily unless clearance <60mL/min or pregnancy wish • Low dose acetylsalicylic acid if aPL positive • Coumarines considered if nephrotic syndrome with albumin <20g/L (C) • Lifestyle	
Treatment for refractory disease	• Switch from ivCYC to MMF or vice versa (4C), or rituximab (4C) • Other options: CNIs, ivIg, plasma exchange for rapid progressive glomerulonephritis, or immunoadsorption	• Switch from ivCYC to MMF or vice versa accompanied by 3 pulses MP (C) • In some cases rituximab can be used • No consensus on CNI	• In patients with worsening sCr and/or proteinuria after completing one of the initial treatment regimens, consider performing a repeat biopsy to distinguish active LN from scarring (NG) → if active LN, treat with alternative induction therapy (NG) • Responders who	• Switch from CYC to MMF or vice versa (1A) • Alternative treatments (if above fails): rituximab (2B), CNIs (2B), ivIg (2C) or combining drugs (2B) • Change treatment scheme if there are no sign of response before completing the sixth month of induction (1B) • Rule out presence of other diseases and ensure compliance (NG) • If nothing works, consider a new biopsy	• Switch from ivCYC to MMF or vice versa accompanied by 3 pulses MP (C) • Consider: rituximab, tacrolimus, NIH ivCYC	Not provided

Supplemental Table 1. Continued

	EULAR/ERA-EDTA	ACR	KDIGO	GEAS	DWP	CARRA
			have failed more than one induction may be treated with rituximab, ivlg or CNIs (2D)			
Pregnancy	• If stable (uPCR <50 mg/mmol, GFR preferably over 50 mL/min) for 6 months (2B) • Acceptable medications: HCQ (3B), low dose prednisone (4C), azathioprine (4C) or CNIs (4C) • Intensity of treatment should not be reduced in anticipation of pregnancy (C) • Consider acetylsalicylic acid to reduce risk of pre-eclampsia (3C) • Assess at least every 4 weeks (C)	• Prior LN but no current systemic or renal activity: no nephritis medication • Mild systemic activity: HCQ (200-400 mg daily) • Clinically active nephritis or substantial extrarenal disease: oral glucocorticoids, if necessary AZA (max 2 mg/kg) (C)	• Preferably delay pregnancy until in complete remission (2D) • Don't use CYC, MMF, ACE-I and ARBs during pregnancy (1A) • Continue HCQ (2B) • If pregnant while on MMF, switch to AZA (1B) • Relapse: corticosteroids possibly with AZA (1B) • Don't taper corticosteroids or AZA during pregnancy or within the 3 months after (2D) • Low dose aspirin to decrease risk of fetal loss (2C)	• Plan after at least 6 months of (partial) remission (1B) • Monitor closely by multi-disciplinary team (NG) • For blood pressure control suspend RAAS inhibitors and use methyldopa, labetalol or nifedipine (1B) • Avoid teratogenic drugs (CYC, MMF, MTX); AZA safe • Continue HCQ during pregnancy • Aspirin at low doses (100 mg/day) before week 12 to reduce risk of pre-eclampsia and fetal loss (1A)	Not provided	Not provided
Vascular	• In patients with	Treat TMA with plasma	• APS involving	• Maintain indefinite	Not provided	Not provided

Supplemental Table 1. Continued

	EULAR/ERA-EDTA	ACR	KDIGO	GEAS	DWP	CARRA
complications	APSN; consider HCQ (C) and/or antiplatelet/ anticoagulant treatment (C) • Definite APS → anticoagulant treatment	exchange therapy	the kidney (APSN) with or without LN → anticoagulation INR 2-3 (2D) • If TTP → plasma exchange as in patients without lupus (2D)	anticoagulant treatment in patients with ASPN (2C) • Treat thrombosis of major renal vessels with prolonged anticoagulation (1B), as in non-APS associated thrombosis		
Monitoring	• Active LN should be regularly monitored by determining at each visit body weight, BP, sCr and eGFR, serum albumin, proteinuria, urinary sediment (microscopic evaluation), serum C3 and C4, serum anti-dsDNA and complete blood cell count • Anti-phospholipid antibodies and lipid profile should be measured at baseline and monitored intermittently • Visits should be scheduled every 2-4 weeks for the	• Active nephritis at onset of treatment: BP 1, urine 1, uPCR 1, sCr 1, C3/C4 2, anti-DNA 3 (monthly intervals) • Previous active nephritis, none currently: BP 3, urine 3, uPCR 3, sCr 3, C3/C4 3, anti-DNA 6 (monthly intervals) • Pregnant with active GN at onset of treatment: BP 1, urine 1, uPCR 1, sCr 1, C3/C4 1, anti-DNA 1 (monthly intervals) • Pregnant with previous nephritis, none currently: BP 1, urine 1, uPCR 3, sCr 3, C3/C4 3, anti-DNA 3	Not provided.	• Every 3 months by determining creatinine proteinuria, anti-dsDNA, C3 and C4 (NG) • Proteinuria should be measured in 24-hour urine, follow-up may only include protein/creatinine ratio in first morning urine (NG) • At baseline more lab tests particularly those relevant for assessing cardiovascular risks (see detailed scheme in table 8 (in original article))	Not provided	Not provided

ivCYC high dose (NIH regimen) = 0.5-1 g/m2 monthly for 6 months; ivCYC low dose (Eurolupus regimen) = 500 mg every 2 weeks for 3 months. A, active; (A)/(B)/(2A), level of evidence (for criteria see table S3); Ag, silver staining; aPL, anti-phospholipid antibodies; APS, antiphospholipid syndrome; APSN, anti-phospholipid syndrome-associated nephropathy; AZA, azathioprine; BP, blood pressure; C, chronic; CNI, calcineurin inhibitor; (e)GFR, (estimated) glomerular filtration rate; EM, electron microscopy; FSGS, focal segmental glomerulosclerosis; GnRH, gonadotrophin-releasing hormone; HCQ, hydroxychloroquine; HD,

Supplemental Table 1. Continued

	EULAR/ERA-EDTA	ACR	KDIGO	GEAS	DWP	CARRA
	first 2-4 months after diagnosis or flare (C) and every 3-6 months for life (C)	(monthly intervals) • No prior or current nephritis: BP 3, urine 6, uPCR 6, sCr 6, C3/C4 6, anti-DNA 6 (monthly intervals) (C)				
Management of ESRD	• Renal replacement therapy: possible increased risk of infection in patients still on immunosuppressives (2B) and of vascular access thrombosis in patients with aPL (3C) • Transplantation: – If lupus activity absent or low for at least 3-6 months (3C) – Determine aPL; associated with increased risk of vascular events in the transplant (2B)	Not provided	Not provided	• ESRD: – If reached during flare, induction treatment should be continued for 4-6 months after beginning dialysis, until lack of recovery is observed (NG) – Decrease immunosuppressives to levels required for extrarenal lupus (1B) • Renal replacement therapy: – Inactive lupus → offer PD; active lupus → offer HD (2C) – Increase prophylaxis against infections for PD and HD • Transplantation: – If lupus activity absent or low for 6-12 months (NG) – Determine aPL; associated with increased risk of vascular events in the transplant (NG)	Not provided	Not provided

haemodialysis; HE, haematoxylin and eosin staining; IF, immunofluorescence; ISN/RPS, International Society of Nephrology/Renal Pathology Society; ivCYC, intravenous cyclophosphamide; ivIg, intravenous immunoglobulins; LN, lupus nephritis; MCD, minimal change disease; MMF, mycophenolate mofetil; MP, methylprednisolone; NG, not graded (level of evidence); NSAID, non-steroidal anti-inflammatory drug; PAS, periodic acid Schiff staining; PD, peritoneal dialysis; RBC, red blood cell; sCr, serum creatinine; TMA: thrombotic microangiopathy; TTP, thrombocytopenic purpura; uPCR, urine protein-creatinine ratio.

Supplemental Table 2. Definitions of response to treatment and flares

	EULAR	KDIGO	GEAS	DWP	CARRA
Complete response	• uPCR <50 mg/mmol [approx. <0.5 g/24h] • Plus (near) normal (within 10% of normal GFR) renal function	• A decline in the uPCR to <500mg/g • Plus return of sCr to previous baseline	• Proteinuria ≤0.5 g/24h • Plus sCr <1.2 mg/dL (or decrease to initial values or ±15% of baseline value in patients with sCr ≥1.2 mg/dL [106 µmol/L]) • Plus inactive urinary sediment (≤5 RBCs/HPF (debatable), ≤5 WBCs/HPF, 0 RBC casts) • Plus serum albumin >3g/dL	• Proteinuria <0.5 g/24h • And/or sCr within 125% of the baseline value at 6 to 12 months after the start of induction therapy	• uPCR <200 mg/g or age appropriate • Plus normalization of renal function • Plus inactive urine sediment (<5 WBCs/HPF, <5 RBCs/HPF, and no casts)
Partial response	• ≥50% reduction in proteinuria to subnephrotic levels • Plus (near) normal renal function • It should be achieved preferably by 6 months but no later than 12 months following treatment initiation	• ≥50% decrease in uPCR • If there was nephrotic-range proteinuria (uPCR ≥3000mg/g), improvement requires a ≥50% reduction in uPCR, and a uPCR <3000 mg/g • Plus stabilization (±25%), or improvement of sCr, but not to normal	• In patients with baseline proteinuria <3.5g/24h, >50% reduction in proteinuria compared to initial values • In patients with ≥3.5 g/24h, decreased proteinuria <3.5 g/24h • Plus stabilization (±25%) or improvement in serum creatinine with regard to initial values	• Reduction of proteinuria of >50% (and at least <3 g/24h) • Plus sCr within 125% of the baseline value at 6 to 12 months after the start of the induction therapy	Moderate response • At least 50% improvement in 2 core renal parameters (with max uPCR ≤1000 mg/g) without clinically relevant worsening of the remaining renal core parameter Mild response • 30–50% improvement in 2 core renal parameters without clinically relevant worsening of the remaining renal core parameter *Renal core parameters:* proteinuria (uPCR), renal function (creatinine clearance or sCr) and urine sediment (WBCs, RBCs, and casts)
Flare	Nephritic flare • Reproducible	Mild kidney relapse • ↑ glomerular hematuria	Mild recurrence • ↑ RBCs in sediment from <5	• An increase in disease activity that requires intensification of the	Nephritic renal flare • Increase or recurrence of

Supplemental Table 2. Continued

	EULAR	KDIGO	GEAS	DWP	CARRA
	increase of serum creatinine by ≥30% (or, decrease in GFR by ≥10%) • Plus active urine sediment with increase in glomerular hematuria by ≥10 RBCs/HPF • Irrespective of changes in proteinuria Proteinuric flare • Reproducible doubling of uPCR to >100 mg/mmol after complete response • Or reproducible doubling of uPCR to >200 mg/mmol after partial response	from <5 to >15 RBC/hpf, with ≥ 2 acanthocytes/HPF • And/or recurrence of ≥1 RBC cast, WBC cast (no infection), or both Moderate kidney relapse • If baseline sCr is: – <2 mg/dL [<177 mmol/L]; increase of 0.2–1.0 mg/dL [17.7–88.4 mmol/L] – ≥2 mg/dL [≥177 mmol/L]; increase of 0.4–1.5 mg/dL [35.4–132.6 mmol/L] • And/or if baseline uPCR is: – <500 mg/g; increase to ≥1000 mg/g – 500–1000 mg/g; increase to ≥2000 mg/g, but less than absolute increase of <5000 mg/g – >1000 mg/g; increase of ≥2-fold with absolute uPCR <5000 mg/g Severe kidney relapse • If baseline sCr is: – <2 mg/dL [<177 mmol/L]; increase of >1.0 mg/dL [>88.4 mmol/L] – ≥2 mg/dL [≥177 mmol/L]; increase of >1.5 mg/dL [>132.6 mmol/L] – *and/or* an absolute increase of uPCR >5000 mg/g	to >15/HPF with ≥2 dimorphic RBCs/HPF • And/or ≥1 cast, leukocyte count (in the absence of urinary infection), or both Moderate recurrence • If baseline sCr is: – <2 mg/dL, ↑ by 0.2-1 mg/dL – >2 mg/dL, ↑ by 0.4-1.5 mg/dL • And/or if the uPCR is: – <500 mg/g , ↑ by ≥1000 mg/g – 500-1000 mg/g, ↑ by ≥2000 mg/g – But with an absolute increase < 5000 mg/g Severe recurrence • If baseline sCr is: – <2 mg/dL, ↑ by >1 mg/dL – ≥2 mg/dL, ↑ by >1.5 mg/dL • And/or a uPCR >5000 mg/g **NB:** in case of relapse rule out non-compliance.	therapy, defined as: – An increase of ≥25% in the lowest sCr measured during the period of induction therapy – And/or the development of either a nephrotic syndrome (proteinuria >3.5 g/24h and serum albumin <30 g/L), while the lowest protein excretion so far has been ≤2.0 g/24h repeatedly, or proteinuria >1.5 g/24h in a previous non-proteinuric patient	active urinary sediment (increased hematuria with or without reappearance of cellular casts) • With or without a concomitant increase in proteinuria Proteinuric/nephrotic renal flare • A persistent increase in uPCR >500 mg/g after achieving complete response • Or a doubling of proteinuria with uPCR >1000 mg/g, after achieving a partial response

Supplemental Table 2. Continued

	EULAR	KDIGO	GEAS	DWP CARRA
Refractory disease	• Failing to improve within 3–4 months • No partial response after 6–12 months of treatment • No complete response after 2 years of treatment	No consensus definition	Resistance to treatment is defined as an absence of complete or partial response after completing the induction therapy phase. But there is no consensus on how to define the minimum time for the induction therapy phase or the minimum cumulative dose of immunosuppressive drugs needed to consider the disease resistant to treatment	**DWP**: Persistent or worsening renal disease activity as manifested by progressive deterioration of renal function and/or proteinuria despite optimal immunosuppressive therapy and supportive treatment, and involving at least one of the following conditions: I) failure of the initial induction treatment at three months, for which a switch to another induction therapy regime has already been carried out; II) intolerance for CYC and MMF; III) exceeding a cumulative dose of 15 gram of cyclophosphamide, IV) a second relapse within two years after start of the initial induction therapy, and V) a relative contraindication for high-dose oral or intravenous (iv) prednisone, such as avascular osteonecrosis, previous psychosis on corticosteroids, osteoporosis and/or severe obesity (BMI ≥35 kg/m^2)

ACR: "Definitions of response, degree of response, flare, severity of flare, and remission vary significantly in the literature and depend on the starting point in each individual patient; therefore, an exact definition of these terms was not included in the scenarios. Identification of response, flare, and failure to respond were based on the experienced clinician's opinion, and it is intended that the treating clinician make similar judgments in employment of the recommendations outlined here"

uPCR 100 mg/mmol ≡ 1000 mg/g ≡ 1(g/g) ≈ 1 g/24 h.55 ACR, American College of Rheumatology; CARRA, Childhood Arthritis and Rheumatology Research Alliance; DWP, Dutch Working Party on Systemic Lupus Erythematosus; EULAR/ERA-EDTA, European League Against Rheumatism and European Renal Association–European Dialysis and Transplant Association; GEAS, Systemic auto-immune disease group of the Spanish Society of Internal Medicine and Spanish Society of Nephrology; GFR, glomerular filtration rate; HPF, high power field; KDIGO, Kidney Disease: Improving Global Outcomes Glomerulonephritis Work Group; RBC, red blood cell; sCr, serum creatinine; uPCR, urine protein-creatinine ratio.

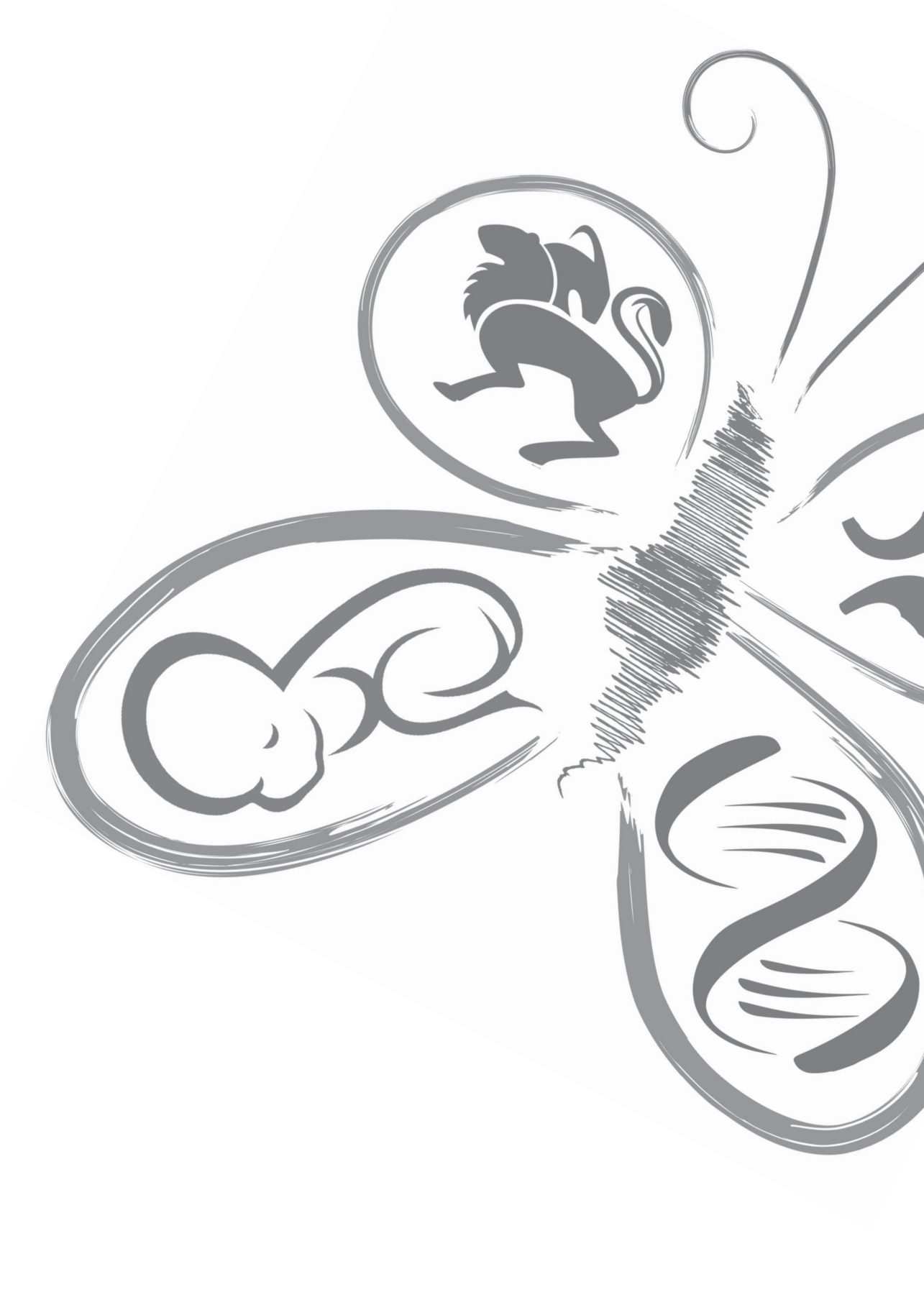
Supplemental Table 3. Quality of evidence and strength of recommendations as applied by the different guidelines

EULAR/ERA-EDTA: European League Against Rheumatism and European Renal Association- European Dialysis and Transplant Association			
Level of evidence	Diagnosis/Monitoring/Prognosis	Treatment	
1	The available evidence is *strong* and includes consistent results from well-designed, well–conducted studies	Meta-analysis of randomized controlled trial (RCT) or >1 RCTs	
2	The available evidence is *sufficient* to determine effects, but confidence in the estimate is constrained by such factors as: the number, size, or quality of individual studies, inconsistency of findings across individual studies, limited generalizability of findings	Single RCT; long-term follow-up study of primary/secondary outcomes or post-hoc analysis based on the original randomization allocation	
3	The available evidence is *weak* due to the limited number or size of studies, important flaws in study design or methods, inconsistency of findings across individual studies, gaps in the chain of evidence, lack of information on important outcomes	Non-randomized controlled study (prospective or retrospective)	
4	-	Uncontrolled studies (case series)	
Strength of statements			
A	Based on level 1 evidence	Based on level 1 or 2 evidence without concerns for the validity of the evidence	
B	Based on level 2 evidence; or extrapolated recommendations from category 1 evidence	Based on level 1 or 2 evidence but with concerns about the validity of the evidence; or level 3 evidence without major concerns about the validity of the evidence	
C	Based on category 3; or extrapolated recommendations from category 2 evidence; or no data (expert opinion); or extrapolation from non-SLE literature	Based on level 3 evidence with concerns about the validity of the evidence; or level 4 evidence; or no data (expert opinion); or extrapolation from non-SLE literature	
ACR/DWP: American College of Rheumatology/Dutch Working Party on SLE			
A	Evidence represents data derived from multiple randomized controlled trials (RCTs) or meta-analysis		
B	Evidence from a single RCT or non-randomized study		
C	Evidence from consensus, expert opinion or case series		
KDIGO: Kidney Disease: Improving Global Outcomes Glomerulonephritis Work Group			
Strength of recommendation	Patients	Clinicians	Policy
1 (recommend)	Most people in your situation would want the recommended course of action and only a small proportion would not	Most patients should receive the recommended course of action	The commendation can be evaluated as a candidate for developing a policy or performance measure

Supplemental Table 3. Continued

2 (suggest)	The majority of people in your situation would want the recommended course of action, but many would not	Different choices will be appropriate for different patients; each patient needs help to arrive at a management decision consistent with her or his values and preferences	The recommendation is likely to require substantial debate and involvement of stakeholders before policy can be determined

Grade	Quality of evidence	Meaning
A	High	We are confident that the true effect lies close to that of the estimate of the effect
B	Moderate	The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different
C	Low	The true effect may be substantially different from the estimate of the effect
D	Very low	The estimate of effect is very uncertain, and often will be far from the truth
GEAS: Systemic auto-immune disease group of the Spanish Society of Internal Medicine and the Spanish Society of Nephrology		
Quality level of evidence		
A	High	
B	Moderate	
C	Low	
D	Very low	
Recommendations grade		
1	Strong	
2	Weak	
NG	Not graded	
CARRA: Childhood Arthritis and Rheumatology Research Alliance		
A	Supported by randomized clinical trials (RCTs)	
B	Supported by non-randomized controlled studies or extrapolations from RCTs	
C	Supported by uncontrolled studies, extrapolations from non-randomized controlled studies, or marked extrapolation from RCTs (e.g. adults to pediatrics)	
D	Based on expert opinion	



5

Increased Microchimerism in Peripheral Blood of Women with Systemic Lupus Erythematosus

Suzanne Wilhelmus, Emilie C. Rijnink, Malu Zandbergen, Juan D.N. Diaz de Pool, Mathilde M. Almekinders, Jo H.M. Berden, Marc Bijl, Ernst C. Hagen, Gerda M. Steup-Beekman, Hans J. Baelde, Jan A. Bruijn, Ingeborg M. Bajema

Abstract

Objectives

Past research suggests that microchimerism plays a role in systemic lupus erythematosus (SLE). In this study, we aimed to determine the presence and amount of microchimerism in peripheral blood of women with SLE as compared to control subjects. Additionally, we investigated the origin of chimeric cells and the relationship between microchimerism and disease onset, disease activity, and accumulated damage.

