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STUDIES ON LUPUS NEPHRITIS

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To Gustav, Joakim, Sofia and Peter

ABSTRACT

Systemic Lupus Erythematosus (SLE) is a chronic inflammatory autoimmune disease characterized by multiple organ involvement, production of autoantibodies to nuclear components and local formation or deposition of immune complexes in different organs. Lupus nephritis (LN) is a common and severe manifestation of SLE. A renal biopsy is the “gold standard” for diagnosis of LN and the basis for treatment strategies. However there is no consensus whether a repeat biopsy should be performed to define response to treatment. Biomarkers available for renal disease activity are insufficient and LN patients may have inflammatory lesions in renal tissue despite of clinical quiescent disease.

The aim of this thesis was to study clinical, laboratory and histopathological findings in LN-patients with repeated renal biopsies performed after immunosuppressive treatment. I aimed to investigate the role of second renal biopsies in evaluation of treatment response, and to identify novel biomarkers for renal disease activity. We also studied long-term outcome and predictors of response in a subset of patients with severe LN who were treated with B-cell depletion therapy (rituximab).

In paper I we studied renal expression and serum levels of High Mobility Group Box 1 protein (HMGB1), a nuclear protein that can act as a proinflammatory mediator and is proposed to be involved in multiple inflammatory diseases. We found high serum levels and increased expression in renal tissue of HMGB1 in LN at both active disease and after immunosuppressive treatment. The study indicates a role for HMGB1 in LN and also supports previous findings of persistent inflammation in the renal tissue despite treatment.

In paper II we compared clinical and histopathological findings in LN patients with repeated renal biopsies performed after induction immunosuppressive treatment. A substantial proportion of patients had persistent inflammatory lesions in renal tissue despite an apparent clinical good response. Repeated biopsies may thus add important information that is not captured by routine laboratory markers which in turn may have impact on long-term renal outcome.

In paper III we studied serum cytokines in association to clinical and histopathological response in LN. We found high baseline levels of interleukin (IL)-17 in patients with a poor histopathological outcome and high IL-23 in clinical non-responding patients. Immunostainings revealed increased expression of IL-17 in areas with inflammatory CD3+ T-cell infiltrates in renal tissue. The study indicates a Th-17 phenotype in a subset of patients with severe LN.

In paper IV we studied long-term (mean 36 months) renal outcome in 25 patients, with previously refractory or relapsing LN, who had been treated with rituximab (RTX). A majority of the patients achieved a complete remission. A long time of B-cell depletion was associated with a faster response. The study supports the use of RTX in patients with refractory LN.

In conclusion, repeated renal biopsies after induction treatment revealed persisting active nephritis in many patients despite clinically inactive disease. Consistently, HMGB1 was increased in renal tissue at both active disease and after treatment. A subset of patients with severe LN had high levels of Th-17 associated cytokines which may be of use as biomarkers. LN patients refractory to standard therapy had overall good response at long term follow-up after B-cell depleting therapy.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

- I. **Zickert A**, Palmblad K, Sundelin B, Chavan S, Tracey KJ, Bruchfeld A, Gunnarsson I. Renal expression and serum levels of high mobility group box 1 protein in lupus nephritis. *Arthritis Res Ther.* 2012 Feb 20;14(1):R36
- II. **Zickert A**, Sundelin B, Svenungsson E, Gunnarsson I. Role of early repeated renal biopsies in lupus nephritis. Submitted.
- III. **Zickert A**, Amoudruz P, Sundström Y, Rönnelid J, Malmström V, Gunnarsson I. IL-17 and IL-23 in lupus nephritis - association to histopathology and response to treatment. Submitted.
- IV. Jónsdóttir T, **Zickert A**, Sundelin B, Welin Henriksson E, van Vollenhoven R, Gunnarsson I. Long-term follow-up in Lupus nephritis patients treated with rituximab- clinical and histopathological response. *Rheumatology (Oxford).* 2013 Jan 3. (Epub ahead of print)

Related publication based on the patient cohort, not included in the thesis

Sjöwall C, **Zickert A**, Skogh T, Wetterö J, Gunnarsson I. Serum levels of autoantibodies against C-reactive protein correlate with renal disease activity and response to therapy in lupus nephritis. *Arthritis Res Ther.* 2009;11(6):R188.

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LIST OF ABBREVIATIONS

ACE	Angiotensin Converting Enzyme
ACR	American College of Rheumatology
ANA	Antinuclear Antibodies
ANCA	Anti-Neutrophil Cytoplasmic Antibodies
Anti-dsDNA	Antibodies to double stranded DNA
APC	Antigen Presenting Cell
ARB	Angiotensin-II Receptor Blocker
AZA	Azathioprin
BAFF	B-cell Activating Factor
BILAG	British Isles Lupus Assessment Group
CKD	Chronic Kidney Disease
C	Complement Component
CBA	Cytometric Bead Array
CR	Complete Response / complete responder
CRP	C-reactive protein
CS	Corticosteroids
CsA	Cyklosporin-A
CYC	Cyclophosphamide
CVD	Cardiovascular Disease
DAMP	Damage Associated Molecular Pattern
DC	Dendritic Cell
DNT-cells	Double Negative T-cells
ELNT	Euro-Lupus Nephritis Trial
ELISA	Electron-Linked Immunosorbent Assay
EM	Electron Microscopy
ESRD	End Stage Renal Disease
eGFR	Estimated Glomerular Filtration Rate
GWAS	Genome Wide Association Studies
HLA	Human Leukocyte Antigen
HMGB1	High Mobility Group Box protein 1
HNR	Histopathological Non-Response/ non-responder
HR	Histopathological Response/ responder
IC	Immune Complex
IFL	Immunofluorescence
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ISN/RPS	International Society of Nephrology/ Renal Pathology Society
LN	Lupus Nephritis
LUNAR	Lupus Nephritis Assessment with Rituximab study
MAC	Membrane-Attack Complex
MCP-1	Monocyte Chemoattractant Protein 1
MDRD	Modification of Diet in Renal Disease
MMF	Mycophenolate Mophetil

MN	Membranous Nephritis
NGAL	Neutrophil Gelatinase-Associated Lipocalin
NET	Neutrophil Extra Cellular Traps
NIH	National Institute of Health
NK	Natural Killer
NR	Non-Response / non-responder
PAMP	Pathogen Associated Molecular Pattern
pDC	Plasmacytoid Dendritic Cell
PN	Proliferative Nephritis
PR	Partial Response / partial responder
PRR	Pattern Recognition Receptors
RTX	Rituximab
SLE	Systemic Lupus Erythematosus
SLEDAI	Systemic Lupus Erythematosus Disease Activity Index
SLICC	Systemic Lupus International Collaborating Clinics
TCR	T-cell Receptor