Methods

We performed a case-control study with 11 female SLE patients and 22 control subjects. Their children (both male and female) and, if possible, their mothers were also included. Quantitative PCR for insertion-deletion polymorphisms and null alleles was used to detect microchimerism in peripheral blood mononuclear cells and granulocytes.

Results

Microchimerism was detected more often in patients than control subjects (54.4% versus 13.6%, respectively; P=0.03). When present, microchimerism was fetal in origin in almost all cases, and the median total number of fetal chimeric cells was $5/10^6$ in patients and $2.5/10^6$ in control subjects (P=0.048). Maternal microchimerism was detected in one patient and one control subject. In 50% of patients with microchimerism, it originated from multiple relatives, whereas in control subjects, microchimerism was always derived from one relative. We found no relationship between microchimerism and clinical or laboratory parameters related to SLE.

Conclusions

SLE patients had microchimerism in peripheral blood more often and at higher levels than control subjects. In both patients and control subjects, microchimerism was predominantly fetal in origin. This study provides the first evidence that microchimerism in SLE can be derived from multiple relatives.

Introduction

Microchimerism (Mc) refers to the presence in an individual of a small number of genetically distinct cells of any type, originating from a different zygote. The most common (physiologic) source of Mc is pregnancy,[1] including both miscarriages and pregnancies resulting in live birth. [2-5] It can occur when fetal cells enter the maternal circulation, causing fetal Mc (FMc) in the mother. It may also develop in the opposite direction, with maternal cells crossing the placental barrier to the fetus, leading to maternal Mc (MMc).

The role of Mc in health and disease is unclear. Mc has been suggested to play a role in several autoimmune diseases, including systemic lupus erythematosus (SLE).[6-9] SLE primarily affects women and has a peak incidence in the reproductive years.[10] Studies in mice showed that, in selected parent-to-F1 combinations, injection of parental lymphocytes in their offspring led to a graft-versus-host response and a lupus-like disease.[11 12] These data suggest that pregnancy-acquired Mc may be of pathogenic significance in the development of SLE.

Women with SLE have a significantly higher prevalence of fetal Y chromosome-positive chimeric cells in tissue than healthy control subjects.[13-15] There is conflicting research as to there is an increased frequency of FMc in the peripheral blood of SLE patients as compared to control subjects.[16 17 18 19] Previous studies on FMc in SLE were limited to the detection of male Mc, thereby underestimating the total amount of Mc. Furthermore, because Mc was mostly studied in whole blood, the phenotype of the chimeric cells could not be determined. MMc in SLE in peripheral blood was studied by Kanold *et al.* and they did not find a difference between patients and control subjects.[20] However, their sensitivity of detecting chimeric cells was relatively low. None of these studies investigated FMc and MMc together.

The aim of our study was to determine the presence and amount of Mc in peripheral blood of SLE patients and compare it to healthy control subjects. We studied peripheral blood mononuclear cells (PBMCs) and granulocytes separately to determine if Mc was present in either subset independently, or in both. We used insertion-deletion polymorphisms (indels) or null alleles for the detection of Mc, enabling us to study the origin of the chimeric cells as either fetal, maternal, or both. We were also able to establish whether Mc was derived from one relative or from multiple relatives. To understand the role of Mc in SLE, we investigated the relationship between disease activity or accumulated damage since the onset of SLE, and the presence of Mc. Finally, the temporal relationship between the chimerism-causing pregnancy and disease onset in SLE patients was studied.

Materials and methods

Ethics statement

This study was approved by the Medical Ethics Committee of the Leiden University Medical Center (LUMC) (P09.047). Informed consent was obtained from all participants. Parents of minors gave written consent on their behalf.

Patients and control subjects

Participants included 11 female SLE patients and 22 female control subjects. From 2010 to 2015 SLE patients were recruited from four hospitals in the Netherlands: University Medical Center Groningen, Radboud University Medical Center Nijmegen, Bronovo Hospital The Hague and Meander Medical Center Amersfoort. All participants fulfilled at least four of the 1982 revised American College of Rheumatology Criteria for the classification of SLE.[21] SLE disease activity was determined using the SLE Disease Activity Index 2000 (SLEDAI-2K).[22] Accumulated damage since SLE onset was measured using the Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index (SDI).[23] The control group consisted of women with no history of autoimmune disease. For inclusion in the study, probands (SLE patients and control subjects) were required to have at least one child of at least 18 years old. Probands' children and mothers were invited to participate. Peripheral blood samples were gathered from the probands; either peripheral blood samples or buccal mouth swabs were collected from their children and mothers. All probands were asked to fill out a questionnaire including their age, ethnicity, reproductive history, history of blood transfusion, use of immunosuppressive medication and medical history.

Isolation of peripheral blood subsets

Peripheral venous blood samples were drawn in sodium-heparine solution vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) and processed to isolate peripheral blood mononuclear cells (PBMCs) by Ficoll amidotrizoate (pharmacy LUMC) with density gradient centrifugation 1.077 g/mL. Erythrolysis (Qiagen, Hilden, Germany) was performed to remove the erythrocytes from the remaining granulocytes. Samples were stored in 10% dimethyl sulfoxide in fetal bovine serum at -180 °C until DNA extraction.

DNA extraction

DNA was extracted from PBMCs and granulocytes using the QIAamp DNA Blood Mini Kit (Qiagen), according to the manufacturer's instructions with a few modifications. We added

40 µL of proteinase K to 5 x 10^6 cells suspended in 200 µL phosphate-buffered saline. After adding 400 µL AL buffer, the suspension was incubated for 30 min (PBMCs) or overnight (granulocytes) at 56 °C. After adding 200 µl of ethanol, the mixture was applied to the Mini spin column. Buffers AW1 and AW2 were used to wash the column, after which 100 µl AE buffer was added and incubated at 70 °C for 10 minutes to elute the DNA. The eluate was reapplied for an optimal yield. DNA samples were stored at 4 °C until quantitative PCR (qPCR). DNA extraction from buccal sterile OmniSwabs (GE Healthcare Life Sciences, Little Chalfont, United Kingdom) was performed with the same kit according to the manufacturer's instruction.

Allele informativity and genotyping

A set of previously published indels and null allelles was used for the detection of FMc and MMc.[24-26] In order to detect both FMc and MMc in the proband, and to discriminate between the proband's children, informative alleles were required to distinguish between the different family members. Maternal DNA was available for six of 11 patients and eight of 22 control subjects. There was no fetal DNA available from any of the miscarriages. Genotyping by qPCR was performed with the same protocol described below, but with a DNA input of 20 ng. Of the published sets of null alleles and indels, 19 were informative in our study population: GSTM1, GSTT1, SRY, RhD (null alleles), and S01a, S01b, S03, S04a, S04b, S05b, S07b, S08b, S09b, S10a, S10b, S11a, S11b, Xq28 and R271 (indels). Primer sequences are listed in Table 1.

Chimerism detection by qPCR

FMc and MMc were detected and quantified by qPCR. In all assays iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) was used, with 7.5 µM of each amplification primer. Amplification and melting conditions for all primers consisted of incubation at 96.5 °C for 10 min, followed by 44 cycles of 96.5 °C for 30 s and 60 °C for 1 min. The melting curve started at 65 °C for 5 s followed by 0.2 °C incremental increase, each lasting 5 s, to 95 °C. Amplification and melting data were collected by a Bio-Rad CFX96 detector and analyzed by Bio-Rad CFX Manager version 3.1.

Primer specificity was ensured by Sanger sequencing of the amplification product and comparing the sequences to known genomic DNA sequences. Sensitivity was determined by testing serial dilutions of DNA positive for the indel or null allele in a background of DNA negative for the respective indel or null allele. A sensitivity of one genome equivalent (gEq,

Table 1. Primers

Marker name	Position	5' Primer 3'
S01a	F	GGT ACC GGG TCT CCA CAT GA
S01b	F	GTA CCG GGT CTC CAC CAG G
S01a/b	R*	GGG AAA GTC ACT CAC CCA AGG
S03	F	CTT TTG CTT TCT GTT TCT TAA GGG C
S03	R	TCA ATC TTT GGG CAG GTT GAA
S04a/b	F*	CTG GTG CCC ACA GTT ACG CT
S04a	R	AAG GAT GCG TGA CTG CTA TGG
S04b	R	AGG ATG CGT GAC TGC TCC TC
S05b	F	AGT TAA AGT AGA CAC GGC CTC CC
S05b	R	CAT CCC CAC ATA CGG AAA AGA
S07b	F	GGT ATT GGC TTT AAA ATA CTC AAC C
S07b	R	CAG CTG CAA CAG TTA TCA ACG TT
S08b	F	GCT GGA TGC CTC ACT GAT GTT
S08b	R	TGG GAA GGA TGC ATA TGA TCT G
S09b	F	GGG CAC CCG TGT GAG TTT T
S09b	R	CAG CTT GTC TGC TTT CTG CTG
S10a	F	GCC ACA AGA GAC TCA G
S10b	F	TTA GAG CCA CAA GAG ACA ACC AG
S10a/b	R*	TGG CTT CCT TGA GGT GGA AT
S11a	F	TAG GAT TCA ACC CTG GAA GC
S11b	F	CCC TGG ATC GCC GTG AA
S11a/b	R*	CCA GCA TGC ACC TGA CTA ACA
GSTM1	F	GAA CTC CCT GAA AAG CTA AAG CT
GSTM1	R	GTT GGG CTC AAA TAT ACG GTG G
GSTT1	F	TCC TTA CTG GTC CTC ACA TCT C
GSTT1	R	TCC CAG CTC ACC GGA TCA T
RhD	F	GCC TGC ATT TGT ACG TGA GA
RhD	R	CAA AGA GTG GCA GAG AAA GGA
Xq28	F	TGG GTT CCA ACC AGC A
Xq28	R	ACT GAC AAT TAT CAC AGC TT
R271	F	AGA GGA TTG ACT CGG G
R271	R	GTT ACG TCT TAG ATG CCA G
SRY	F	TGG CGA TTA AGT CAA ATT CGC
SRY	R	CCC CCT AGT ACC CTG ACA ATG TAT T

F, forward; R, reverse, *common primer.

based on 6.6 pg DNA content per cell) in 100 000 gEq was reached for all primersets. Four aliquots, each containing 660 ng DNA (100 000 gEq), were tested in each subset (PBMC or granulocytes) for every proband. A standard curve for the specific assay was included to quantify the chimeric cells and validate the assay on each plate. It consisted of 100, 10, and 1 gEq spiked DNA per 100 000 gEq background DNA. Every sample was tested for the housekeeping gene GAPDH. Results were expressed as the gEq of chimeric cells per one million gEq (gEq/10^6). The qPCR plate included negative controls consisting of either a water control or background DNA not carrying the indel or null allele tested. Negative

controls were consistently negative across all experiments. If there was any doubt as to the specificity of the amplification product, the length of the PCR product was compared to that of the positive control using QIAxcel Advanced System (Qiagen) according to the manufacturer's protocol.

Anti-contamination procedures

Strict anti-contamination procedures were employed during blood work-up, DNA extraction, and qPCR preparation. Aerosol-resistant pipette tips and clean gloves were used in every stage and blood work-up was performed in a laminar flow cabinet. Before DNA extraction or preparation of the qPCR, the cabinet used was thoroughly cleaned with DNA decontamination reagent (Sigma-Aldrich, St. Louis, MO), and irradiated with UV light for one hour. All lab consumables were certified DNA free, and also irradiated with UV light for one hour. For the qPCR 8-well strips with individual lids were used.

Statistical analysis

For comparison of categorical data a Fisher's exact test was used (history of blood transfusion, presence of Mc). A Student's *t*-test was used to compare normally distributed data (age proband, age eldest child, age youngest child, SDI). For comparison of non-normally distributed numerical data a Mann-Whitney U test was used (number of pregnancies, number of children, number of chimeric cells, SLEDAI-2K). A *P*-value ≤0.05 was considered statistically significant. All analyses were performed using SPSS Statistics 20.0 (IBM, Armonk, NY).

Table 2. Baseline characteristics of SLE patients and controls

Parameter	SLE patients (n=11)	Controls (n=22)	*P*-value
Age proband (y)	56.6 ± 5.5	57.2 ± 5.5	0.79[a]
Age eldest child (y)	31.4 ± 5.2	28.9 ± 5.7	0.24[a]
Age youngest child (y)	27.5 ± 5.3	24.4 ± 4.9	0.11[a]
Number of children	2 (1)	2 (1)	0.60[b]
Number of pregnancies	3 (2)	2.5 (1)	0.37[b]
History of blood transfusion (%)	72.7	13.6	0.001[c]
SLEDAI-2K	0 (4)	-	n/a
SDI	2.2 ± 2.3	-	n/a

Results are shown as mean ±SD or as median (interquartile range), unless otherwise specified. *P*-values were assessed with [a] Student's *t*-test, [b] Mann-Whitney U test, or [c] Fisher's exact test. SLE, systemic lupus erythematosus; SLEDAI-2K, SLE Disease Activity Index 2000; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index; y, years.

Table 3. Microchimerism in SLE patients and controls

Parameter	SLE patients (n=11)	Controls (n=22)	*P*-value
Mc present in PBMCs or granulocytes (%)	54.5	13.6	0.03[a]
Mc present in PBMCs (%)	36.4	9.1	0.15[a]
Mc present in granulocytes (%)	40.0 (n=10)	10.0 (n=20)	0.14[a]
Total number of fetal chimeric cells/proband, when Mc is present (gEq/10^6 gEq)	5 (8.1) (n=6)	2.5 (n/a) (n=3)	0.048[b]

Results are shown as mean ±SD or as median (interquartile range), unless otherwise specified. *P*-values were assessed with [a] Fisher's exact test or [b] Mann-Whitney U test. gEq, genome equivalents; Mc, microchimerism; n/a, not applicable because number of cases is too low to provide an interquartile range; PBMCs, peripheral blood mononuclear cells; SLE, systemic lupus erythematosus.

Table 4. Comparison of SLE patients and controls with and without microchimerism

Parameter	SLE patients			Controls		
	No Mc (n=5)	Mc (n=6)	*P*-value	No Mc (n=19)	Mc (n=3)	*P*-value
Age proband (y)	57.4 ± 5.2	56.0 ± 6.3	0.70[a]	57.6 ± 5.6	54.3 ± 4.3	0.33[a]
Number of children	2 (1)	2.5 (1.25)	0.84[b]	2 (1)	3 (n/a)	0.44[b]
Number of pregnancies	2 (1.5)	3.5 (2)	0.33[b]	2 (1)	3 (n/a)	0.71[b]
Blood transfusion (%)	60.0	83.3	0.55[c]	10.5	33.3	0.37[c]
SLEDAI-2K	0 (2.5)	2 (5.5)	0.37[b]	-	-	-
SDI	1.8 ± 2.0	2.5 ± 2.6	0.64[a]	-	-	-

Results are shown as mean ±SD or as median (interquartile range), unless otherwise specified. *P*-values were assessed with [a] Student's *t*-test, [b] Mann-Whitney U test, or [c] Fisher's exact test. Mc, microchimerism; n/a, not applicable because number of cases is too low to provide an interquartile range; SLE, systemic lupus erythematosus; SLEDAI-2K, SLE Disease Activity Index 2000; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index; y, years.

Results

Baseline characteristics of patients and control subjects are shown in Table 2. SLE patients had Mc more often than control subjects (54.4% versus 13.6%, respectively; *P*=0.03). When Mc was present, the median total number of fetal chimeric cells per proband was higher in the patient group than in the control group (5 gEq/10^6 versus 2.5 gEq/10^6, respectively; *P*=0.048) (Table 3). When comparing patients and control subjects with and without Mc, there was no significant difference in age, number of children, number of pregnancies, history of blood transfusion, disease activity (SLEDAI-2K) or accumulated damage since onset of disease (SDI) (Table 4). No difference was found in the use of immunosuppressive medication between patients with and without Mc (data not shown).

With one exception, all patients and control subjects with detectable Mc had FMc (Table 5). Of the eight control subjects with maternal DNA available, one had detectable MMc. Of the

Table 5. Origin of microchimerism in patients and controls

	Relatives[a]	Chimerism in PBMCs		Chimerism in granulocytes		Blood transfusion in history	SLEDAI-2K	SDI
		Origin	Amount (gEq/10^6)	Origin	Amount (gEq/10^6)			
Patients								
1	Mother, daughter 1, miscarriage (n/a), daughter 2, son	Mother Daughter 1 or 2 Son	16.5 2.5 2.5	n/a	n/a	Yes	10	4
2	Mother, daughter 1, 2 and 3	Daughter 1	5	Daughter 3 Daughter 2 or mother Possibly daughter 1	7.5 2.5	Yes	4	0
3	Mother, daughter, son	Son -	2.5	Daughter, and possibly mother	2.5	Yes	0 (1st blood draw[b])	2
				-			0 (2nd blood draw[b])	2
4	Mother (n/a), miscarriages 1, 2 and 3 (n/a), daughter	-	-	Daughter	15	Yes	4	7
5	Mother (n/a), daughter 1 (deceased, n/a), miscarriage (n/a), daughter 2 (n/a), daughter 3	-	-	Daughter 3	5	Yes	0	1
6	Mother (n/a), son, daughter	Daughter	5	-	-	No	0	1
Controls								
1	Mother (n/a), daughter 1 and 2, son	Daughter 2	2.5	Daughter 2	2.5	Yes	-	-
2	Mother (n/a), daughter, son	-	-	Daughter	2.5	No	-	-
3	Mother, daughter 1, daughter 2, son	Mother	2.5	-		No	-	-

[a] chronologically from old to young based on year of birth/miscarriage

[b] due to technical problems with the material from the first blood draw, a second blood draw was done approximately 1 year later

gEq, genome equivalent; n/a, no DNA available; PBMCs, peripheral blood mononuclear cells; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index.

six SLE patients with maternal DNA available, one had detectable MMc and three did not. Of two patients the possible MMc was indistinguishable from the FMc that was present, due to an overlap in indels and null alleles.

Additionally, we looked at whether the Mc originated from one relative or more. In all three control subjects with detectable Mc, it originated from one relative. In contrast, in at least three of the six SLE patients, the Mc originated from more than one relative, either from multiple children or from a child and mother (Table 5).

In patients with detectable Mc, we did not find a pattern in the temporal relationship between chimerism-causing pregnancies and the start of symptoms or diagnosis of SLE. One patient who experienced prior symptoms had an exacerbation of symptoms during her first pregnancy. This pregnancy resulted in a spontaneous miscarriage. Two patients experienced their first symptoms in their second pregnancy. In one of these patients, this pregnancy resulted in a spontaneous miscarriage. It could not be determined if this pregnancy resulted in long-lasting Mc. In the other patient, both her first and second pregnancy resulted in long-lasting Mc. Finally, three of six patients experienced their first symptoms one, seven and ten years after the birth of their youngest child of whom they carried chimeric cells.

Discussion

Our study demonstrates that female SLE patients are more likely to have detectable Mc in their peripheral blood than female control subjects. In almost all cases with detectable Mc, the origin of the chimeric cells was fetal. Additionally, MMc was detected in one patient and one control. The median total number of fetal chimeric cells in individuals with detectable Mc was higher in patients than in control subjects. Also, SLE patients often had chimeric cells originating from multiple relatives, in contrast to the control subjects, in whom the chimeric cells originated from only one relative.

Our results add support to two prior studies that found FMc more often in the peripheral blood of SLE patients than in control subjects,[16 17] contradicting other studies suggesting that there is no significant difference between the groups.[18 19] Differences in the blood compartment tested (PBMCs or whole blood), the specificities and sensitivities of the different techniques used, and the numbers of patients and control subjects included, may account for these conflicting results. In contrast to our study, previous studies did not demonstrate a difference between SLE patients and control subjects in the number

of chimeric cells present. However, a limitation of these studies was that they exclusively investigated the presence of the Y chromosome, limiting their findings to the detection of male Mc. Our approach allowed us to detect both male and female FMc, as well as MMc. If, in our present study, we had only investigated Mc using the Y chromosome in women with at least one son, we would not have found a statistically significant difference in the occurrence of Mc between patients and control subjects (data not shown). Only one study investigated the presence of MMc in SLE and found no difference between patients and control subjects, MMc occurring in 6% and 3%, respectively.[20] This low prevalence of MMc is in accordance with our results.

In literature, there are indications that within one individual some sources of Mc lead to persistent Mc, while others do not. In one case report about a woman with hepatitis C, the detected chimerism in the liver appeared to originate from only one of her five pregnancies.[27] After blood transfusions, it has been shown that, in the majority of cases with transfusion-associated Mc, there was evidence of only one or two non-recipient HLA-DR alleles, suggesting that the Mc commonly involves only one donor despite some patients receiving blood products from multiple donors.[28] However, in women with multiple children, it has not been systematically studied if there is a "favoured-child" with regard to the persistence of Mc, i.e. if FMc usually originates from one of the children, or from more children. Because we used indels and null alleles for the detection of Mc, we were able to show that at least half of the patients had persistent Mc from multiple relatives while all control subjects only had persistent Mc from one relative. The cause of this phenomenon is largely unknown. Studies in animals have demonstrated that syngenic or congenic matings resulted in more chimerism than allogenic matings, suggesting a role for HLA (mis)matches.[29 30] In humans, in certain autoimmune diseases mothers and children were shown to have fewer HLA disparities,[31 32] but these have not yet been significantly correlated to the presence of Mc.[31] Nevertheless, having a certain HLA allele (HLA DQA1*0501) appears to be associated with the presence of FMc.[33 34] Interestingly, HLA DQA1*0501 has been associated with SLE.[35]

The phenotype of a chimeric cell may affect its potential to lead to persistent Mc. We detected Mc in both PBMCs and granulocytes. Considering the relatively short half-life of granulocytes,[36] it is likely that the chimeric cells detected in this compartment are derived from stem cells. The existence of chimeric fetal progenitor cells was demonstrated in several studies (for review, see Seppanen *et al.*[37]). A higher prevalence of Mc in SLE patients than in control subjects can either mean that (a) more chimeric cells were acquired during pregnancy, (b) more chimeric cells persisted after pregnancy, (c) chimeric stem cells gave rise

to more chimeric cells due to an unknown trigger, or (d) a combination of aforementioned possibilities.

SLE patients were significantly more likely to have a prior history of blood transfusion than were control subjects. However, within the groups of SLE patients or control subjects, we did not find a difference in blood transfusion history between subjects with and subjects without detectable Mc. In literature, persistent chimerism was only described after blood transfusion following traumatic injury (for review, see Bloch *et al.*[38]), a condition that was not the indication for a blood transfusion in any of our subjects. Furthermore, a recent study in patients having received a blood transfusion in the peripartum period, like some of our subjects, did not show Mc at six weeks and six months after pregnancy.[39] Therefore, it is unlikely that the difference in blood transfusion history between patients and control subjects explains our results.

In our study there was no difference in disease activity (SLEDAI-2K) or accumulated damage (SDI) between patients with and without Mc. The former result is in line with previous research.[19] This finding may be a result of our small sample size. Future research will be required to further study the possible association. Additionally, many of the SLE patients who participated in our study were in clinical remission, which may have influenced results.

Our study had a few limitations. Because we did not have maternal DNA available for all subjects, we could not exclude a maternal source of the Mc in all cases. In cases where maternal DNA was available, it was not always possible to distinguish MMc from the detected FMc, due to an overlap in genetic markers. Furthermore, it was not possible to formally exclude all possible sources of Mc, such as unrecognized pregnancies or spontaneous abortions.

In summary, we detected Mc in peripheral blood more often and in higher numbers in female SLE patients than in female control subjects. The Mc detected was predominantly fetal in origin and was found in both PBMCs and granulocytes. This study provides the first evidence that SLE patients can have chimeric cells from more than one relative, while all of the chimeric control subjects had chimeric cells from only one relative. Any attempts to explain the phenomenon at this time are speculative. It may depend on the immune response evoked by specific chimeric cells, possibly relating to HLA, or on the activation status of the immune system of the recipient in general. Future studies addressing the immunological aspects of this phenomenon are necessary to improve our understanding of the process. The exact role of chimeric cells in SLE is still unknown, but our data substantiate the hypothesis that chimeric cells do play a role in SLE.

Acknowledgements

We thank J. Vork for her technical assistance.

Funding

This research was partly funded by Ars Donandi - Schokkenkamp Wegener Lonzieme foundation.

References

1. Walknowska J, Conte FA, Grumbach MM. Practical and theoretical implications of fetal-maternal lymphocyte transfer. *Lancet* 1969;293(7606):1119-22.

2. Sato T, Fujimori K, Sato A*, et al.* Microchimerism after induced or spontaneous abortion. *Obstet Gynecol* 2008;112(3):593-97.

3. Peterson SE, Nelson JL, Guthrie KA*, et al.* Prospective assessment of fetal-maternal cell transfer in miscarriage and pregnancy termination. *Hum Reprod* 2012;27(9):2607-12.

4. Ariga H, Ohto H, Busch MP*, et al.* Kinetics of fetal cellular and cell-free DNA in the maternal circulation during and after pregnancy: implications for noninvasive prenatal diagnosis. *Transfusion* 2001;41(12):1524-30.

5. Rijnink EC, Penning ME, Wolterbeek R*, et al.* Tissue microchimerism is increased during pregnancy: a human autopsy study. *Mol Hum Reprod* 2015;21(11):857-64.

6. Artlett CM, Smith JB, Jimenez SA. Identification of Fetal DNA and Cells in Skin Lesions from Women with Systemic Sclerosis. *N Engl J Med* 1998;338(17):1186-91.

7. Reed AM, Picornell YJ, Harwood A*, et al.* Chimerism in children with juvenile dermatomyositis. *Lancet* 2000;356(9248):2156-57.

8. Nelson JL, Gillespie KM, Lambert NC*, et al.* Maternal microchimerism in peripheral blood in type 1 diabetes and pancreatic islet beta cell microchimerism. *Proc Natl Acad Sci U S A* 2007;104(5):1637-42.

9. Johnson KL, McAlindon TE, Mulcahy E*, et al.* Microchimerism in a female patient with systemic lupus erythematosus. *Arthritis Rheum* 2001;44(9):2107-11.

10. Lisnevskaia L, Murphy G, Isenberg D. Systemic lupus erythematosus. *Lancet* 2014;384(9957):1878-88.

11. Via CS, Shearer GM. T-cell interactions in autoimmunity: insights from a murine model of graft-versus-host disease. *Immunol Today* 1988;9(7-8):207-13.

12. Gleichmann E, Van Elven EH, Van der Veen JP. A systemic lupus erythematosus (SLE)-like disease in mice induced by abnormal T-B cell cooperation. Preferential formation of autoantibodies characteristic of SLE. *Eur J Immunol* 1982;12(2):152-9.

13. Kremer Hovinga I, Koopmans M, Baelde HJ*, et al.* Chimerism occurs twice as often in lupus nephritis as in normal kidneys. *Arthritis Rheum* 2006;54(9):2944-50.

14. Kremer Hovinga I, Koopmans M, Baelde HJ*, et al.* Tissue chimerism in systemic lupus erythematosus is related to injury. *Ann Rheum Dis* 2007;66(12):1568-73.

15. Florim GM, Caldas HC, de Melo JC*, et al.* Fetal microchimerism in kidney biopsies of lupus nephritis patients may be associated with a beneficial effect. *Arthritis Res Ther* 2015;17(1):101.

16. Abbud Filho M, Pavarino-Bertelli EC, Alvarenga MP*, et al.* Systemic lupus erythematosus and microchimerism in autoimmunity. *Transplant Proc* 2002;34:2951-52.

17. Kekow M, Barleben M, Drynda S*, et al.* Long-term persistence and effects of fetal microchimerisms on disease onset and status in a cohort of women with rheumatoid arthritis and systemic lupus erythematosus. *BMC Musculoskelet Disord* 2013;14:325.

18. Gannage M, Amoura Z, Lantz O*, et al.* Feto-maternal microchimerism in connective tissue diseases. *Eur J Immunol* 2002;32(12):3405-13.

19. Mosca M, Curcio M, Lapi S*, et al.* Correlations of Y chromosome microchimerism with disease activity in patients with SLE: analysis of preliminary data. *Ann Rheum Dis* 2003;62(7):651-4.

20. Kanold AMJ, Svenungsson E, Gunnarsson I*, et al.* A Research Study of the Association between Maternal Microchimerism and Systemic Lupus Erythematosus in Adults: A Comparison between Patients and Healthy Controls Based on Single-Nucleotide Polymorphism Using Quantitative Real-Time PCR. *PLoS One* 2013;8(9):e74534.

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. Gladman DD, Ibanez D, Urowitz MB. Systemic lupus erythematosus disease activity index 2000. *J Rheumatol* 2002;29(2):288-91.

23. Gladman D, Ginzler E, Goldsmith C*, et al.* The development and initial validation of the Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index for systemic lupus erythematosus. *Arthritis Rheum* 1996;39(3):363-9.

24. Alizadeh M, Bernard M, Danic B*, et al.* Quantitative assessment of hematopoietic chimerism after bone marrow transplantation by real-time quantitative polymerase chain reaction. *Blood* 2002;99:4618-25.

25. Jimenez-Velasco A, Barrios M, Roman-Gomez J*, et al.* Reliable quantification of hematopoietic chimerism after allogeneic transplantation for acute leukemia using amplification by real-time PCR of null alleles and insertion/deletion polymorphisms. *Leukemia* 2005;19(3):336-43.

26. Pujal JM, Gallardo D. PCR-based methodology for molecular microchimerism detection and quantification. *Exp Biol Med* 2008;233(9):1161-70.

27. Johnson KL, Samura O, Nelson JL*, et al.* Significant fetal cell microchimerism in a nontransfused woman with hepatitis C: Evidence of long-term survival and expansion. *Hepatology* 2002;36(5):1295-7.

28. Utter GH, Owings JT, Lee TH*, et al.* Blood transfusion is associated with donor leukocyte microchimerism in trauma patients. *J Trauma* 2004;57(4):702-7.

29. Khosrotehrani K, Johnson KL, Guegan S*, et al.* Natural history of fetal cell microchimerism during and following murine pregnancy. *J Reprod Immunol* 2005;66(1):1-12.

30. Bonney EA, Matzinger P. The maternal immune system's interaction with circulating fetal cells. *J Immunol* 1997;158:40-47.

31. Nelson JL, Furst DE, Maloney S*, et al.* Microchimerism and HLA-compatible relationships of pregnancy in scleroderma. *Lancet* 1998;351(9102):559-62.

32. Stevens AM, Tsao BP, Hahn BH*, et al.* Maternal HLA class II compatibility in men with systemic lupus erythematosus. *Arthritis Rheum* 2005;52(9):2768-73.

33. Lambert NC, Evans PC, Hashizumi TL*, et al.* Cutting edge: persistent fetal microchimerism in T lymphocytes is associated with HLA-DQA1*0501: implications in autoimmunity. *J Immunol* 2000;164(11):5545-48.

34. Reed AM, McNallan K, Wettstein P*, et al.* Does HLA-dependent chimerism underlie the pathogenesis of juvenile dermatomyositis? *J Immunol* 2004;172(8):5041-46.

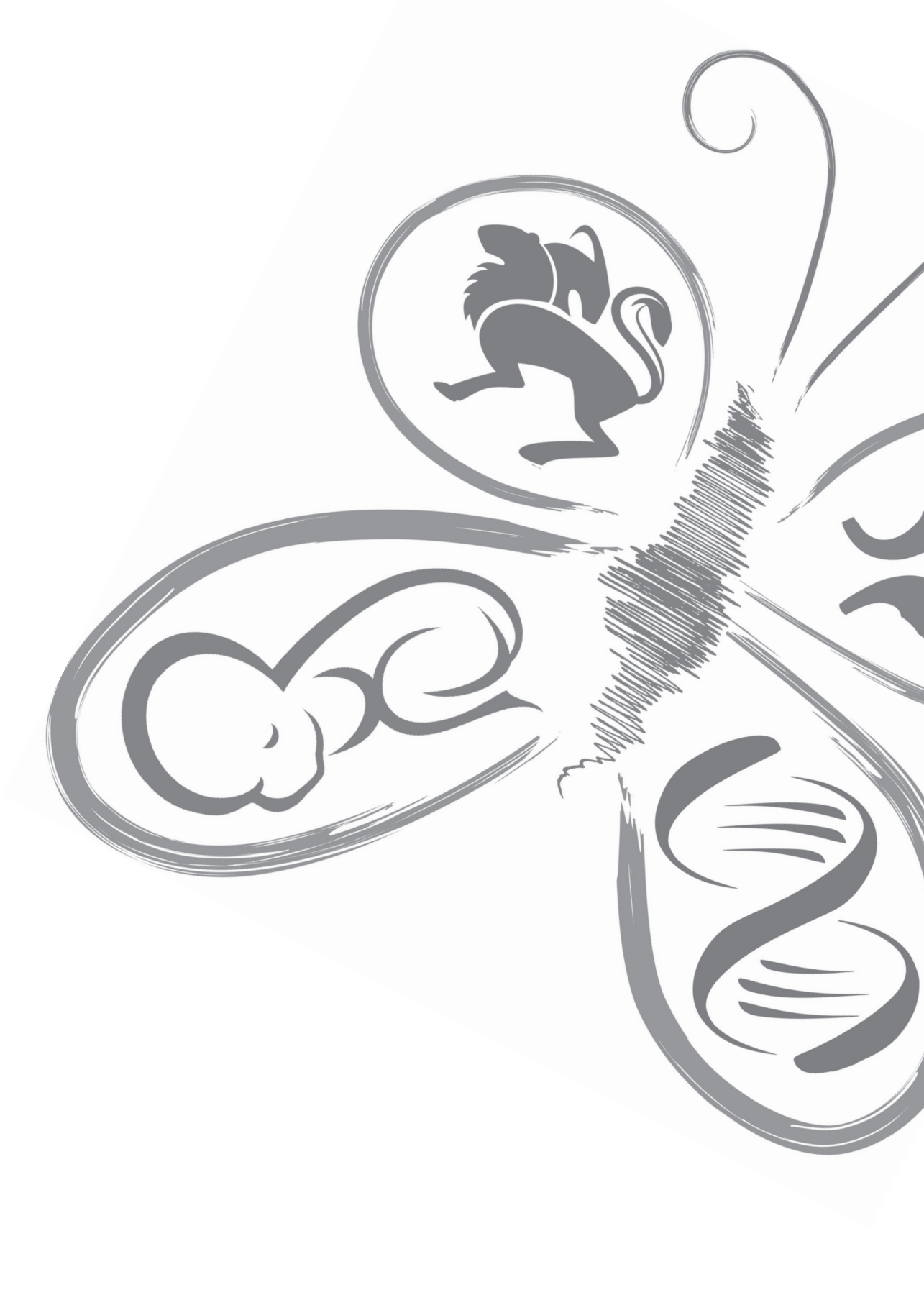
35. Martin-Villa JM, Martinez-Laso J, Moreno-Pelayo MA*, et al.* Differential contribution of HLA-DR, DQ, and TAP2 alleles to systemic lupus erythematosus susceptibility in Spanish patients: role of TAP2*01 alleles in Ro autoantibody production. *Ann Rheum Dis* 1998;57(4):214-9.

36. Tak T, Tesselaar K, Pillay J*, et al.* What's your age again? Determination of human neutrophil half-lives revisited. *J Leukoc Biol* 2013;94(4):595-601.

37. Seppanen E, Fisk NM, Khosrotehrani K. Pregnancy-acquired fetal progenitor cells. *J Reprod Immunol* 2013;97(1):27-35.

38. Bloch EM, Jackman RP, Lee TH*, et al.* Transfusion-associated microchimerism: the hybrid within. *Transfus Med Rev* 2013;27(1):10-20.

39. Bloch EM, Busch MP, Lee TH*, et al.* Microchimerism in the transfused obstetric population. *Vox Sang* 2014;107(4):428-30.



6

Microchimerism in Peripheral Blood of Patients with Systemic Lupus Erythematosus during and after Pregnancy

Suzanne Wilhelmus, Malu Zandbergen, Juan D.N. Diaz de Pool, Ron Wolterbeek, Hans J. Baelde, Clara Kolster-Bijdevaate, Kitty W. Bloemenkamp, Jan A. Bruijn, Ingeborg M. Bajema

Abstract

Objectives

Microchimerism has been shown to be increased in peripheral blood of women with systemic lupus erythematosus (SLE) many years after pregnancy. We hypothesized that either SLE patients accrue more microchimerism during pregnancy or clear chimeric cells less efficiently after pregnancy. Therefore, we studied the kinetics of microchimerism in peripheral blood from 30 weeks of gestation until six months postpartum in SLE patients and control subjects.

Methods

Peripheral blood was drawn from six pregnant SLE patients and eleven control subjects at 30 weeks of gestation, just after delivery, and one week, six weeks, three months and six months postpartum. Quantitative PCR for insertion-deletion polymorphisms and null alleles was used to detect microchimerism in peripheral blood mononuclear cells and granulocytes. Disease activity was monitored.

Results

SLE patients had a significantly higher median number of fetal chimeric cells in the granulocyte fraction just after delivery than control subjects (7.5 gEq/10^6 versus 0 gEq/10^6, respectively; P=0.02). At three and six months postpartum neither patients nor control subjects had detectable microchimerism. A relationship between microchimerism and disease activity was not found.

Conclusions

Although just after delivery SLE patients have more microchimerism than control subjects do, this difference cannot be demonstrated thereafter. Interestingly, many years after pregnancy SLE patients have been shown to have more microchimerism than control subjects, shedding new light on the dynamics of microchimerism during and after pregnancy.

Introduction

Microchimerism (Mc) refers to the presence in an individual of a small number of genetically distinct cells, originating from a different zygote. Transplantation of solid organs[1] or bone marrow,[2] blood transfusions,[3] and pregnancies[4] are possible sources of Mc, the latter being the most common.

Pregnancy can have an effect on the symptomatology of several autoimmune diseases, such as systemic lupus erythematosus (SLE). Pregnant SLE patients are more likely to experience a flare of disease activity than non-pregnant SLE patients.[5] Since Mc is known to be increased in pregnancy,[6] it is possible that Mc plays a role in these flares..

Indeed, there are also indications that Mc plays a role in disease development. SLE mainly affects women and has a peak incidence in the reproductive years.[7] Second, studies in mice demonstrated that injection of parental lymphocytes in their offspring, in selected parent-to-F1 combinations, leads to a graft-versus-host response and a lupus like disease.[8 9] Finally, we (manuscript submitted) and others[10 11] have shown an increase in Mc in peripheral blood of SLE patients compared to control subjects. The cause of this increase is unknown and since these chimeric cells are most likely derived from pregnancy, we hypothesized that either SLE patients accrue more Mc during pregnancy, or they clear chimeric cells less efficiently after pregnancy, or both.

Several studies investigated the kinetics of Mc during and after pregnancy in healthy individuals.[12-16] In these studies it was demonstrated that Mc tended to increase with gestational age and disappeared in the months postpartum. However, Mc has not been studied yet in pregnant patients with an autoimmune disease.

Therefore, our aim was to study the kinetics of Mc in peripheral blood mononuclear cells (PBMCs) and granulocytes from 30 weeks of gestation to six months postpartum in SLE patients and control subjects. Furthermore, we collected clinical data to study the relationship between disease activity and the amount of Mc detected.

Materials and methods

Ethics statement

This study was approved by the Medical Ethics Committee of the Leiden University Medical Center (LUMC) (P09.047). Informed consent was obtained from all participants. If DNA from an infant was required, the parents gave written consent on his/her behalf.

Patients and control subjects

Six pregnant SLE patients and 11 pregnant control subjects were studied. The pregnant SLE patients were recruited from the Obstetrics department at the LUMC. All included patients fulfilled at least four of the 1982 revised American College of Rheumatology Criteria for the classification of SLE.[17] The control group consisted of women without a history of autoimmune disease. Of both patients and control subjects peripheral blood samples were drawn at 30 weeks of gestation, just after delivery, and 1 week, 6 weeks, 3 months, and 6 months postpartum. Disease activity was monitored during the study period. To acquire DNA of the infant either umbilical cord blood was used or a buccal mouth swab from the infant was obtained. All subjects were asked to fill out a questionnaire including their age, ethnicity, reproductive history, history of blood transfusion, and medical history.

Isolation of peripheral blood subsets

Peripheral venous blood samples were drawn in sodium-heparine solution vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) and processed to isolate peripheral blood mononuclear cells (PBMCs) by Ficoll amidotrizoate (pharmacy LUMC) with density gradient centrifugation 1.077 g/mL. To remove the erythrocytes from the remaining granulocytes erythrolysis was applied. Until DNA extraction, samples were stored at -180°C in 10% dimethyl sulfoxide in fetal bovine serum.

DNA extraction

DNA was extracted from PBMCs and granulocytes using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with modifications. Briefly, 40 µL of proteinase K was added to 5 x 10^6 cells suspended in 200 µL phosphate-buffered saline. After the addition of 400 µL AL buffer, the suspension was incubated for 30 min (PBMCs) or overnight (granulocytes) at 56 °C. The mixture was applied to the Mini spin column and centrifuged, after adding 200 µl of ethanol. Buffers AW1 and AW2 were used to wash the column. To elute the DNA 100 µl AE buffer was added and incubated at 70 °C for 10 minutes. For an optimal yield, the eluate was reapplied. DNA concentration was measured using Nanodrop (Thermo Scientific, Wilmington, DE). All DNA samples were stored at 4 °C until quantitative PCR (qPCR). DNA extraction from buccal sterile OmniSwabs (GE Healthcare Life Sciences, Little Chalfont, United Kingdom) was performed with the same kit and according to the manufacturer's instruction.

Table 1. Primers

Marker name	Position	5' Primer 3'
S04b	F	CTG GTG CCC ACA GTT ACG CT
S04b	R	AGG ATG CGT GAC TGC TCC TC
S10b	F	TTA GAG CCA CAA GAG ACA ACC AG
S10b	R	TGG CTT CCT TGA GGT GGA AT
S11a	F	TAG GAT TCA ACC CTG GAA GC
S11a	R	CCA GCA TGC ACC TGA CTA ACA
FVII	F	CCC AAC TTA CAT TCC TAT ATC CT
FVII	R	GGG ACA GGA GAA AGG TCA
GSTT1	F	TCC TTA CTG GTC CTC ACA TCT C
GSTT1	R	TCC CAG CTC ACC GGA TCA T
SRY	F	TGG CGA TTA AGT CAA ATT CGC
SRY	R	CCC CCT AGT ACC CTG ACA ATG TAT T

F, forward; R, reverse.

Allele informativity and genotyping

For the detection of FMc a set of previously published insertion-deletion polymorphisms (indels) and null allelles was used.[18-20] For the quantification of FMc an informative difference between the patient or control and her infant was required. For genotyping qPCR was performed with the same protocol as described below, only with a DNA input of 20 ng. Of the published sets of indels and null alleles, six were informative in our study population: GSTT1, SRY (null alleles), and S04b, S10b, S11a and FVII (indels). The primer sequences are listed in Table 1.

Chimerism detection by qPCR

FMc was detected and quantified by qPCR. In all assays iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) was used, with 7.5 µM of each amplification primer. The amplification and melting conditions for all primers consisted of incubation at 96.5 °C for 10 min, followed by 44 cycles of 96.5 °C for 30 s and 60 °C for 1 min. The melting curve started at 65 °C for 5 s followed by 0.2 °C incremental increase, each lasting 5 s, to 95 °C. Amplification and melting data were collected by a Bio-Rad CFX96 detector and analyzed by Bio-Rad CFX Manager version 3.1.

Sanger sequencing of the amplification product was performed and compared with known genomic DNA sequences to ensure primer specificity. Serial dilutions of DNA positive for the indel or null allele in a background of DNA negative for the respective indel or null allele was tested to determine sensitivity. A sensitivity of one genome equivalent (gEq, based on 6.6 pg DNA content per cell) in 100 000 gEq was reached for all primer sets. For every patient and

control, for every time point during or after pregnancy, four aliquots containing 660 ng DNA (100 000 gEq) were tested, in both granulocytes and PBMCs. In order to quantify the chimeric cells and validate the assay on each plate a standard curve for the specific assay was included in each run. This standard curve consisted of 100, 10, and 1 gEq spiked DNA per 100 000 gEq background DNA. In addition, every sample was tested for the housekeeping gene GAPDH. Results were expressed as the gEq of chimeric cells per one million gEq (gEq/10^6). Negative controls consisted of either a water control or background DNA not carrying the indel or null allele tested. Negative controls were consistently negative across all experiments. If the specificity of the amplification product was questioned, the length of the PCR product was compared to that of the positive control using QIAxcel Advanced System (Qiagen) according to the manufacturer's protocol.

Anti-contamination procedures

Strict anti-contamination procedures were applied during blood work-up, DNA extraction and qPCR preparation. The isolation of PBMCs and granulocytes from peripheral blood was performed in a laminar flow cabinet. DNA decontamination reagent (Sigma-Aldrich, St. Louis, MO) was used to clean the cabinet where the DNA was extracted and the qPCR prepared. Furthermore, this cabinet was irradiated with UV light for one hour. All lab consumables were certified DNA free. We used aerosol-resistant pipette tips and clean gloves for all procedures. Eight-wells strips with individual lids were used for the qPCR experiments.

Statistical analysis

For comparison of categorical data, a Fisher's exact test was used (history of blood transfusion, presence of Mc at any time point during or after pregnancy, male fetus, mode of delivery). A Student's *t*-test was used to compare normally distributed data (age proband, gestational age at delivery). For comparison of non-normally distributed numerical data a Mann-Whitney U test was used (number of pregnancies, number of children, number of chimeric cells). A *P*-value ≤0.05 was considered statistically significant. All analyses were performed using SPSS Statistics 20.0 (IBM, Armonk, NY).

Results

Characteristics of patients and control subjects are shown in Table 2; no differences were found between the groups with respect to age, number of children and pregnancies at time

Table 2. Characteristics of SLE patients and control subjects

Parameter	SLE patients (n=6)	Control subjects (n=11)	*P*-value
Age (y)	31.0 ± 4.6	31.1 ± 3.5	0.97[a]
Number of children at time of pregnancy	0 (0.25)	0 (1)	0.38[b]
Number of pregnancies at time of pregnancy	1 (1)	1 (1)	0.72[b]
Blood transfusion (%)	16.7	0	0.35[c]
Gestational age at delivery (weeks)	37.5 ± 2.2	39.9 ± 1.2	0.01[a]
Male fetus	84%	46%	0.30[c]
Delivery mode			
Vaginal delivery	83%	100%	0.35[c]
Cesarean section	17%	0%	

Results are shown as mean ±SD or as median (interquartile range), unless otherwise specified. *P*-values were assessed with [a] Student's *t*-test, [b] Mann-Whitney U test, or [c] Fisher's exact test. SLE, systemic lupus erythematosus; y, years.

Table 3. Microchimerism at any time point during or after pregnancy in patients and control subjects

Parameter	SLE patients (n=6)	Control subjects (n=11)	*P*-value
Mc present in PBMCs or granulocytes (%)	72.7	83.3	1.0
Mc present in PBMCs (%)	54.5	66.7	1.0
Mc present in granulocytes (%)	45.5	83.3	0.30

P-values were assessed with Fisher's exact test. Mc, microchimerism; PBMCs, peripheral blood mononuclear cells; SLE, systemic lupus erythematosus.

of pregnancy, history of blood transfusion, sex of the infant and mode of delivery. However, patients delivered at an earlier gestational age than control subjects (average gestational age 37.5 versus 39.9 weeks, respectively). The majority of both patients and control subjects had detectable Mc at one time point during or after pregnancy (Table 3). Just after delivery, the median number of fetal chimeric cells in the granulocyte fraction was significantly higher in the patient group than in the control group (7.5 gEq/10^6 versus 0 gEq/10^6, respectively; *P*=0.02). This difference was not found in the PBMC fraction (2.5 gEq/10^6 in patients versus 0 gEq/10^6 in control subjects; *P*=0.13). Figure 1 shows the dynamics of the Mc detected during the study period. The demonstrated difference just after delivery disappeared quickly and was no longer present one week after delivery. In both patients and control subjects there was no detectable Mc at three and six months postpartum.

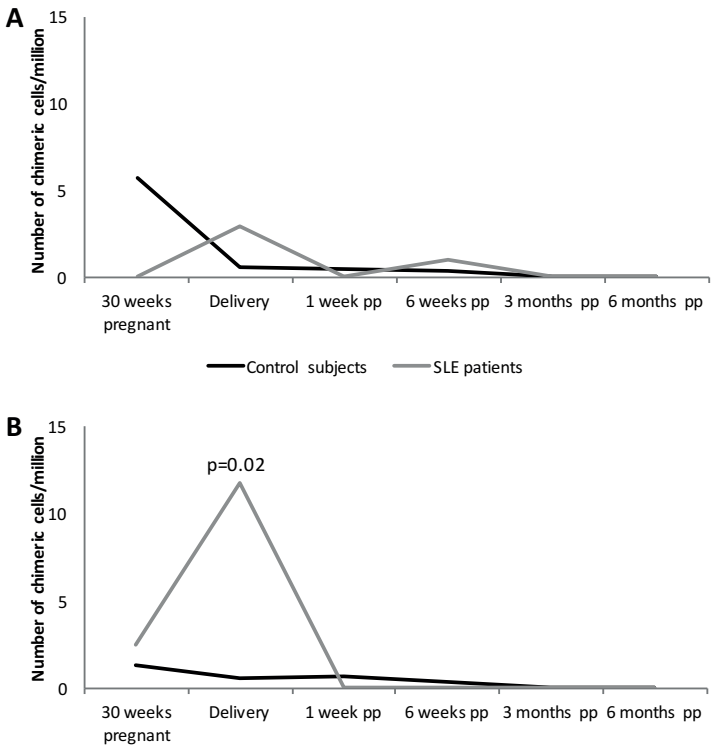


Figure 1. Microchimerism dynamics during and after pregnancy in patients and control subjects
Average number of chimeric cells per million peripheral blood mononuclear cells (panel A) and granulocytes (panel B). Just after delivery SLE patients have more chimeric cells in their granulocyte fraction than control subjects (*P*=0.02, Mann-Whitney U test). At other time points and in the PBMC fraction there are no statistical differences. Pp, postpartum; SLE, systemic lupus erythematosus.

Only one of the patients experienced an increase in disease activity during the course of the study; she had new onset arthritis in seven joints in the postpartum period. Both at the time of disease activity as well as thereafter, there was no detectable Mc.

All but one patient used anti-inflammatory or immunosuppressive medication (hydroxychloroquine, prednisone and/or azathioprine). Interestingly, the one patient without medication was also the only patient without detectable Mc at any of the time points. One of the patients and one of the control subjects developed preeclampsia. They showed Mc at one time point, only in the PBMC fraction, at 6 weeks and one week postpartum, respectively.

Discussion

Our study demonstrates that pregnant women with SLE had more chimeric cells circulating in their peripheral blood just after delivery than pregnant control subjects. Furthermore, these chimeric cells were mainly present in the granulocyte fraction rather than in the PBMCs. They quickly disappeared, and none of the patients and control subjects had detectable Mc three months after pregnancy.

We are the first to study Mc in pregnant SLE patients. The observed difference between patients and control subjects in the blood samples just after delivery was striking, and could bear important implications for the perceived role of Mc in the pathogenesis of SLE. It is particularly interesting that this difference was demonstrated in the granulocyte fraction. Neutrophils are capable of a form of cell death called NETosis (formation of neutrophil extracellular traps, or NETs) in which the neutrophils extrude their chromatin; an autoantigen in SLE.[21] It is conceivable that chimeric neutrophils undergoing NETosis are more likely to illicit an immune response than "regular" neutrophils undergoing NETosis, which may have an effect on the development of SLE or activity of the disease. Furthermore, defects in clearance of apoptotic debris in SLE (for review, see Rekvig *et al*.[22]) may lead to an increased exposure to 'chimeric' chromatin. We can only speculate about the cause of the observed difference. Because the difference was observed in the hours after delivery it may be that a minor feto-maternal hemorrhage is responsible for the increase in detectable Mc, although in this scenario, an increase of Mc in both PBMCs and granulocytes would be expected.

Our results concerning Mc in uncomplicated pregnancies are comparable to those obtained by Ariga *et al.*[15] and Adams Waldorf *et al.*.[14] Although we did find a difference between patients and control subjects in our study, we could not confirm our hypothesis that either SLE patients accrue more Mc during pregnancy or clear chimeric cells less efficiently after pregnancy: *i.e.*, at 30 weeks pregnancy we did not find a difference between patients and control subjects, and the higher level of Mc in SLE patients just after pregnancy was quickly cleared. The quick clearance of the Mc in the granulocytes was not unexpected, because they have a short half-life.[23] However, all Mc was cleared to the extent that three and six months after pregnancy neither patients nor control subjects showed any Mc. This result is striking since our previous study (manuscript submitted) showed that over 50% of women with SLE have detectable Mc more than 20 years after their last pregnancy. A previous study on Mc in SLE suggested that the number of chimeric cells may slowly increase over the years after pregnancy[10] in SLE patients but not in healthy control subjects. Thus, rather

than a decreased clearance of chimeric cells, it is possible that chimeric stem cells obtained during pregnancy are the supply for the higher number of chimeric cells many years after pregnancy, *i.e.*, that persistent Mc in stem cells generates *de novo* Mc in peripheral blood (and solid organs).

Our study has some limitations. First, the number of included SLE patients was small. However, all participants were prospectively followed in the same study protocol. Second, not all participants were primigravid. Nevertheless, because none of the participants had any detectable Mc after three and six months postpartum, it is unlikely that this influenced our results. Finally, all patients in our study were already diagnosed with SLE at inclusion. Strictly speaking, the results of this study are not suitable to draw any conclusions about the pathogenic role of Mc in SLE. In a recent study, however, it was shown that even before the diagnosis of SLE, SLE-associated pregnancy complications occur more frequently than in the general population, suggesting similarities between pregnancies before and after the diagnosis of SLE.[24]

In summary, we found that pregnant women with SLE have more peripheral blood Mc than pregnant control subjects just after delivery. This increase was mostly due to the increased presence of chimeric cells in the granulocyte fraction. Although both cause and consequence of this observation are speculative, it can be hypothesized that these chimeric cells modulate the disease through “chimeric” NETosis and defects in the clearance of apoptotic chimeric cells.

Acknowledgements

We thank J. Vork for her technical assistance.

Funding

This research was partly funded by Ars Donandi - Schokkenkamp Wegener Lonzieme foundation.

References

1. Lagaaij EL, Cramer-Knijnenburg GF, van Kemenade FJ*, et al.* Endothelial cell chimerism after renal transplantation and vascular rejection. *Lancet* 2001;357(9249):33-37.

2. Korbling M, Katz RL, Khanna A*, et al.* Hepatocytes and epithelial cells of donor origin in recipients of peripheral-blood stem cells. *N Engl J Med* 2002;346(10):738-46.

3. Lee TH, Paglieroni T, Ohto H*, et al.* Survival of donor leukocyte subpopulations in immunocompetent transfusion recipients: frequent long-term microchimerism in severe trauma patients. *Blood* 1999;93(9):3127-39.

4. Walknowska J, Conte FA, Grumbach MM. Practical and theoretical implications of fetal-maternal lymphocyte transfer. *Lancet* 1969;293(7606):1119-22.

5. Ruiz-Irastorza G, Lima F, Alves J*, et al.* Increased rate of lupus flare during pregnancy and the puerperium: a prospective study of 78 pregnancies. *Br J Rheumatol* 1996;35(2):133-8.

6. Rijnink EC, Penning ME, Wolterbeek R*, et al.* Tissue microchimerism is increased during pregnancy: a human autopsy study. *Mol Hum Reprod* 2015;21(11):857-64.

7. Lisnevskaia L, Murphy G, Isenberg D. Systemic lupus erythematosus. *Lancet* 2014;384(9957):1878-88.

8. Via CS, Shearer GM. T-cell interactions in autoimmunity: insights from a murine model of graft-versus-host disease. *Immunol Today* 1988;9(7-8):207-13.

9. Gleichmann E, Van Elven EH, Van der Veen JP. A systemic lupus erythematosus (SLE)-like disease in mice induced by abnormal T-B cell cooperation. Preferential formation of autoantibodies characteristic of SLE. *Eur J Immunol* 1982;12(2):152-9.

10. da Silva Florim GM, Caldas HC, Pavarino EC*, et al.* Variables associated to fetal microchimerism in systemic lupus erythematosus patients. *Clin Rheumatol* 2016;35(1):107-11.

11. Kekow M, Barleben M, Drynda S*, et al.* Long-term persistence and effects of fetal microchimerisms on disease onset and status in a cohort of women with rheumatoid arthritis and systemic lupus erythematosus. *BMC Musculoskelet Disord* 2013;14:325.

12. Hamada H, Arinami T, Hamaguchi H*, et al.* Fetal nucleated cells in maternal peripheral blood after delivery. *Am J Obstet Gynecol* 1994;170(4):1188-93.

13. Hamada H, Arinami T, Kubo T*, et al.* Fetal nucleated cells in maternal peripheral blood: frequency and relationship to gestational age. *Hum Genet* 1993;91(5):427-32.

14. Adams Waldorf K. Dynamic Changes in Fetal Microchimerism in Maternal Peripheral Blood Mononuclear Cells, CD4+ and CD8+ cells in Normal Pregnancy. *Placenta* 2010;31(7):589-94.

15. Ariga H, Ohto H, Busch MP*, et al.* Kinetics of fetal cellular and cell-free DNA in the maternal circulation during and after pregnancy: implications for noninvasive prenatal diagnosis. *Transfusion* 2001;41(12):1524-30.

16. Hsieh TT, Pao CC, Hor JJ*, et al.* Presence of fetal cells in maternal circulation after delivery. *Hum Genet* 1993;92(2):204-05.

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

18. Alizadeh M, Bernard M, Danic B*, et al.* Quantitative assessment of hematopoietic chimerism after bone marrow transplantation by real-time quantitative polymerase chain reaction. *Blood* 2002;99:4618-25.

19. Jimenez-Velasco A, Barrios M, Roman-Gomez J*, et al.* Reliable quantification of hematopoietic chimerism after allogeneic transplantation for acute leukemia using amplification by real-time PCR of null alleles and insertion/deletion polymorphisms. *Leukemia* 2005;19(3):336-43.

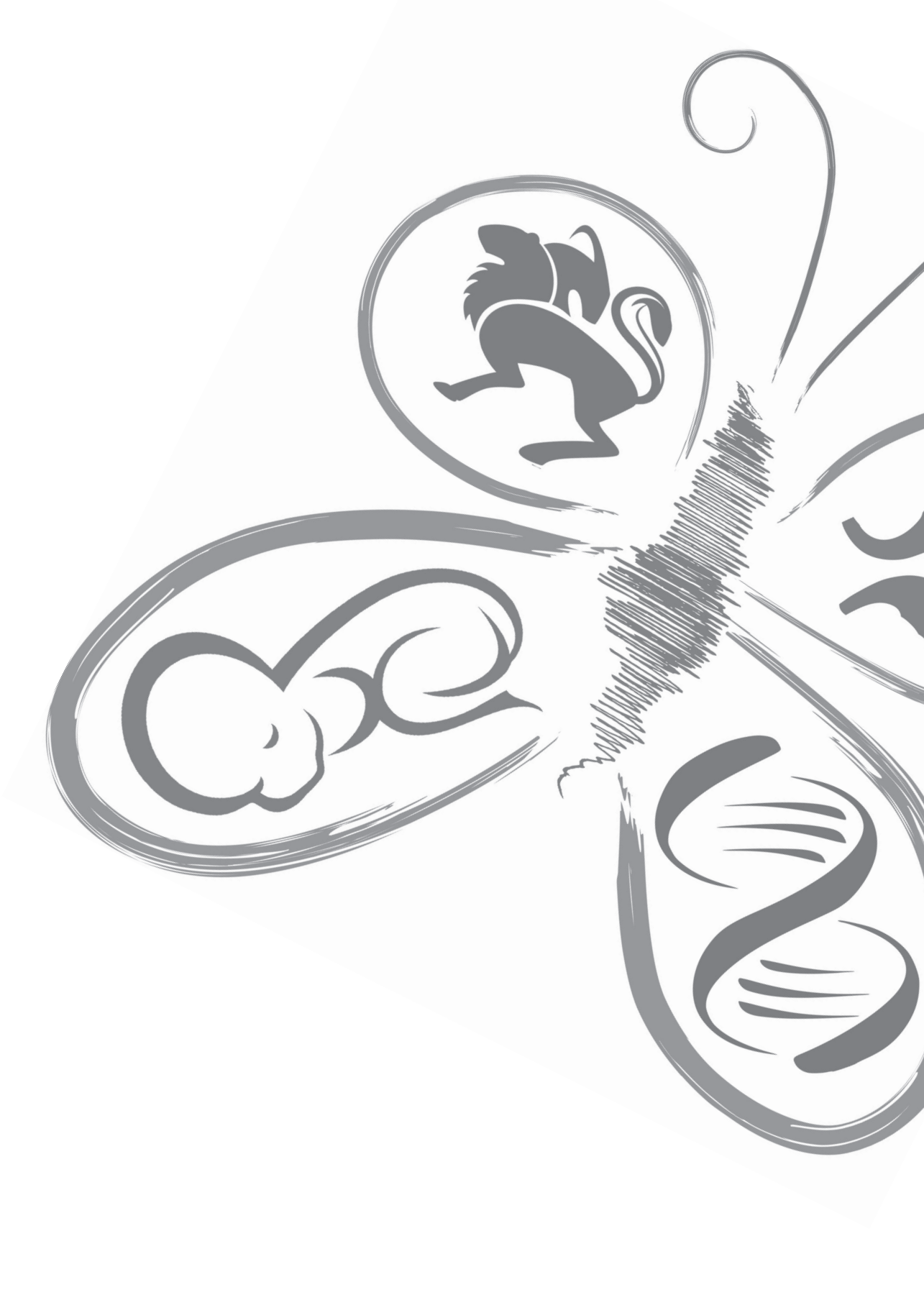
20. Pujal JM, Gallardo D. PCR-based methodology for molecular microchimerism detection and quantification. *Exp Biol Med* 2008;233(9):1161-70.

21. Smith CK, Kaplan MJ. The role of neutrophils in the pathogenesis of systemic lupus erythematosus. *Curr Opin Rheumatol* 2015;27(5):448-53.

22. Rekvig OP, Van der Vlag J. The pathogenesis and diagnosis of systemic lupus erythematosus: still not resolved. *Semin Immunopathol* 2014;36(3):301-11.

23. Tak T, Tesselaar K, Pillay J*, et al.* What's your age again? Determination of human neutrophil half-lives revisited. *J Leukoc Biol* 2013;94(4):595-601.

24. Arkema EV, Palmsten K, Sjöwall C*, et al.* What to expect when expecting with SLE: A population-based study of maternal and fetal outcomes in SLE and pre-SLE. *Arthritis Care Res* 2015.



7

Familial and Sporadic Lupus Nephritis Compared: Genetics, Clinical Characteristics, Histology, and Renal Outcome

Natasha P. Jordan, Suzanne Wilhelmus, Urmas Roostalu, Ingeborg M. Bajema, H. Terence Cook, David A. Isenberg, David P. D'Cruz, Frederic Geissmann

Abstract

Systemic lupus erythematosus is an autoimmune disease which is thought to have a significant genetic contribution in its aetiology. Either susceptibility alleles identified in GWAS, such as in those involved in monocyte/macrophage function, or rare variants may play a role in familial disease. Therefore, the aim of our study was to compare patients with familial and sporadic lupus nephritis (LN) with respect to clinical parameters, serology, histological class, activity and chronicity indices (AI and CI), the number of glomerular monocytes/macrophages, and the contribution of known lupus susceptibility polymorphisms.

Our cohort consisted of 154 patients of which 16 patients had a first-degree relative with LN. Age, sex, ancestry, progression to advanced renal impairment, serology, histological class, AI, CI, and glomerular CD16 and CD68 counts were determined. Also, we calculated a polygenic risk score based on the number of selected lupus susceptibility alleles carried.

We found that patients with familial LN more often had juvenile onset disease (50% vs 22%, respectively; *P*=0.03), were more often male (44% vs 12% male, respectively; *P*=0.004), had a higher frequency of progressing to advanced renal impairment (25% vs 7%, respectively; *P*=0.03) and had different ancestral backgrounds than patients with sporadic LN (*P*=0.002). The serology was not different, neither was the distribution among the histological classes, the AI and CI, and the number of glomerular CD16 (0.9 vs 1.4, respectively; *P*=0.23) and CD68 (10.1 vs 6.2, respectively; *P*=0.12) positive cells. Familial LN patients did not have a statistically significant higher polygenic risk score than patients with sporadic LN.

In conclusion, although we did find a worse renal outcome in familial LN compared to sporadic LN, we did not find a difference in histological parameters or genetic background. Therefore, the cause of the observed differences remains unknown. Whole exome sequencing in families with multiple affected members to search for rare variants may provide new leads for future research.

Introduction

Systemic Lupus Erythematosus (SLE) is considered to be the prototypic autoimmune disease with aberrations throughout the immune system resulting in diverse clinical manifestations. Lupus nephritis (LN) is one of the most severe clinical manifestations of SLE, with an estimated 10-15% of patients progressing to end-stage renal disease (ESRD).[1 2] Renal damage is the overall most important predictor of mortality in SLE patients.[3 4]

Epidemiologic studies suggest a significant contribution of genetic factors in the aetiology of SLE. Disease concordance in SLE is higher in monozygotic twins (25-50%) than in dizygotic twins (2%) and there is a high sibling risk ratio (λs) of 20-29.[5-7] Although most studies found similar clinical presentations in patients with familial and sporadic SLE,[8-12] one small study in children reported an increase in all-cause mortality in familial SLE.[13] However, other studies did not show a difference in outcome.[14 15]

The genetics involved in familial SLE might include a clustering of multiple common risk alleles in families, or the presence of a rare variant with a large effect, such as in *DNASE1*.[16] Linkage analysis, candidate gene studies and genome wide-association studies (GWAS) have led to the identification of several candidate polymorphisms, including those effecting monocyte/macrophages function.[17 18] Also, monocytes/macrophages have been suggested to play a role in the pathogenesis of SLE in general,[19] and in LN in particular.[20 21]

The aim of this research was to explore the differences between familial and sporadic LN with respect to clinical parameters, serology, histological class, glomerular influx of monocytes/macrophages, and the contribution of known lupus susceptibility polymorphisms.

Methods

Study population

From July 2010 to January 2012, 160 patients with LN were recruited from three designated clinical centres. Inclusion criteria were as follows; a definite diagnosis of SLE in accordance with the American College of Rheumatology (ACR) revised classification criteria,[22] biopsy-proven LN, and the ability to provide written informed consent. Enquiry into the family history resulted in four additional cases with biopsy-proven LN, leading to a total of 164 cases. In families with clustering of LN, unaffected family members were also recruited where available (Figure 1). All study work was conducted in accordance with the requirements of the Helsinki Declaration and this study was approved by the Outer South East London and the London City Road and Hampstead Research Ethics Committees.

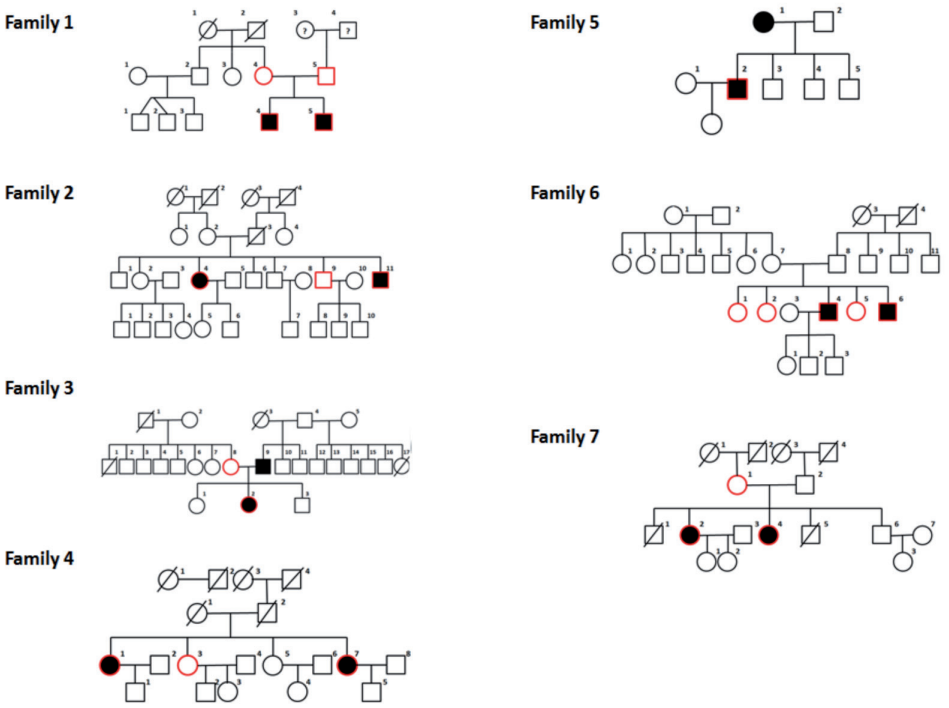


Figure 1. Families with lupus nephritis in two first degree family members
Seven families with clustering of lupus nephritis within our study cohort. Circles indicate females and squares indicate males. Filled circles/squares are probands. A red outline indicates that DNA was available for genotyping. A stoke through the circle/square signifies that the person has died. Family 1 and 5 are of South Asian ancestry, families 2, 3, 4, and 7 are of African ancestry and family 6 is of East Asian ancestry.

Clinical variables

Clinical variables explored in this study included gender, age at diagnosis of nephritis, family history of SLE, and ancestral background. Ancestry was self-reported by patients recruited to the study. To avoid bias due to population stratification in assessment of the frequency of susceptibility polymorphisms, Multidimensional Scaling was carried out using PLINK to identify outliers from the main ancestral groups.[23]

Autoantibody profiles were performed in all patients at the designated clinical centres including ANA (anti-nuclear antibodies), anti-double stranded DNA, anti-Ro (SS-A), anti-RNP (ribonucleoproteins) and anti-Sm (Smith antigen) antibodies using a standardized counterimmunoelectrophoresis.

Long-term renal outcomes in LN patients were assessed using the National Kidney

Foundation Kidney Disease Outcomes Quality Initiative scoring system.[24] Patients with Stage 4, severe reduction in glomerular filtration rate (GFR) (15-29 mL/min) and Stage 5, kidney failure (GFR<15 or dialysis) were classified as having advanced renal impairment.

Renal histology

Paraffin-embedded renal biopsy tissue was available from 77% (n=126) of LN patients recruited to this study. Biopsies were traced back to the time of the patients' original diagnosis of LN (n=107) or when this was not possible biopsies taken at the onset of a new nephritis flare before induction immunosuppression was commenced were obtained (n=19). Biopsies were reclassified independently by two renal histopathologists as per the 2004 ISN/RPS classification system.[25 26] Discrepancies in classes were resolved during a consensus meeting. For purpose of analyses, cases with class III or IV LN combined with class V LN were considered as class III or IV LN. In addition, the activity and chronicity indices (AI and CI) were obtained from the original pathology report and were available for 51 cases (all class III or IV LN). Furthermore, immunohistochemical staining was performed for CD16 and CD68. Slides were deparaffinised and subjected to antigen retrieval (Tris/EDTA buffer). After blocking endogenous peroxidase, the sections were incubated with either mouse anti-human CD16 (MS1085; Thermo Scientific, Waltham, MA, USA) for 2 hours, or mouse anti-human CD68 (KP-1; Dako, Glostrup, Denmark) for 1 hour. Sections were then counterstained with haematoxylin. Once mounted and dried, the slides were scanned and the number of CD16 and CD68 positive cells in the glomerular tuft was counted (viewer software: 3DHISTECH Pannoramic Viewer or Philips Digital Pathology Solution). Results are presented as average number of positive cells per glomerular tuft in a biopsy. During analysis, slides with < 7 glomeruli present were excluded, leaving 105 biopsies for ISN/RPS classification, 69 biopsies for CD16 analysis (only cases with either class III or IV LN were stained) and 91 biopsies for CD68 analysis.

Genotyping by ImmunoChip

Genomic DNA was extracted from thawed frozen whole blood using the GenElute™ Blood Genomic DNA Kit (Sigma Aldrich, St Louis, MO, USA) as per the manufacturer's instructions. Genotyping was performed using the Illumina ImmunoChip. Analysis and genotype calling was performed using Illumina GenomeStudio software. Four patients' genotyping results did not meet quality control standards leaving 160 patients results suitable for analysis.

A polygenic risk score was calculated using 20 common nucleotide polymorphisms (SNPs)

Table 1. Lupus susceptibility polymorphisms included in polygenic risk score

Gene	SNPs
HLA region	rs3135394, rs9271366
IRF5	rs2070197, rs10954213
IRAK1	rs2269368
PTPN22	rs2476601
ITGAM	rs1143679
IRF7	rs4963128
IRF8	rs2280381
NCF2	rs10911363
STAT4	rs7574865
IKZF1	rs4917014
IFIH1	rs1990760
TNFAIP3	rs6920220, rs5029939
TNFSF4	rs2205960
ETS1	rs6590330
BLK	rs2736340
BANK1	rs10516487
LYN	rs7829816

that represent confirmed SLE susceptibility loci (Table 1). Inclusion of SNPs that may in linkage disequilibrium with one another was avoided. The polygenic risk score was assessed by two methods, a simple polygenic risk score and a weighted polygenic risk score. The simple polygenic risk score (count genetic risk score, cGRS) was calculated by counting the number of risk allele carried by an individual. In the weighted polygenic risk score (wGRS), the risk allele is weighted by the logarithmic odds ratio (log OD) for that allele. The overall wGRS is the sum of the log OD for each individual risk allele included in the score divided by the number of alleles.

Statistical analysis

Categorical variables were compared using Pearson's Chi-squared ($\chi 2$) test, except in instances where expected counts were <5, when a Fisher's exact test was used. Continuous variables with a normal distribution were described as mean with standard deviation (SD) and continuous variables without a normal distribution as median with an interquartile range (IQR). Student's *t*-tests or Mann Whitney *U* tests were used to compare continuous variables that were normally or non-normally distributed, respectively. Correlations were

tested with Spearman's rank correlation test. A value of $P \leq 0.05$ was considered to be significant. Analyses were carried out using SPSS Statistics 20.0 (IBM, Armonk, NY).

Results

Demographics of overall patient cohort

Twenty-six patients reported a family history of SLE. Of these, 16 patients had a first-degree family history, defined as having a parent, sibling or children affected with LN. All self-reported first degree family relatives were confirmed clinically. Five patients reported a second degree family history and five a third degree family history. Only patients with a first-degree family member with LN were considered as familial cases. The remaining ten patients with a positive family history were excluded from the analyses, as they were considered as neither familial nor sporadic, leaving 154 patients for analysis. Of these 154 patients 130 (84%) patients were female and 24 (16%) were male. The mean age at diagnosis of LN was 26.0 ± 11.2 years. The mean disease duration was 11.7 ± 7.3 years. Twenty-five percent (n=38) of the study group were of juvenile onset as defined by diagnosis of nephritis before 18 years of age.

Forty-two percent (n=64) of the cohort were of European origin, predominantly from the United Kingdom. Thirty-two percent (n=49) were of African ancestry, the majority being of Afro-Caribbean descent and others from Nigeria, Ghana, Sierra Leone and Uganda. Fourteen percent (n=21) were of South Asian extraction, all from India and Pakistan. Eight percent (n=12) were East Asian, from China, Vietnam and Singapore. Five percent (n=8) were classified as outliers from the 4 main ancestral groups.

Comparison of familial and sporadic LN

Of the cases with familial LN 56% (n=9) were female as compared to 88% (n=121) of sporadic cases (P=0.004). Familial cases were younger, although just not statistically different (17 vs 26 years, P=0.07). However, 50% (n=8) of familial cases had juvenile onset disease as opposed to 22% (n=30) in the sporadic group (P=0.03). The distribution among the different ancestries was different in familial and sporadic LN (P= 0.001) due to a relatively high percentage of patients of African descent in the familial group and the absence of familial cases of European descent. Twenty-five percent (n=4) of familial LN cases had progressed to advanced renal disease while 7% (n=9) had done so in the sporadic patient cohort (P=0.03). The autoantibody profile did not differ significantly between familial and sporadic cases (Table 2).

Table 2. Patient sociodemographics, clinical, laboratory and histologic features in familial and sporadic lupus nephritis

	Familial nephritis (n=16)	Sporadic nephritis (n=138)	*P*-value
Female (% (n))	56% (9)	88% (121)	0.004[a]
Age at diagnosis (years) (Median (IQR) (n))	17 (15) (15)	26 (13) (127)	0.07[b]
Juvenile (<18 y) onset (% (n))	50% (8)	22% (30)	0.03[c]
European (% (n))	0%	46% (64)	-
African (% (n))	69% (11)	28% (38)	-
South Asian (% (n))	19% (3)	13% (18)	-
East Asian (% (n))	13% (2)	7% (10)	-
Outliers from main ancestral groups (% (n))	-	6% (8)	0.002[c]
Duration of follow-up (years) (Median (IQR) (n))	13 (9) (15)	10 (10.5) (125)	0.03[b]
Progression to advanced renal impairment (% (n))	25% (4)	7% (9)	0.03[a]
ANA (% (n))	94% (15/16)	97% (132/136)	0.43[a]
Anti-dsDNA (% (n))	67% (10/15)	71% (96/135)	0.77[a]
Anti-Ro (% (n))	40% (6/15)	36% (46/128)	0.76[c]
Anti-Sm (% (n))	36% (5/14)	18% (23/128)	0.15[a]
Anti-RNP (% (n))	43% (6/14)	36% (46/128)	0.61[c]
Class I (% (n))	0%	2% (2/95)	-
Class II (% (n))	0%	5% (5/95)	-
Class III (% (n))	40% (4/10)	27% (26/95)	-
Class IV-S (% (n))	20% (2/10)	30% (28/95)	-
Class IV-G (% (n))	30% (3/10)	22% (21/95)	-
Class V (% (n))	10% (1/10)	13% (13/95)	0.87[c]
Activity index (Mean ± SD (n))	10.4 ± 5.1 (7)	9.1 ± 3.8 (44)	0.42[d]
Chronicity index (Median (IQR) (n))	3.0 (1.0) (7)	3.0 (2.0) (44)	0.87[b]
Glomerular CD16 count (Median (IQR) (n))	0.9 (1.3) (6)	1.4 (2.3) (63)	0.23[b]
Glomerular CD68 count (Median (IQR) (n))	10.1 (16.2) (9)	6.2 (12.6) (82)	0.12[b]

P-values were assessed with [a] Fisher's exact test, [b] Mann-Whitney U test, [c] Pearson Chi-square test or [d] Student's t-test. ANA, anti-nuclear antibody; anti-dsDNA, anti-double stranded DNA antibody; anti-RNP, anti-ribonucleoprotein antibody; anti-Sm, anti-Smith; IQR, interquartile range; SD, standard deviation.

Histopathology

The distribution among the ISN/RPS classes was similar in familial and sporadic cases (Table 2). Also, the AI and CI were not different. The median number of glomerular CD68 positive cells was 10.1 in familial cases and 6.2 in sporadic cases, but this was not significantly different (*P*=0.12). CD16 staining showed the opposite result with 0.9 positive cells in familial cases and 1.4 positive cells in sporadic cases, but this was also not significantly different (*P*=0.23). In addition, within class III and IV LN, there was no difference in the number of glomerular CD68 positive cells (*P*=0.49) between familial and sporadic cases.

Overall, we did see a difference in the number of glomerular CD68 positive cells between classes, with class I, II and V LN having few CD68 positive cells and class IV LN having the most (Figure 2). There was no difference in the number of glomerular CD68 positive cells between IV-S and IV-G (*P*=0.93), in contrast to previous literature.[27] The number of CD68 positive cells was correlated with the AI (r=0.49, *P*=0.000).

Polygenic risk scores

Given the varying frequencies of risk alleles in different ancestral groups, polygenic risk scores were compared on an ancestry-by-ancestry basis, in which ancestry outliers were

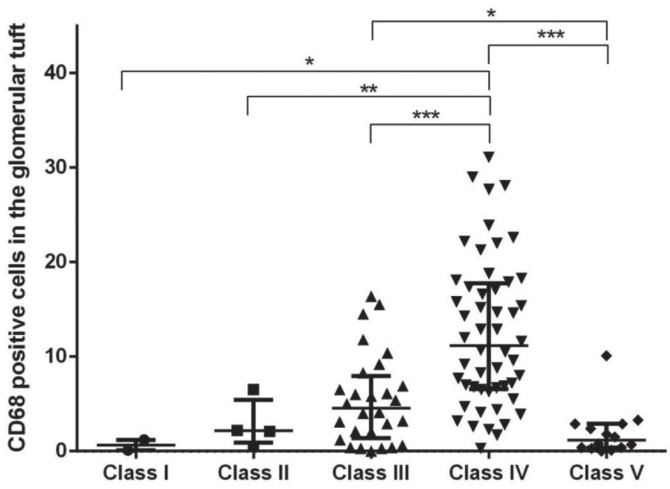


Figure 2. Number of glomerular CD68 positive cells in relation to the ISN/RPS class
Average number of CD68 positive cells in the glomerular tuft in relation to the ISN/RPS class. *P≤0.05; **P≤0.01, ***P≤0.001

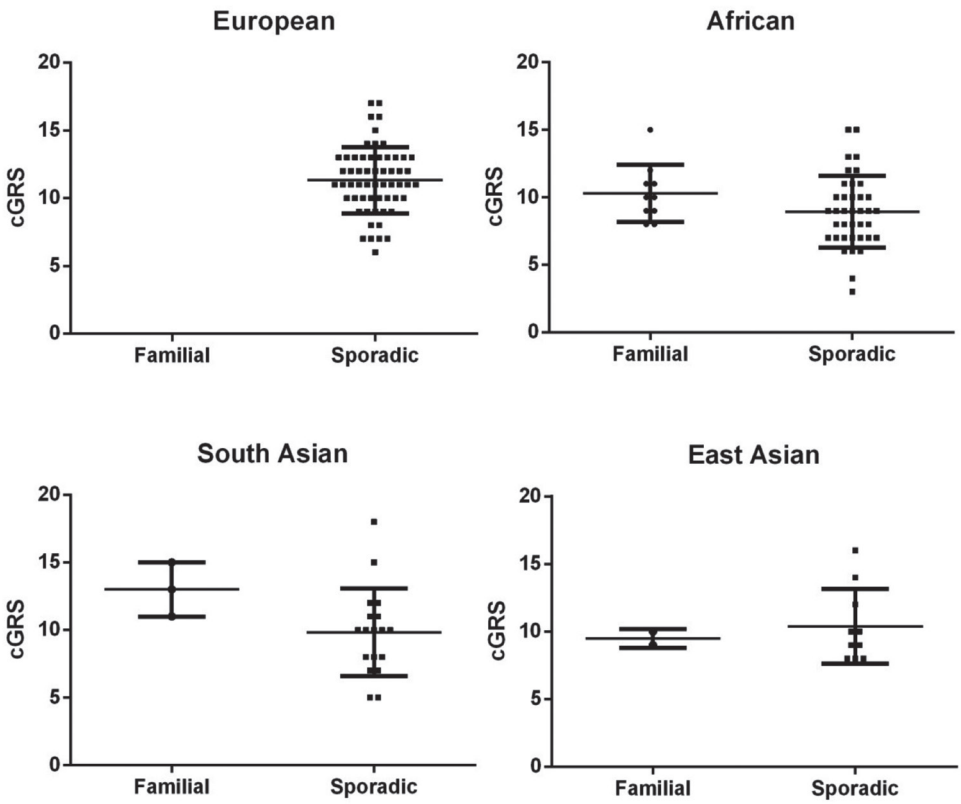


Figure 3. A comparison of polygenic risk score of lupus susceptibility alleles in familial and sporadic lupus nephritis patients of different ancestries

None of the comparisons between familial and sporadic lupus nephritis cases show a statistically significant difference.

excluded. European LN patients had the highest mean cGRS at 11.3 ± 2.5 while African patients had the lowest mean score at 9.2 ± 2.6 (*P*=0.000). South Asian and East Asian patients had similar mean cGRS, 10.3 ± 3.3 and 10.3 ± 2.5 respectively.

Mean cGRS in African familial cases was 10.3 ± 2.1 in comparison to 8.9 ± 2.7 in sporadic African patients (*P*=0.14). South Asian familial patients' mean cGRS was 13.0 ± 2.0 while the sporadic patients mean score was 9.8 ± 3.2 (*P*=0.12). East Asian familial mean cGRS was 9.5 ± 0.71 and 10.4 ± 2.8 in sporadic patients (*P*=0.67). There were no cases of familial nephritis in patients of European ancestry to enable a comparison of familial and sporadic disease (Figure 3).

Mean wGRS in African familial cases was 0.69 ± 0.14 as compared to 0.59 ± 0.18 in sporadic

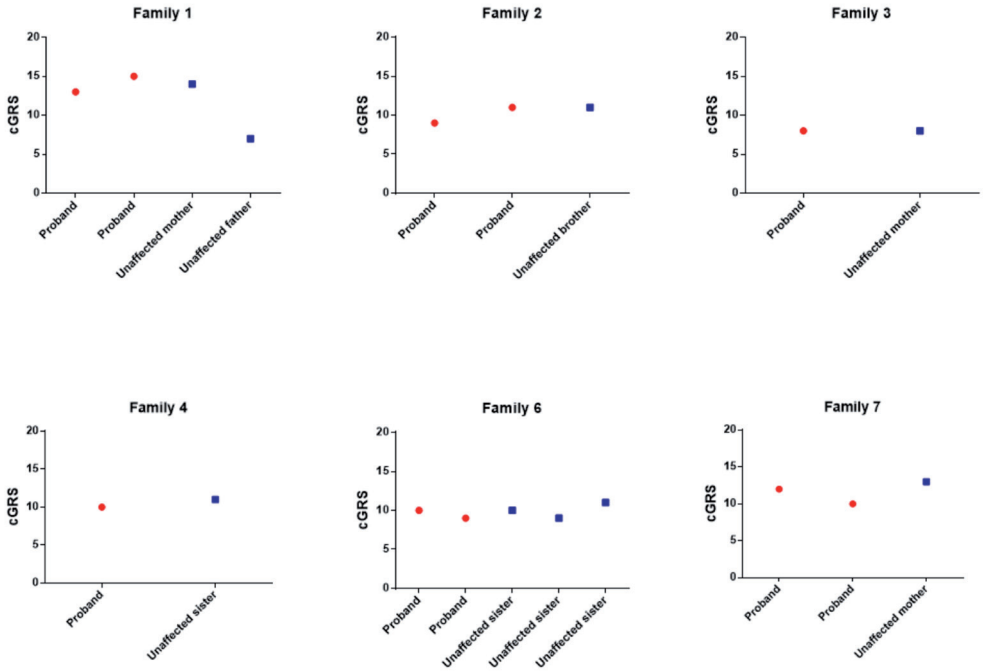


Figure 4. Polygenic risk score in probands and unaffected family members.
A comparison of cGRS (count genetic risk score) in probands and unaffected family members in families with clustering of lupus nephritis. Family 1 is of South Asian ancestry, families 2, 3, 4, and 7 are of African ancestry and family 6 is of East Asian ancestry. Of family 5 there was no DNA available of unaffected family members. Circles indicate family members with LN, squares indicate unaffected family members.

patients (*P*=0.16). South Asian familial patients had a mean wGRS of 0.83 ± 0.13 while sporadic patients of this ancestry scored 0.66 ± 0.22 (*P*=0.22). East Asian familial cases scored 0.60 ± 0.03 in comparison to 0.63 ± 0.18 in sporadic disease (*P*=0.848).
When both mean cGRS and wGRS of probands were compared to their unaffected relatives, their scores were found to be similar (Figure 4).

Discussion

We found that patients with familial LN were more often male, younger, had a different ancestral background and progressed to advanced renal impairment more often than patients with sporadic LN. However, we did not find a difference in their antibody profile, the distribution among the ISN/RPS classes, or the number of CD16 and CD68 positive

cells in the glomeruli. Furthermore, familial LN patients did not have more risk alleles than sporadic LN patients.

When comparing clinical characteristics, the familial patients in our cohort were younger than the sporadic patients, although this did not reach statistical significance (*P*=0.07). Nevertheless, familial cases did have juvenile onset disease more often (*P*=0.03). In a small study in paediatric SLE in Saudi Arabia familial cases were found to be younger[13]: 6.8 years old in familial patients and 10.2 years old in sporadic patients. However, this was not confirmed in other, larger, studies from France,[9] the US (multiracial)[11] and China.[12] Similar to other studies,[10 11] the antibody profile was not different in familial and sporadic cases. However, there was a clear difference in racial distribution between familial and sporadic cases.

We showed that familial patients have a worse renal outcome than sporadic patients. One other study addressing renal outcome did not find a difference.[14] However, in that study patients with a general family history of autoimmune disease and not SLE specifically were included. Since a significantly larger proportion of SLE patients have a family history of autoimmune disease in general than of SLE specifically, this may account for this difference.[28] A likely explanation for the difference in renal outcome would have been that renal disease was more severe in familial cases. We did not, however, find a difference in the distribution among the ISN/RPS classes or in activity or chronicity index. Because of the possible role of monocytes/macrophages in the pathogenesis of LN[20 21] and identified SLE susceptibility alleles involving monocyte/macrophage function, we investigated if there was a difference between familial and sporadic cases in the number of glomerular CD16 or CD68 positive cells. However, there were no statistically significant differences. This could be related to the number of cases included, or because changes are functional rather than numerical.

With regard to the polygenic risk score, there was minimal difference in the outcomes of our analysis whether cGRS or wGRS were used, presumably due to the modest odds ratios of most lupus susceptibility alleles. European LN patients in general had the highest polygenic risk scores. A possible explanation is that the majority of variants tested were identified in GWAS of SLE patients of European ancestry. However, many of these loci have been confirmed in East Asian populations and had similar effect sizes in both European and East Asian populations. [29-31] Nevertheless, SLE is known to be more prevalent in patients of African, Asian and Hispanic descent than in those of European ancestry.[32-34] In addition to being more frequent, the clinical phenotype of SLE is usually more severe in non-Europeans with younger onset disease and higher frequency of disease manifestations such as LN.[35-39] These observations indicate potential genetic heterogeneity for SLE between populations.

Due to a paucity of GWAS data in South Asian and African populations, it is unknown if these susceptibility alleles studied here even confer a higher risk of SLE in these populations.
When examining the polygenic risk score in familial LN, a trend was seen towards higher risk scores in African and South Asian familial cases as compared to sporadic disease but these did not reach statistical significance. Interestingly, in families with clustering of LN, probands and unaffected relatives had a similar GRS. This also argues against an accumulation of susceptibility alleles in familial cases as a cause of LN.
There are a number of factors to take into consideration when interpreting these results. First, our cohort consists of a relatively small number of patients, in particular when studying common variants with low effect sizes in different ancestries. In addition, follow up was 3 years longer in familial cases than in sporadic cases. However, it is unlikely that this explains the observed difference in renal outcome. Furthermore, the susceptibility alleles studied here were selected from GWAS data of SLE patients versus controls, and not from GWAS data of SLE patients with LN versus SLE patients without LN. Some of these alleles, however, have recently also been identified as LN predisposing loci.[40] Other complexities when studying genetics are the possibility of incomplete penetrance, and gene-gene and gene-environment interactions.
In summary, we report that patients with familial LN progressed to advanced renal impairment more often than patients with sporadic LN. Furthermore, familial LN patients were more often male, younger and had a different ancestral background. However, patients did not differ with respect to the histological severity of LN at presentation as determined by the ISN/RPS classification and activity and chronicity indices, and the count of CD16 and CD68 positive cells in the glomerulus. Also, familial LN cases did not show increased clustering of SLE susceptibility alleles. Therefore, the cause of the differences between familial and sporadic LN remains unknown. Performing whole exome sequencing on families with multiple affected members may identify variants for further exploration and may eventually lead to identification of factors involved.

References

1. Cervera R, Khamashta MA, Font J*, et al.* Morbidity and mortality in systemic lupus erythematosus during a 10-year period: a comparison of early and late manifestations in a cohort of 1,000 patients. *Medicine (Baltimore)* 2003;82(5):299-308.

2. Adler M, Chambers S, Edwards C*, et al.* An assessment of renal failure in an SLE cohort with special reference to ethnicity, over a 25-year period. *Rheumatology (Oxford)* 2006;45(9):1144-7.

3. Danila MI, Pons-Estel GJ, Zhang J*, et al.* Renal damage is the most important predictor of mortality within the damage index: data from LUMINA LXIV, a multiethnic US cohort. *Rheumatology (Oxford)* 2009;48(5):542-5.

4. Mak A, Cheung MW, Chiew HJ*, et al.* Global trend of survival and damage of systemic lupus erythematosus: meta-analysis and meta-regression of observational studies from the 1950s to 2000s. *Semin Arthritis Rheum* 2012;41(6):830-9.

5. Block SR, Winfield JB, Lockshin MD*, et al.* Studies of twins with systemic lupus erythematosus. A review of the literature and presentation of 12 additional sets. *Am J Med* 1975;59(4):533-52.

6. Deapen D, Escalante A, Weinrib L*, et al.* A revised estimate of twin concordance in systemic lupus erythematosus. *Arthritis Rheum* 1992;35(3):311-8.

7. Alarcon-Segovia D, Alarcon-Riquelme ME, Cardiel MH*, et al.* Familial aggregation of systemic lupus erythematosus, rheumatoid arthritis, and other autoimmune diseases in 1,177 lupus patients from the GLADEL cohort. *Arthritis Rheum* 2005;52(4):1138-47.

8. Koskenmies S, Widen E, Kere J*, et al.* Familial systemic lupus erythematosus in Finland. *J Rheumatol* 2001;28(4):758-60.

9. Michel M, Johanet C, Meyer O*, et al.* Familial lupus erythematosus. Clinical and immunologic features of 125 multiplex families. *Medicine (Baltimore)* 2001;80(3):153-8.

10. Gourley IS, Cunnane G, Bresnihan B*, et al.* A clinical and serological comparison of familial and non-familial systemic lupus erythematosus in Ireland. *Lupus* 1996;5(4):288-93.

11. Sestak AL, Nath SK, Kelly JA*, et al.* Patients with familial and sporadic onset SLE have similar clinical profiles but vary profoundly by race. *Lupus* 2008;17(11):1004-9.

12. Wang Z, Tang Z, Zhang HT*, et al.* Clinicopathological characteristics of familial SLE patients with lupus nephritis. *Lupus* 2009;18(3):243-8.

13. Al-Mayouf SM, Al Sonbul A. Juvenile systemic lupus erythematosus in multicase families from Saudi Arabia: comparison of clinical and laboratory variables with sporadic cases. *Lupus* 2006;15(9):616-8.

14. Apenteng T, Kaplan B, Meyers K. Renal outcomes in children with lupus and a family history of autoimmune disease. *Lupus* 2006;15(2):65-70.

15. Burgos PI, McGwin G, Jr., Reveille JD*, et al.* Is familial lupus different from sporadic lupus? Data from LUMINA (LXXIII), a multiethnic US cohort. *Lupus* 2010;19(11):1331-6.

16. Yasutomo K, Horiuchi T, Kagami S*, et al.* Mutation of DNASE1 in people with systemic lupus erythematosus. *Nat Genet* 2001;28(4):313-4.

17. Deng Y, Tsao BP. Advances in lupus genetics and epigenetics. *Curr Opin Rheumatol* 2014;26(5):482-92.

18. Byrne JC, Ni Gabhann J, Lazzari E*, et al.* Genetics of SLE: functional relevance for monocytes/macrophages in disease. *Clin Dev Immunol* 2012;2012:582352.

19. Orme J, Mohan C. Macrophages and neutrophils in SLE-An online molecular catalog. *Autoimmun Rev* 2012;11(5):365-72.

20. Rogers NM, Ferenbach DA, Isenberg JS*, et al.* Dendritic cells and macrophages in the kidney: a spectrum of good and evil. *Nat Rev Nephrol* 2014;10(11):625-43.

21. Davidson A, Bethunaickan R, Berthier C*, et al.* Molecular studies of lupus nephritis kidneys. *Immunol Res* 2015;63(1-3):187-96.

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

23. Purcell S, Neale B, Todd-Brown K*, et al.* PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 2007;81(3):559-75.

24. Collins AJ, Roberts TL, St Peter WL*, et al.* United States Renal Data System assessment of the impact of the National Kidney Foundation-Dialysis Outcomes Quality Initiative guidelines. *Am J Kidney Dis* 2002;39(4):784-95.

25. Weening JJ, D'Agati VD, Schwartz MM*, et al.* The classification of glomerulonephritis in systemic lupus erythematosus revisited. *J Am Soc Nephrol* 2004;15(2):241-50.

26. Weening JJ, D'Agati VD, Schwartz MM*, et al.* The classification of glomerulonephritis in systemic lupus erythematosus revisited. *Kidney Int* 2004;65(2):521-30.

27. Hill GS, Delahousse M, Nochy D*, et al.* Class IV-S versus class IV-G lupus nephritis: clinical and morphologic differences suggesting different pathogenesis. *Kidney Int* 2005;68(5):2288-97.

28. Watson L, Leone V, Pilkington C*, et al.* Disease activity, severity, and damage in the UK Juvenile-Onset Systemic Lupus Erythematosus Cohort. *Arthritis Rheum* 2012;64(7):2356-65.

29. Han JW, Zheng HF, Cui Y*, et al.* Genome-wide association study in a Chinese Han population identifies nine new susceptibility loci for systemic lupus erythematosus. *Nat Genet* 2009;41(11):1234-7.

30. Yang W, Shen N, Ye DQ*, et al.* Genome-wide association study in Asian populations identifies variants in ETS1 and WDFY4 associated with systemic lupus erythematosus. *PLoS Genet* 2010;6(2):e1000841.

31. Wang C, Ahlford A, Jarvinen TM*, et al.* Genes identified in Asian SLE GWASs are also associated with SLE in Caucasian populations. *Eur J Hum Genet* 2013;21(9):994-9.

32. Feldman CH, Hiraki LT, Liu J*, et al.* Epidemiology and sociodemographics of systemic lupus erythematosus and lupus nephritis among US adults with Medicaid coverage, 2000-2004. *Arthritis Rheum* 2013;65(3):753-63.

33. Lim SS, Bayakly AR, Helmick CG*, et al.* The incidence and prevalence of systemic lupus erythematosus, 2002-2004: The Georgia Lupus Registry. *Arthritis Rheumatol* 2014;66(2):357-68.

34. Somers EC, Marder W, Cagnoli P*, et al.* Population-based incidence and prevalence of systemic lupus erythematosus: the Michigan Lupus Epidemiology and Surveillance program. *Arthritis Rheumatol* 2014;66(2):369-78.

35. Alarcon GS, McGwin G, Jr., Bartolucci AA*, et al.* Systemic lupus erythematosus in three ethnic groups. IX. Differences in damage accrual. *Arthritis Rheum* 2001;44(12):2797-806.

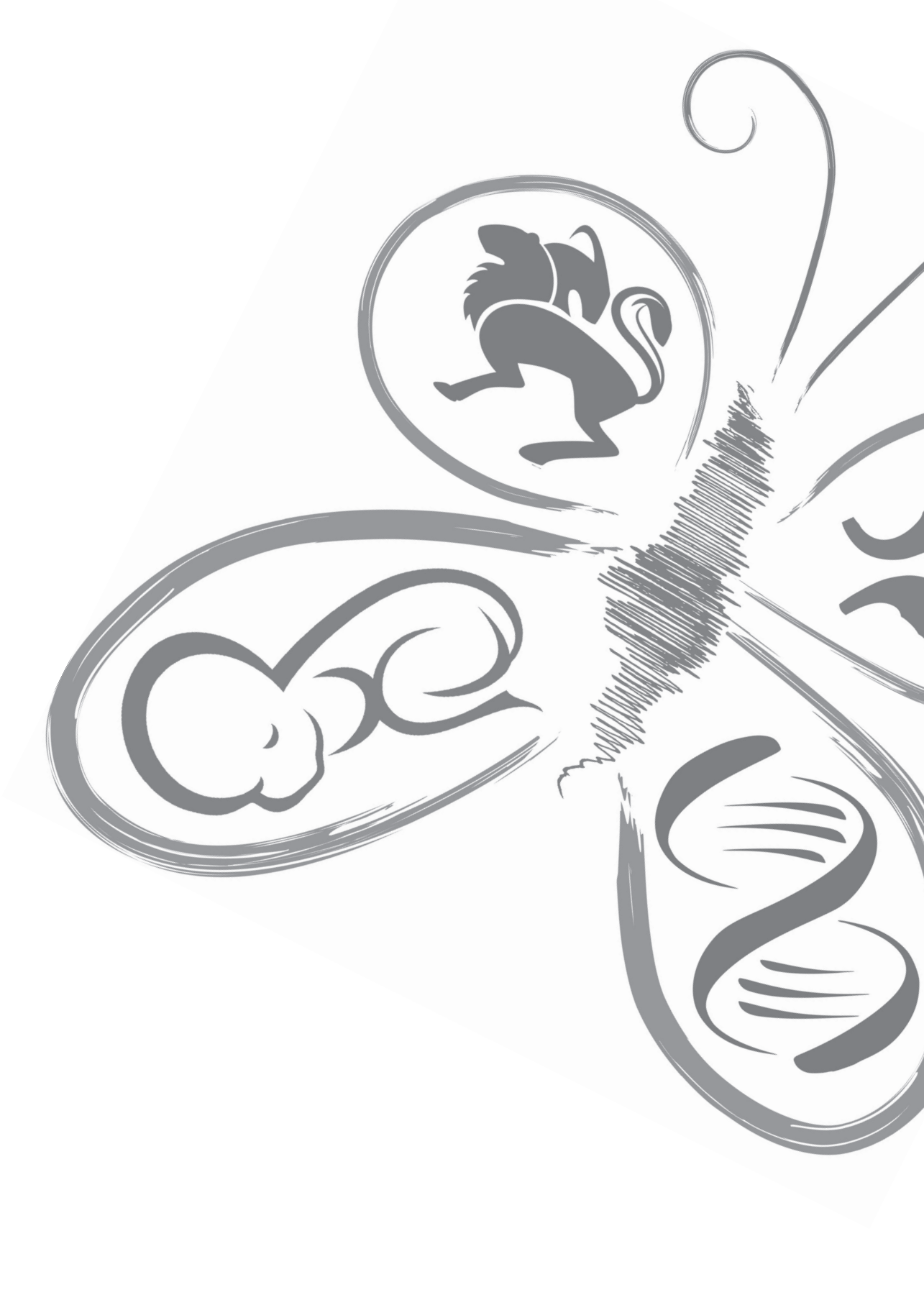
36. Hopkinson ND, Jenkinson C, Muir KR*, et al.* Racial group, socioeconomic status, and the development of persistent proteinuria in systemic lupus erythematosus. *Ann Rheum Dis* 2000;59(2):116-9.

37. Thumboo J, Uramoto K, O'Fallon WM*, et al.* A comparative study of the clinical manifestations of systemic lupus erythematosus in Caucasians in Rochester, Minnesota, and Chinese in Singapore, from 1980 to 1992. *Arthritis Rheum* 2001;45(6):494-500.

38. Johnson SR, Urowitz MB, Ibanez D*, et al.* Ethnic variation in disease patterns and health outcomes in systemic lupus erythematosus. *J Rheumatol* 2006;33(10):1990-5.

39. Peschken CA, Katz SJ, Silverman E*, et al.* The 1000 Canadian faces of lupus: determinants of disease outcome in a large multiethnic cohort. *J Rheumatol* 2009;36(6):1200-8.

40. Chung SA, Brown EE, Williams AH*, et al.* Lupus nephritis susceptibility Loci in women with systemic lupus erythematosus. *J Am Soc Nephrol* 2014;25(12):2859-70.



8

Summary and Discussion

Systemic lupus erythematosus (SLE) is an autoimmune disease that affects a variety of organs and therefore includes a wide range of symptoms. SLE affects primarily women, with peak incidence in the reproductive years. Because the first symptoms of SLE usually manifest at a relatively young age, and because SLE currently has no cure, developing an effective therapy—preferably with few adverse effects—is essential for increasing the likelihood of achieving long-term remission. In addition to establishing an accurate diagnosis of SLE, it is also necessary to determine if, how, and to what extent various organs are involved in the disease process in order to select an appropriate treatment strategy. Further insight into the pathogenesis of SLE may provide novel targets for new therapeutic approaches.

Diagnosis

All current guidelines for managing SLE recommend performing a renal biopsy when renal involvement is suspected, as clinical and laboratory parameters are not sufficient for accurately assessing the histologic class of lupus nephritis (LN). As discussed below, the class of LN, which is determined by renal biopsy, guides the choice of treatment. The class of LN is determined primarily by the glomerular lesions present in the biopsy and is described in the current classification system for LN, which was published in 2004.[1 2]

In **Chapter 2** of this thesis, we report the results of our study of interobserver agreement with respect to the histopathologic lesions in class III and class IV LN. We focused on these two classes because these classes of LN present with the most severe renal involvement and are typically treated with aggressive immunosuppressive therapy. We took images of glomeruli reflecting the range of lesions that can be encountered in LN, and we distributed these pictures to the members of the Renal Pathology Society. We then asked participating nephropathologists whether glomerular lesions were present that would categorize the biopsy as class III/class IV. Our analysis revealed poor agreement among nephropathologists in terms of recognizing class III/class IV lesions. Importantly, the more experienced nephropathologists had a higher level of agreement for all lesions investigated, suggesting improvement can be made by training of pathologists. Other factors may also have influenced interobserver disagreement, including ambiguous definitions and non-adherence to classification methodology. The most ambiguous definition in the 2004 classification guidelines is the definition of “endocapillary proliferation”. Poor interobserver agreement was also observed with respect to assigning the distribution of glomerular lesions as either segmental (S) or global (G). Current guidelines and definitions on this

subject are both incomplete and inconsistent, possibly explaining the poor agreement among nephropathologists. The relevance of subdividing class IV LN into class IV-S and IV-G is the subject of ongoing debate. Haring *et al*. [3] performed a meta-analysis and found no difference in clinical outcome between patients with class IV-S LN and patients with class IV-G LN; nevertheless, some researchers argue that class IV-S LN and IV-G LN represent two distinct biological entities and should therefore remain separate in the classification.[4] Lastly, many of the respondents in our study did not appear to adhere to the definition of extracapillary proliferation, which requires involvement of at least one quarter of the glomerular capsular circumference.

These observations led us to re-evaluate the current classification of glomerulonephritis in SLE. In **Chapter 3**, we critically discuss all aspects of the current classification system, and we make suggestions for steps to improve the system. We also summarize the history of the classification system in order to provide insight into how the system evolved into its current form. In the current classification system, the is a lack of guidelines regarding how to approach certain aspects (for example, small or incomplete glomeruli), how to apply the classification system when evaluating multiple levels, and how to score extraglomerular lesions. Furthermore, the cutoffs separating class II from class I or class III are ambiguous. Our suggestions for improvement are based partly on expert opinion, partly on currently available new evidence, and partly on the future acquisition of new evidence. To improve the current classification system further, the goals of a classification system in general should be kept in mind.[5] Specifically, the classification system should: *i*) improve the quality of communication both between and among renal pathologists and clinical nephrologists; *ii*) provide a logical structure for categorizing groups of patients for epidemiological, prognostic (outcome), or intervention studies (*i.e.*, clinical trials); and *iii*) assist in the clinical management of individual patients in terms of therapeutic decision-making and prognostication. With respect to the first goal, clear and unambiguous definitions and guidelines should be provided; clear definitions may also improve interobserver agreement. With respect to the third goal, the current classification system certainly helps facilitate clinical decision-making. However, improvements can be made with respect to prognostication, particularly within class III and class IV LN. In order to achieve this, more evidence regarding the prognostic effects of individual histologic lesions such as fibrinoid necrosis is needed. For class III/IV LN, nearly all patients are treated with immunosuppressive therapy; therefore, it is not currently possible to study the natural course of individual histologic lesions in relation to outcome. However, one can study which lesions respond to therapy—perhaps even to a

specific therapy—and which lesions do not respond to therapy. For this purpose, repeat biopsies—although usually not available—would be extremely useful. Ideally, studies that relate histologic lesions to clinical outcome should be conducted in a group of patients who are treated using a similar protocol. Such studies may also help achieve a more evidence-based system for classifying LN.

Treatment

LN is one of the most severe manifestations of SLE and occurs in 20-60% of patients with SLE. To avoid end-stage renal disease and the resulting need for renal replacement therapy, LN must be treated both immediately and effectively. In **Chapter 4,** we compare, summarize, and discuss the current national and international guidelines for managing LN, which were published in 2012;[6-11] it is important to note that the principal statements were similar among all guidelines. With respect to class II LN, the focus of the therapeutic strategy should be on reducing proteinuria by inhibiting the renin-angiotensin-aldosterone system (RAAS). Moreover, some guidelines recommend the use of additional immunosuppressive medication in cases with high levels of proteinuria. To achieve remission in patients with class III or class IV LN, induction treatment should consist of intravenous cyclophosphamide (ivCYC) or mycophenolate mofetil (MMF) in combination with oral glucocorticoids, either with or without three pulses of intravenous methylprednisolone at the start of induction treatment. The optimal dosages of ivCYC and oral glucocorticoids, however, are less clear. Some guidelines base their recommendations on disease severity (*e.g.*, the presence of crescents in the renal biopsy), race (Caucasian or non-Caucasian), or the specific drug combinations used. Some guidelines also explicitly state that only patients with “active” lesions visible on renal biopsy should be treated. Although this may seem obvious, it should nevertheless be explicitly discussed between the nephrologist and nephropathologist. All guidelines recommend including either MMF or azathioprine (AZA) in the maintenance phase of treatment, although some guidelines prefer MMF over AZA.

For the treatment of class V LN less robust evidence is available, which is reflected in the recommendations. Although most guidelines recommend RAAS inhibitors with the addition of immunosuppressive medication in case of nephrotic-range proteinuria, one guideline advises immunosuppressive medication irrespective of the level of proteinuria. Furthermore, which immunosuppressive medication is preferred—if any—is unclear. As adjunct therapy to the specific strategies outlined above, controlling blood pressure, treating hyperlipidemia

with statins, and treating proteinuria with RAAS inhibitors are recommended. In addition, hydroxychloroquine is recommended for all SLE patients, despite a lack of randomized controlled trials to support its use in LN. Despite the lack of clinical trial-based evidence for treating refractory LN, the guidelines generally recommend switching from MMF to ivCYC—or from ivCYC to MMF, if appropriate—if induction treatment fails. If this strategy fails, one of the recommendations is the use of rituximab, a humanized antibody directed against the B cell antigen CD20. However, given that the LUNAR trial, which included rituximab as an addition to steroid-MMF combination therapy, failed to reach the study endpoint, the efficacy of rituximab in this context has not yet been demonstrated in a randomized clinical trial.[12]

Designing a successful randomized clinical trial with SLE patients poses many challenges. First, selecting the study population can be difficult, particularly given the extremely heterogeneous disease manifestations among patients. Even though LN is only one such disease manifestation, patients with LN are a heterogeneous population with respect to renal involvement. Second, the disease manifestations, disease severity, and response to treatment differ between races, further increasing the clinical heterogeneity of the study population. Selection of the treatment and control regimens is also a key factor when designing a trial. The control regimen should leave room for measurable and meaningful improvement. Finally, selecting appropriate response criteria is essential to the outcome of a trial. However, as reflected by the differences in response criteria among the guidelines discussed above, no consensus has been reached with respect to what these criteria should be. Measures of irreversible damage (for example, the extent of chronic changes observed on renal biopsy) may be utilized to either stratify patients or balance randomization at baseline. These measurements can also be incorporated in the endpoint analyses to ensure that treatment- and/or disease-related deterioration—which can be overlooked when scoring disease activity alone—has not occurred.[13 14] Performing a post-treatment renal biopsy may also provide additional insight into which histologic lesions respond to therapy and which lesions do not. Evidence also suggests that gene expression profiles may in the future be used to predict which patients will likely respond to therapy and which patients will likely not respond.[15] Given the high heterogeneity of SLE patients, developing patient-tailored treatments is essential, but will be extremely difficult to achieve. Therefore, large, collaborative studies that involve all relevant medical disciplines are needed.

Pathogenesis

To investigate the pathogenesis of SLE and LN, we focused on DNA. First, we studied microchimerism (Mc), which is the presence of a small number of genetically distinct cells (of any type and originating from a different zygote) in an individual. Fetal Mc arises from fetal cells that enter the maternal circulation. We used differences in genetic polymorphisms between individuals to detect Mc. Second, we studied the contribution of known lupus susceptibility polymorphisms in familial lupus nephritis. Both of these approaches are discussed below.

SLE

Mc has been implicated in the pathogenesis of SLE (for review, see Kremer Hovinga *et al*.[16]). Although the precise role of Mc in SLE is unclear, three hypotheses have been suggested: *i*) the chimeric cells induce a graft-versus-host response; *ii*) the chimeric cells induce a host-versus-graft response; and *iii*) chimeric cells play a beneficial role in repair mechanisms. Further studies regarding the role of Mc in SLE are described in **Chapter 5** and **Chapter 6**. In **Chapter 5,** we report the occurrence and number of chimeric cells in the peripheral blood of SLE patients and control subjects. Our analysis revealed that SLE patients have a significantly higher prevalence of Mc compared to control subjects (54.5% versus 12.6%, respectively; P=0.03). Furthermore, when analyzing only patients and control subjects with Mc, the median number of fetal chimeric cells was significantly higher in SLE patients compared to control subjects (with 5 and 2.5 chimeric cells per 10^6 cells, respectively; P=0.046).

In previous studies, the detection of Mc was limited to the detection of male Mc (by identifying the Y chromosome). Here, using insertion-deletion polymorphisms and null alleles, in addition to the Y-chromosome, we were able to detect and distinguish Mc from different sources. We found that when present, Mc was usually fetal in origin in both patients and control subjects. Strikingly, we also found that in SLE patients with Mc, the chimeric cells originated from several relatives in 50% of cases; in contrast, in control subjects with Mc, the chimeric cells originated from only one relative in 100% of cases. We found no correlation between Mc and either clinical or laboratory parameters related to SLE. Because the transfer of fetal chimeric cells occurs during pregnancy (when the mother is exposed to the fetus), we reasoned that the higher prevalence of Mc in SLE patients occurred either because SLE patients acquire more fetal cells than control subjects during pregnancy, or because Mc is cleared to a lesser extent in SLE patients. To test these two

possibilities, we compared pregnant SLE patients with healthy pregnant control subjects (**Chapter 6**). We measured the level of Mc in the peripheral blood of pregnant women at 30 weeks of gestation, just after delivery, and 1 week, 6 weeks, 3 months, and 6 months after delivery. Compared to control subjects, SLE patients had a significantly higher number of fetal chimeric cells in the granulocyte fraction just after delivery; no difference was observed at any other time point measured. Importantly, at both 3 and 6 months after delivery, no fetal chimeric cells were detected in either SLE patients or control subjects. This finding is in contrast to the Mc detected in both patients and control subjects many years after their last pregnancy (as described in **Chapter 5**), shedding new light on the dynamics of fetal Mc. This finding also argues against our notion that the increased prevalence of Mc among patients with SLE years after their last pregnancy is due to the acquisition of more chimeric cells during pregnancy or reduced clearance of chimeric cells after pregnancy. Rather, it suggests that chimeric cells are cleared from the peripheral blood rapidly after pregnancy and then reappear years later, possibly originating from non-circulating fetal chimeric stem cells. Although the trigger for the reappearance of chimeric cells in the peripheral blood is unknown, it may be related to disease activity and/or tissue damage.

With respect to Mc in the peripheral blood mononuclear cell fraction, we found no difference between patients and control subjects at any time points examined. The role of fetal chimeric cells in the granulocyte fraction in SLE remains unclear. One possibility is that the chimeric neutrophils may undergo NETosis (the formation of neutrophil extracellular traps, or NETs), leading to the presentation of chromatin to the immune system. This “chimeric NETosis” may be more immunogenic than “self NETosis”. Nevertheless, it should be noted that the patients in this study were already diagnosed with SLE, rather than being in a preclinical phase of the disease. Therefore, this increase in Mc may be either a consequence or cause of the disease—or possibly both. Regarding the role of Mc in SLE in general, Kremer Hovinga *et al*. proposed three hypotheses, two in which Mc plays a pathogenic role and one in which increased Mc is a side effect of SLE. This putative side effect could be the result of repair following damage, or it could be the result of an altered immune system (either intrinsic or iatrogenic in nature). However, none of the aforementioned hypotheses stand out in terms of supportive evidence obtained to date. Thus, the chimeric cells could be beneficial, detrimental, or even inconsequential to the host. To determine whether Mc is a cause or consequence of SLE, it would be interesting to test whether SLE patients have more fetal chimeric cells than healthy control subjects *before* their first symptoms occur. Unfortunately, however, this would require repeated blood draws from a large number of healthy women

over a prolonged period of time, which is simply not feasible. To gain further insight into the role of Mc in SLE, it would also be interesting to determine the precise identity (*i.e.*, cell type) of the chimeric peripheral blood mononuclear cells. Furthermore, to determine whether chimeric granulocytes undergo NETosis, an animal model could be developed in which the chimeric cells are labeled (for example, with GFP). Moreover, the hypothesis that the increased prevalence of Mc in SLE is due to damage repair during SLE disease activity could be tested by following subjects over time, collecting clinical data, and then correlating these data with sequential data regarding Mc in the same patients. This approach could be performed in SLE patients and/or an animal model. If the results indicate that chimeric cells play a role in initiating and/or maintaining SLE, these chimeric cells could then be targeted (for example, using anti-HLA antibodies) and removed from the patient, providing a strategy for treating SLE in these patients.

The role of Mc in disease can also be examined from beyond the field of SLE, as the prevalence of Mc is also increased in several other autoimmune diseases.[17-19] This suggests that these autoimmune diseases have a common pathogenic basis. Alternatively, the increased prevalence of Mc could be a bystander effect. These diseases manifest as a chronic state of inflammation, which could facilitate the recruitment of chimeric stem cells; alternatively, the tissue damage caused by these diseases could lead to repair by chimeric cells (among other cells). In some cancers, chimeric cells are believed to play a beneficial role (for review, see Fugazzola *et al*.[20]) For example, chimeric cells may be involved in the immune surveillance of cancer cells, thereby providing a protective effect. Increased Mc in tumor tissue compared to adjacent benign tissue supports the notion of the recruitment of chimeric cells for tissue repair. If Mc plays a similar role in diseases in general—including various autoimmune diseases, inflammation, and cancer—the most likely role of chimeric cells is to repair damaged tissue. The involvement of chimeric cells in tissue repair may be beneficial to the host, or it may be an "innocent bystander" effect.

Lupus nephritis

Genetic factors are believed to play a significant role in the etiology of SLE. In **Chapter 7**, we compare and contrast familial and sporadic forms of lupus nephritis with respect to clinical parameters, serology, histologic class, the activity and chronicity indices (AI and CI), the number of glomerular monocytes/macrophages, and the contribution of known lupus susceptibility polymorphisms. We found that the frequency of juvenile onset was higher among familial LN patients compared to sporadic LN patients (50% versus 22%, respectively;

P=0.03). In addition, 44% of familial LN patients were male, compared to 12% of sporadic LN patients (*P*=0.004), and familial LN patients had a higher likelihood of progressing to advanced renal disease (25% versus 7% for sporadic LN; *P*=0.03). However, we found no difference in any of the histologic parameters explaining the observed difference in renal outcome between familial LN and sporadic LN. To provide a composite measure of genetic susceptibility, we calculated a genetic risk score (GRS). Our analysis revealed that the GRS did not differ significantly between familial LN patients and sporadic LN patients. Furthermore, in families in which LN clusters, the GRS was similar between each proband and the proband's unaffected relatives, providing further evidence that an accumulation of susceptibility alleles likely does not underlie familial LN. Therefore, the underlying differences between familial LN and sporadic LN remain unknown. Future experiments could include whole-exome sequencing in families with several affected members, which may identify rare genetic variants.

It's all a matter of perception

Perception can be defined in several ways, including *i*) the ability to see, hear, or become aware of something through the senses and *ii*) the way something is regarded, understood,

Figure 1. "My wife and my mother-in-law"
British cartoonist William Ely Hill (1887–1962) published "My Wife and My Mother-in-Law" in *Puck*, an American humor magazine, on 6 November 1915, with the caption "They are both in this picture—Find them" (panel A). However, the oldest known form of this image is an anonymous 1888 German postcard (panel B).

or interpreted. The second definition applies to the classic image "My wife and my mother-in-law" by W.E. Hill (Figure 1), and both definitions pertain to many aspects of this thesis.
Perception plays a major role in diagnosing SLE in general and LN in particular. Because SLE can present clinically with many "faces", combining the right perception of symptoms with other parameters often leads to the eventual diagnosis of SLE. In 2012, a new classification system for use in diagnosing SLE was proposed.[21] One remarkable change was the addition of the criterion that a diagnosis of SLE can be established based on the presence of LN in a renal biopsy combined with the presence of circulating anti-nuclear antibodies (positive ANA test). This criterion—combined with the principal role of a renal biopsy in guiding the treatment of LN—puts additional emphasis on the way in which the pathologist perceives the biopsy results. When evaluating a renal biopsy, both definitions of perception apply. First, all sections, special stains and immunofluorescence must be evaluated carefully in order to obtain a correct diagnosis and classification. Even the presence of focal "proliferative" lesions in only one or a few glomeruli will determine the treatment strategy in an individual patient. Second, interpretation also plays a major role in classifying a biopsy. Even if a new classification system is proposed by a panel of experts, if that classification system—including all of its definitions—is not interpreted by the users as intended by its creators, the system may be useless. Difficulties arising from one or both types of perception can lead to low interobserver agreement. One possible solution is to train pathologists in order to improve their ability to "see". This approach—along with clear, practical, uniform, and careful formulation of definitions in the classification system—may also affect their understanding and/or interpretation of the classification system. This is not an easy task, as most experienced

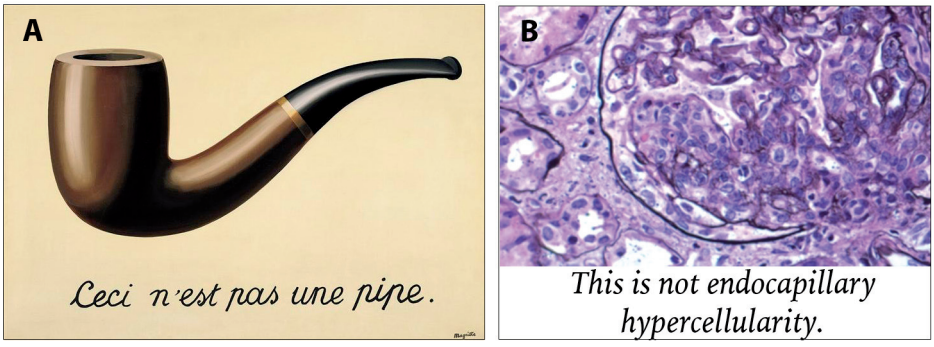


Figure 2. The treachery of images: This is not a pipe
Panel A shows the 1929 painting entitled "*Ceci n'est pas une pipe*" ("This is not a pipe") by René Magritte. Panel B shows an image depicting endocapillary hypercellularity.

pathologists have a preconceived mental image of what they perceive as *e.g.* endocapillary hypercellularity. Can words replace what the pathologist sees in a picture? And which has more authority, the picture or the words? This struggle is represented in the 1929 painting entitled "*Ceci n'est pas une pipe*" ("This is not a pipe"; this painting is commonly referred to as "The Treachery of Images") by René Magritte (Figure 2A). The paragon of complete agreement may only be achieved if the pathologist is replaced by a computer. Although replacing pathologists with computers is not likely to occur in the near future, computer-aided diagnostic technologies (such as automated screening of Pap smear results) are being developed. In breast cancer, a computer model based on a plethora of microscopic features in tissue microarray samples, as analyzed by the computer, was able to predict patient survival more accurately than conventional histologic parameters (*e.g.*, tumor grade).[22] In the future, automated analysis of renal biopsy images may help pathologists obtain a more accurate, more reliable, and more reproducible assessment of specific prognostic features. Alternatively—and analogous to the breast cancer study discussed above—computer models may be able to perceive features relevant to prognosis that are not currently identified by performing a conventional examination.

In clinical trials, the perception and documentation of treatment effects are essential to the development of new treatment strategies. However, determining treatment effect is often hindered by several factors, including the way in which the resulting change in symptoms is both perceived and defined. With respect to lupus, one of the major challenges lies in finding equally effective—or more effective—drugs with fewer and/or less severe side effects. For example, cyclophosphamide, although often administered for a limited period of time, can have severe side effects, including reduced fertility. Although mycophenolate mofetil (MMF) does not have these fertility-related side effects, it does have other side effects, including an increased risk of severe infections. Furthermore, oral glucocorticoids have been the standard treatment for many decades. Despite the existence of steroid-sparing treatment strategies, many SLE patients are treated with long-term courses of oral glucocorticoids, which can have long-lasting side effects, including suppression of the hypothalamic-pituitary-adrenal axis, Cushingoid appearance, hirsutism or virilism, impotence, menstrual irregularities, peptic ulcer disease, cataracts and/or increased intraocular pressure/glaucoma, myopathy, osteoporosis, and vertebral compression fractures. However, before oral glucocorticoids can be eliminated from the standard treatment regimen, new trials must be performed to compare steroid-free regimens with classic steroid-containing regimens. For example, a trial is currently underway (RITUXILUP NCT01773616) comparing the "standard" oral

glucocorticoid/MMF regimen with a regimen of induction therapy that includes two doses of rituximab and methylprednisolone followed by maintenance with MMF. This study also circumvents a problem commonly encountered with studies to test a new drug for LN: many drugs are tested either as an add-on or in refractory disease. In these settings, defining the primary endpoint is extremely important; specifically, it is important to address the following question: What do we *perceive* to be a clinically relevant and reasonable response? One may also wonder whether the clinical parameters that are currently used as the response criteria truly represent the actual disease activity and chronicity, and—consequently—whether protocol biopsies may be a valuable addition for determining renal response.

When studying the role of Mc in SLE, one must always keep in mind that more information might be found beyond the limits of our perception. Although Mc is often reported as a binary outcome (*i.e.*, either present or absent), this view is likely only one part of a much bigger picture. For example, an absence of Mc may indicate that the subject truly does not carry any chimeric cells, or it may mean that chimeric cells are present but are below the current detection limit (*i.e.*, fewer than 1 chimeric cell per 100,000 "host" cells); in other words, absence of proof is not proof of absence. This begs the question of whether the presence of cells that we cannot detect has any biological relevance. As stated by Elliot Eisner, "Not everything that matters can be measured, and not everything that is measured matters."[23] Because the number of chimeric cells in an individual is extremely low, isolating and characterizing these cells can be quite difficult. To determine the phenotype of these chimeric cells, many studies—including those presented in this thesis—use an indirect method in which Mc is detected in a specific subset of cells. Drabbels *et al.* used a method in which fluorescence-activated cell sorting was used to isolate chimeric cells based on HLA mismatch.[24] Some animal studies used a variation of this method by isolating fetal chimeric cells of GFP-positive offspring.[25] Although it is clearly preferable to study Mc in human subjects, animal studies currently offer the only platform for studying the dynamics of Mc, its effects, and factors that influence Mc.

In **Chapter 7**, we report that patients with familial LN are more likely to progress to advanced renal disease compared to patients with sporadic LN. However, none of the parameters investigated were sufficient to explain this perceived difference. For example, biopsies from familial LN patients revealed similar disease severity as biopsies from sporadic LN patients. We also found no difference between familial and sporadic cases with respect to their genetic risk scores, suggesting either that an accumulation of susceptibility alleles does not lead to familial LN, or that risk alleles other than the ones studied here play a role. In this

respect, exome sequencing may be a useful strategy for identifying rare genetic variants that may play a role in familial LN.

Concluding remarks

In daily practice, perception—which is defined both as the ability to see, hear, or become aware of something through the senses and as the way something is regarded, understood, or interpreted—is an essential tool for diagnosing and treating SLE in general and LN in particular. Moreover, research regarding the pathogenesis, diagnosis, and treatment of this disease hinges on how we observe the outcome and results, how we interpret those results, and what we perceive to be clinically relevant. In both clinical practice and research, we should always be aware of the strong influence of our *perception*.

References

1. Weening JJ, D'Agati VD, Schwartz MM*, et al.* The classification of glomerulonephritis in systemic lupus erythematosus revisited. *J Am Soc Nephrol* 2004;15(2):241-50.

2. Weening JJ, D'Agati VD, Schwartz MM*, et al.* The classification of glomerulonephritis in systemic lupus erythematosus revisited. *Kidney Int* 2004;65(2):521-30.

3. Haring CM, Rietveld A, van den Brand JA*, et al.* Segmental and global subclasses of class IV lupus nephritis have similar renal outcomes. *J Am Soc Nephrol* 2012;23(1):149-54.

4. Hill GS, Delahousse M, Nochy D*, et al.* Class IV-S versus class IV-G lupus nephritis: clinical and morphologic differences suggesting different pathogenesis. *Kidney Int* 2005;68(5):2288-97.

5. Glassock RJ. Reclassification of lupus glomerulonephritis: Back to the future. *J Am Soc Nephrol* 2004;15(2):501-03.

6. Bertsias GK, Tektonidou M, Amoura Z*, et al.* Joint European League Against Rheumatism and European Renal Association–European Dialysis and Transplant Association (EULAR/ERA-EDTA) recommendations for the management of adult and paediatric lupus nephritis. *Ann Rheum Dis* 2012;71(11):1771-82.

7. Hahn BH, McMahon MA, Wilkinson A*, et al.* American College of Rheumatology guidelines for screening, treatment, and management of lupus nephritis. *Arthritis Care Res* 2012;64(6):797-808.

8. Kidney Disease: Improving Global Outcomes (KDIGO) Glomerulonephritis Work Group. KDIGO Clinical Practice Guideline for Glomerulonephritis. *Kidney Int Suppl* 2012;2:139-274.

9. Ruiz Irastorza G, Espinosa G, Frutos MA*, et al.* Diagnosis and treatment of lupus nephritis. Consensus document from the systemic auto-immune disease group (GEAS) of the Spanish Society of Internal Medicine (SEMI) and Spanish Society of Nephrology (S.E.N.). *Nefrologia* 2012;32 Suppl 1:1-35.

10. van Tellingen A, Voskuyl AE, Vervloet MG*, et al.* Dutch guidelines for diagnosis and therapy of proliferative lupus nephritis. *Neth J Med* 2012;70(4):199-207.

11. Mina R, von Scheven E, Ardoin SP*, et al.* Consensus treatment plans for induction therapy of newly diagnosed proliferative lupus nephritis in juvenile systemic lupus erythematosus. *Arthritis Care Res (Hoboken)* 2012;64(3):375-83.

12. Rovin BH, Furie R, Latinis K*, et al.* Efficacy and safety of rituximab in patients with active proliferative lupus nephritis: the Lupus Nephritis Assessment with Rituximab study. *Arthritis Rheum* 2012;64(4):1215-26.

13. Strand V. Clinical trial design in systemic lupus erythematosus: lessons learned and future directions. *Lupus* 2004;13(5):406-11.

14. Dall'Era M, Wofsy D. Clinical trial design in systemic lupus erythematosus. *Curr Opin Rheumatol* 2006;18(5):476-80.

15. Parikh SV, Malvar A, Song H*, et al.* Characterising the immune profile of the kidney biopsy at lupus nephritis flare differentiates early treatment responders from non-responders. *Lupus Sci Med* 2015;2(1):e000112.

16. Kremer Hovinga I, Koopmans M, de Heer E*, et al.* Chimerism in systemic lupus erythematosus--three hypotheses. *Rheumatology (Oxford)* 2007;46(2):200-08.

17. Lambert NC, Lo YM, Erickson TD*, et al.* Male microchimerism in healthy women and women with scleroderma: cells or circulating DNA? A quantitative answer. *Blood* 2002;100(8):2845-51.

18. Lepez T, Vandewoestyne M, Hussain S*, et al.* Fetal Microchimeric Cells in Blood of Women with an Autoimmune Thyroid Disease. *PLoS ONE* 2011;6(12):e29646.

19. Reed AM, Picornell YJ, Harwood A*, et al.* Chimerism in children with juvenile dermatomyositis. *Lancet* 2000;356(9248):2156-57.

20. Fugazzola L, Cirello V, Beck-Peccoz P. Fetal cell microchimerism in human cancers. *Cancer Lett* 2010;287(2):136-41.

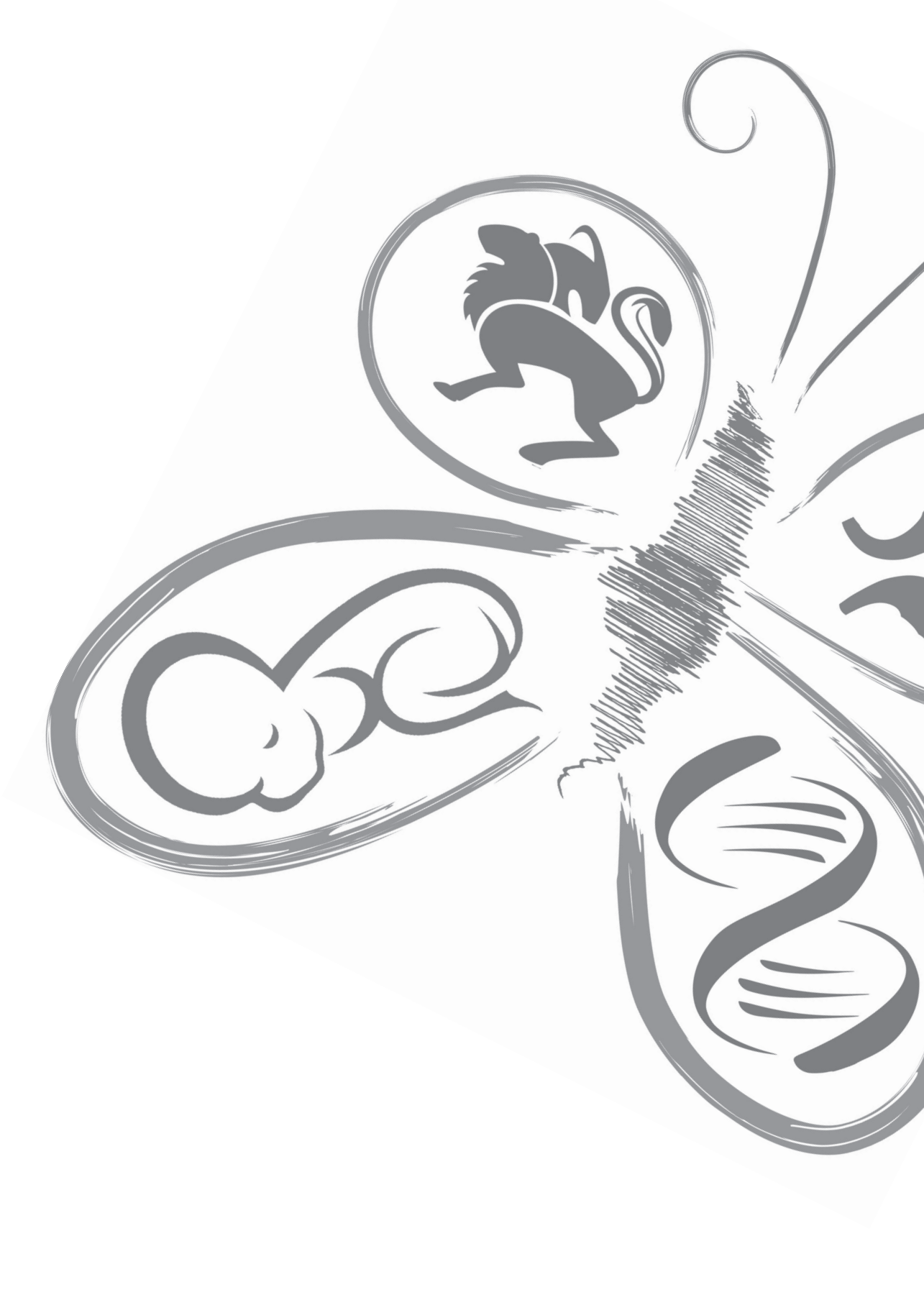
21. Petri M, Orbai AM, Alarcon GS*, et al.* Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus. *Arthritis Rheum* 2012;64(8):2677-86.

22. Beck AH, Sangoi AR, Leung S*, et al.* Systematic analysis of breast cancer morphology uncovers stromal features associated with survival. *Sci Transl Med* 2011;3(108):108ra13.

23. Eisner E. *The arts and the creation of mind*. London: Yale University Press, 2002.

24. Drabbels JJ, van de Keur C, Kemps BM*, et al.* HLA-targeted flow cytometric sorting of blood cells allows separation of pure and viable microchimeric cell populations. *Blood* 2011;118(19):e149-55.

25. Fujiki Y, Johnson KL, Tighiouart H*, et al.* Fetomaternal Trafficking in the Mouse Increases as Delivery Approaches and Is Highest in the Maternal Lung. *Biol Reprod* 2008;79(5):841-48.



Addenda

Nederlandse samenvatting

Systemische lupus erythematosus (SLE) is een auto-immuunziekte waarbij vele organen aangedaan kunnen zijn. SLE-patiënten kunnen zich dan ook, gedurende het beloop van hun ziekte, met veel verschillende symptomen presenteren. SLE-patiënten betreffen meestal vrouwen, waarbij de piekincidentie rond de vruchtbare leeftijd ligt. Aangezien SLE zich vaak op relatief jonge leeftijd presenteert en omdat er geen genezing mogelijk is, is het belangrijk een effectieve behandeling – met weinig bijwerkingen – te ontwikkelen, om langdurige remissie te bewerkstelligen. Behalve het stellen van de diagnose is het belangrijk om te bepalen welke organen in het ziekteproces zijn betrokken, zodat een juist behandelplan opgesteld kan worden. Verder inzicht in de pathogenese van SLE kan bijdragen aan het ontwikkelen van nieuwe behandelmogelijkheden voor deze ziekte.

Diagnose

Alle huidige richtlijnen voor de behandeling van SLE raden aan een nierbiopt te verrichten wanneer er verdenking is op nierbetrokkenheid (lupus nefritis). Het klinisch beeld en laboratoriumuitslagen zijn niet accuraat genoeg om de histologische klasse lupus nefritis te kunnen inschatten. Zoals hieronder toegelicht zal worden, is de klasse lupus nefritis leidend in het bepalen van de behandeling. De klasse lupus nefritis wordt bepaald door de afwijkingen zoals gezien in de glomeruli in het nierbiopt. Deze afwijkingen worden beschreven in het huidige classificatiesysteem, dat in 2004 gepubliceerd is.

In **hoofdstuk 2** van dit proefschrift beschrijven we de resultaten van ons onderzoek naar de inter-beoordelaarsbetrouwbaarheid met betrekking tot de histopathologische laesies in klasse III en klasse IV lupus nefritis. We hebben ons gericht op klasse III en klasse IV, omdat dit de meest ernstige vormen van nierbetrokkenheid betreffen en behandeld worden met agressieve immunosuppressieve therapie. We hebben afbeeldingen van glomeruli, waarin een spectrum van afwijkingen zoals die gezien kunnen worden in lupus nefritis, gedistribueerd onder leden van de Renal Pathology Society. We hebben deelnemende pathologen gevraagd of er een glomerulaire laesie aanwezig was die het biopt als klasse III/IV zou categoriseren. Uit statistische analyse bleek dat er een slechte overeenkomst was tussen het oordeel van de verschillende pathologen. Wel bleek dat de overeenstemming tussen ervaren pathologen beter was dan de overeenstemming tussen minder ervaren pathologen. Dit suggereert dat verbetering mogelijk is door het trainen van pathologen. Ook andere factoren kunnen een rol hebben gespeeld, waaronder onduidelijke definities van laesies in de huidige classificatie

voor lupus nefritis en het zich niet houden aan de afgesproken definities. Eerstgenoemde speelt vooral een rol in de beoordeling van endocapillaire proliferatie/hypercelllulariteit en de beoordeling of de glomerulus segmental of globaal is aangedaan. Ten aanzien van de laatste bleek, dat sommige pathologen zich niet leken te houden aan de definitie voor extracapillaire proliferatie, waarin staat dat dit aanwezig moet zijn in tenminste een kwart van de omtrek van de glomerulus.

Deze observaties hebben ons ertoe gebracht de classificatie voor lupus nefritis, zoals gepubliceerd in 2004, met een internationale groep van ervaren nefropathologen te herevalueren. In **hoofdstuk 3** geven we een kritische bespreking van veel aspecten van de huidige classificatie en doen we voorstellen hoe deze aspecten verbeterd zouden kunnen worden. Daarnaast geven we een overzicht van de geschiedenis van de classificatie om meer inzicht te geven in hoe deze in de loop der jaren tot stand is gekomen. In de huidige classificatie ontbreken op een aantal punten richtlijnen, bijvoorbeeld ten aanzien van het omgaan met kleine of incomplete glomeruli, hoe de classificatie toegepast moet worden als er meerdere niveaus beoordeeld worden en hoe extra-glomerulaire laesies gescoord moeten worden. Ook zijn de afkappunten van klasse II ten opzichte van klasse I en klasse III niet geheel duidelijk. Onze suggesties ter verbetering zijn deels gebaseerd op de ervaring van experts, deels op nieuw beschikbaar bewijs en deels op het toekomstig verkrijgen van bewijs.

Behandeling

Lupus nefritis is één van de ernstigste uitingen van SLE en komt voor in 20-60% van de patiënten met SLE. Om progressie tot nierfalen en de bijkomstige noodzaak tot dialyse of transplantatie te voorkomen, moet zowel snel als effectief behandeld worden. In **hoofdstuk 4** vergelijken we, vatten we samen en bediscussiëren we de huidige nationale en internationale richtlijnen voor de behandeling van lupus nefritis (alle gepubliceerd in 2012). De hoofdlijnen zijn vergelijkbaar in de verschillende richtlijnen. Bij lupus nefritis klasse II ligt de focus op het verminderen van de proteïnurie door middel van het remmen van het renine-angiotensine-aldosteron (RAAS) systeem. Sommige richtlijnen adviseren daarbij het gebruik van immunosuppressieve medicatie in geval van grote hoeveelheden proteïnurie. Voor lupus nefritis klasse III en klasse IV bestaat de behandeling om remissie te bereiken (inductiefase) uit intraveneus cyclofosfamide (ivCYC) of mycofenolaat mofetil (MMF),

in combinatie met orale glucocorticoïden, met of zonder gepulseerd intraveneus methylprednisolon aan het begin van de behandeling. De optimale doseringen voor de ivCYC en orale glucocorticoïden zijn niet geheel duidelijk. Sommige richtlijnen baseren de dosering op de ernst van de ziekte (bijv. de aanwezigheid van crescents in het biopt), ras (Kaukasisch of niet-Kaukasisch) of de specifieke combinatie van medicijnen. Sommige richtlijnen geven specifiek aan, dat alleen patiënten met actieve laesies in het nierbiopt behandeld moeten worden. Hoewel dit voor de hand liggend lijkt, is het wel belangrijk dat dit benadrukt wordt in de dialoog tussen nefroloog en patholoog. In de onderhoudsfase van de behandeling raden alle richtlijnen aan om azathioprine (AZA) of MMF te geven, hoewel sommige richtlijnen de voorkeur geven aan MMF boven AZA. Voor de behandeling van lupus nefritis klasse V is de wetenschappelijke onderbouwing minder sterk. Desondanks adviseren de meeste richtlijnen immunosuppressieve medicatie in het geval van nefrotische proteïnurie, maar welke immunosuppressieve medicatie dan gebruikt moet worden, verschilt per richtlijn. Naast de specifieke therapieën zoals hierboven beschreven, wordt geadviseerd de bloeddruk te reguleren, hyperlipidemie te behandelen met statines en proteïnurie te behandelen met remmers van het RAAS-systeem. Daarnaast wordt het gebruik van hydroxychloroquine geadviseerd, hoewel er geen gerandomiseerd klinisch onderzoek is dat dit ondersteunt in lupus nefritis. Voor refractaire lupus nefritis bestaat geen bewijs uit gerandomiseerd klinisch onderzoek. Desondanks wordt in alle richtlijnen geadviseerd om over te schakelen van MMF naar ivCYC – of van ivCYC naar MMF, indien van toepassing – als de inductiefase van de behandeling niet succesvol was. Als ook dit faalt, is één van aanbevelingen om rituximab, een gehumaniseerd antilichaam tegen het B-cel antigen CD20, te geven. Echter, de LUNAR-studie, waarin rituximab gegeven is als toevoeging aan een MMF-steroïden combinatie therapie, heeft zijn studie eindpunten niet bereikt. Daarom is de effectiviteit van rituximab vooralsnog niet bewezen in deze context.

Pathogenese

Binnen ons onderzoek naar de pathogenese van SLE en lupus nefritis lag de focus op DNA. Ten eerste onderzochten we microchimerisme (Mc): de aanwezigheid van een klein aantal genetisch andere cellen (elk type cel, afkomstig van een andere zygote) in een individu. Foetaal Mc ontstaat, doordat tijdens de zwangerschap cellen van het kind, via de placenta, naar de circulatie van de moeder gaan. Ook gaan er cellen van de moeder naar het kind;

dit wordt maternaal Mc genoemd. Wij maakten gebruik van verschillen in genetische polymorfismen tussen individuen om Mc te detecteren. Daarnaast bestudeerden we onder andere de bijdrage van bekende lupus susceptibiliteitspolymorfismen in familiair en sporadisch voorkomende lupus nefritis. Beide benadering worden hieronder besproken.

SLE

Mc wordt genoemd als mogelijke speler in de pathogenese van SLE. Hoewel deze eventuele pathogenetische rol nog onduidelijk is, zijn er wel verschillende hypothesen: *i*) de chimere cellen lokken een graft-versus-host reactie uit; *ii*) de chimere cellen lokken een host-versus-graft reactie uit en *iii*) de chimere cellen spelen een positieve rol in het herstellen van schade. Nader onderzoek naar de rol van Mc in SLE wordt beschreven in **hoofdstuk 5** en **hoofdstuk 6**. In **hoofdstuk 5** beschrijven we het voorkomen van chimere cellen in het perifere bloed van vrouwen met SLE en in het perifere bloed van controlepersonen. Ons onderzoek wees uit dat vrouwen met SLE een significant hogere prevalentie van chimere cellen in hun bloed hebben dan controlepersonen (respectievelijk 54,5% versus 12,6%; *P*=0,03). Daarnaast was, wanneer alleen patiënten en controlepersonen met Mc geanalyseerd werden, het mediane aantal chimere cellen significant hoger in patiënten met SLE dan in controlepersonen (respectievelijk 5 en 2,5 chimere cellen per 10^6 cellen; *P*=0,046). Eerdere studies naar het voorkomen van Mc in SLE maakten gebruik van de detectie van het Y-chromosoom. Zodoende kon alleen mannelijk Mc onderzocht worden. In dit onderzoek hebben we, naast het Y-chromosoom, gebruik gemaakt van insertie-deletie polymorfismen en nul-allelen, waardoor we in staat waren om Mc van verschillende bronnen te detecteren en te onderscheiden. We constateerden dat, in personen met Mc, dit Mc meestal foetaal in origine was, in zowel patiënten als controlepersonen. Wat ook opviel is, dat in de helft van de gevallen dat Mc werd gedetecteerd in patiënten met SLE, dit afkomstig was van meerdere familieleden. Dit in tegenstelling tot controlepersonen waarbij de chimere cellen afkomstig waren van één familielid in alle gevallen. We vonden geen relatie tussen Mc en klinische parameters of laboratoriumuitslagen. Omdat de chimere cellen tijdens de zwangerschap overgaan van het kind naar de moeder, beredeneerden we dat de toename van Mc in patiënten met SLE zou kunnen ontstaan, doordat er óf meer chimere cellen van het kind naar de moeder gaan tijdens de zwangerschap, óf de chimere cellen na de zwangerschap minder goed geklaard worden in patiënten met SLE. Om deze twee mogelijkheden te onderzoeken, hebben we zwangere patiënten met SLE vergeleken met zwangere controlepersonen (**hoofdstuk 6**). We hebben de hoeveelheid

foetaal Mc in perifeer bloed bepaald bij 30 weken amenorroeduur, direct na de bevalling, en 1 week, 6 weken, 3 maanden en 6 maanden postpartum. Vergeleken met controlepersonen hadden de patiënten met SLE direct na de bevalling een significant hoger aantal chimere cellen in de granulocytenfractie dan de controlepersonen; bij alle andere tijdspunten werd geen verschil aangetoond. Bij 3 en 6 maanden postpartum werden zelfs in zowel SLE-patiënten als controlepersonen geen chimere cellen meer aangetoond. Dit resultaat staat in contrast met het Mc, dat vele jaren na de laatste zwangerschap gedetecteerd werd in zowel SLE-patiënten als controlepersonen (**hoofdstuk 5**), hetgeen nieuwe inzichten geeft in de dynamiek van het foetale Mc. Dit resultaat pleit ook tegen ons idee, dat de toegenomen prevalentie van Mc in SLE-patiënten het gevolg is van het verkrijgen van meer Mc tijdens de zwangerschap of het verminderd klaren van Mc na de zwangerschap. Deze resultaten pleiten ervoor dat chimere cellen snel na de zwangerschap geklaard worden uit het perifere bloed en jaren later opnieuw verschijnen; mogelijk afkomstig van niet-circulerende foetale chimere stamcellen. Hoewel de prikkel die hiertoe zou leiden onbekend is, is het mogelijk gerelateerd aan ziekteactiviteit en/of weefselschade.

Lupus nefritis

Er wordt gedacht dat genetische factoren een belangrijke rol spelen in de etiologie van SLE. In **hoofdstuk 7** vergelijken we familiair en sporadisch voorkomende lupus nefritis, ten aanzien van klinische parameters, serologie, histologische klasse, activiteits- en chroniciteitsindices, het aantal glomerulaire macrofagen/monocyten en de bijdrage van bekende lupus susceptibiliteitspolymorfismen. Onze resultaten lieten zien dat familiaire lupus nefritis vaker op kinderleeftijd (t/m 18 jaar) gediagnosticeerd werd in vergelijking met sporadisch voorkomende lupus nefritis (respectievelijk 50% versus 22%; *P*=0,03). Daarnaast was 44% van de patiënten met familiaire lupus nefritis man, in vergelijking met 12% van de patiënten met sporadisch voorkomende lupus nefritis (*P*=0,004). Voorts hadden de familiaire patiënten een hoger risico op progressie naar vergevorderde nierziekte (25% versus 7% voor sporadisch; *P*=0,03). We vonden echter in geen van de histologische variabelen een verschil tussen familiair en sporadisch voorkomende lupus nefritis, die het geobserveerde verschil in uitkomst van de nierziekte kan verklaren. Om een composietscore voor genetische gevoeligheid te geven, werd een genetisch risico score (GRS) berekend. Deze score bleek niet verschillend tussen beide groepen. Daarnaast zagen we dat, in families met clustering van lupus nefritis, de GRS vergelijkbaar was tussen de patiënt en de onaangedane familieleden van de patiënt. Samen suggereert dit dat een ophoping

van deze susceptibiliteitspolymorfismen niet verantwoordelijk is voor het ontstaan van familiair voorkomende lupus nefritis. Wel is het mogelijk, dat andere risicoallelen dan die hier bestudeerd zijn, een rol spelen. Verder onderzoek, bijvoorbeeld door middel van 'whole exome sequencing' in families met meerdere aangedane familieleden, kan mogelijk zeldzame genetische varianten identificeren.

Curriculum vitae

Suzanne Wilhelmus is op 30 mei 1982 geboren in Zoetermeer. Na het behalen van haar Gymnasium diploma in 2000 aan het Erasmus College te Zoetermeer begon zij aan de studie Biomedische Wetenschappen aan de Universiteit Leiden. Na het *cum laude* behalen van haar bachelordiploma Biomedische Wetenschappen, is zij via de "zij-instroom" begonnen aan de studie Geneeskunde. Ook deed ze naast deze studie onderzoek op de afdeling Neurologie onder begeleiding van prof. dr. J.J.G.M. Verschuuren. Gedurende een deel van haar studie werkte ze als student-assistent, waarbij zij zowel praktisch als theoretisch onderwijs gaf aan (bio)medische studenten over humane fysiologie. In 2006 behaalde zij haar doctoraal Geneeskunde *cum laude*, hetgeen in 2008 gevolgd werd door het artsexamen en een master in Biomedische Wetenschappen. In 2008 is ze begonnen als arts in opleiding tot specialist en klinisch onderzoeker (AIOSKO) op de afdeling Pathologie in het Leids Universitair Medisch Centrum (opleider prof. dr. G.J. Fleuren, opgevolgd door prof. dr. V.T.H.B.M. Smit). Haar promotieonderzoek vond plaats onder de supervisie van prof. dr. J.A. Bruijn en dr. I.M. Bajema. Gedurende haar opleiding heeft ze 3 maanden doorgebracht bij prof. H.T. Cook in Hammersmith Hospital, Imperial College (Londen) en bij prof. dr. W.J. Mooi, VU Medisch Centrum (Amsterdam) voor verdieping in respectievelijk nefropathologie en dermatopathologie, laatstgenoemde met focus op melanocytaire leasies. In juni 2016 heeft zij de opleiding tot klinisch patholoog afgerond en is sindsdien werkzaam in het Academisch Medisch Centrum (Amsterdam).

Suzanne Wilhelmus was born in Zoetermeer, the Netherlands, on May 30th, 1982. She graduated from secondary school (Erasmus College, Zoetermeer, Gymnasium) in 2000. In the same year, she started studying Biomedical Sciences at Leiden University. After completing her bachelor Biomedical Sciences *cum laude* in 2003, she expanded her studies to Medicine and participated in research at the Department of Neurology under supervision of prof. dr. J.J.G.M. Verschuuren. During her studies, she also worked as a student assistant providing practical and theoretical education to (bio)medical students on multiple subjects concerning human physiology. In 2006, she obtained her master in Medicine *cum laude*, in 2008 followed by her medical degree and master Biomedical Sciences. In 2008, she started both her residency in Pathology (program director prof. dr. G.J. Fleuren, succeeded by prof. dr. V.T.H.B.M. Smit), and her PhD research under supervision of prof. dr. J.A. Bruijn and dr. I.M. Bajema, at the Leiden University Medical Center. In this period, she spent 3

months with prof. H.T. Cook at Hammersmith Hospital, Imperial College (London) to further her knowledge on nephropathology, and with prof. dr. W.J. Mooi at the VU Medical Center (Amsterdam) to study dermatopathology with emphasis on melanocytic lesions. In June 2016, she finished her residency in Pathology and has since been working at the Academic Medical Center (Amsterdam).

List of publications

1. Rijnink EC, Teng YKO, **Wilhelmus S**, Almekinders M, Wolterbeek R, Cransberg K, Bruijn JA, Bajema IM. Clinical and histopathologic determinants of renal outcome in lupus nephritis: starting from scratch. *Clin J Am Soc Nephrol* 2017 (accepted for publication)

2. **Wilhelmus S**, Bajema IM, Bertsias GK, Boumpas DT, Gordon C, Lightstone L, Tesar V, Jayne DR. Lupus nephritis management guidelines compared. *Nephrol Dial Transplant* 2016; 31(6): 904-913.

3. **Wilhelmus S**, Alpers CE, Cook HT, Ferrario F, Fogo AB, Haas M, Joh K, Noël LH, Seshan SV, Bruijn JA, Bajema IM. The Revisited Classification of GN in SLE at 10 Years: Time to Re-Evaluate Histopathologic Lesions. *J Am Soc Nephrol* 2015; 26(12): 2938-2946.

4. Rijnink EC, Penning ME, Wolterbeek R, **Wilhelmus S**, Zandbergen M, van Duinen SG, Schutte J, Bruijn JA, Bajema IM. Tissue microchimerism is increased during pregnancy: a human autopsy study. *Mol Hum Reprod* 2015; 21(11): 857-864.

5. Chua JS, Baelde HJ, Zandbergen M, **Wilhelmus S**, van Es LA, de Fijter JW, Bruijn JA, Bajema IM, Cohen D. Complement Factor C4d Is a Common Denominator in Thrombotic Microangiopathy. *J Am Soc Nephrol* 2015; 26(9): 2239-2247.

6. **Wilhelmus S**, Cook HT, Noël LH, Ferrario F, Wolterbeek R, Bruijn JA, Bajema IM. Interobserver agreement on histopathological lesions in class III or IV lupus nephritis. *Clin J Am Soc Nephrol* 2015; 10(1): 47-53.

7. Tiren-Verbeet NL, Van Weers EC, **Wilhelmus S**, Von dem Borne PA. Casuspresentatie beenmergnecrose. *Ned Tijdschr Hematol* 2014; 11: 131-132.

8. Buurma AJ, Penning ME, Prins F, Schutte JM, Bruijn JA, **Wilhelmus S**, Rajakumar A, Bloemenkamp KW, Karumanchi SA, Baelde HJ. Preeclampsia is associated with the presence of transcriptionally active placental fragments in the maternal lung. *Hypertension* 2013; 62(3): 608-13.

Dankwoord

Sinds de start van mijn promotieonderzoek in 2008 zijn er veel mensen die, ieder op zijn of haar eigen manier, hebben bijgedragen aan het tot stand komen van dit proefschrift:

Jan Anthonie Bruijn

Ingeborg Bajema

Nepa's, met het risico mensen te missen: Aletta, Annelies, Annemieke, Antien, Arda, Céline, Chinar, Daan, Daphne, Diego, Emile, Emilie, Emma, Hanneke, Hans, Jamie, Junling, Kimberly, Klaas, Marian, Marije, Marion, Marlies, Nicole, Nina, Pascal, Ramzi, Rosanne

Pathologen en AIOS LUMC

Overige medewerkers afdeling Pathologie LUMC

Natasha Jordan

Ron Wolterbeek

Mijn vele co-auteurs

Afdeling Pathologie AMC

Eelco Roos en Wim van Est

De deelnemers aan het onderzoek beschreven in dit proefschrift

Paranimfen Lianne en Malu

De 'schaapjes'

Mijn (schoon)familie

Last, but not least: Auke en Emma, de liefdes van mijn leven

Bedankt! Zonder jullie was het niet gelukt.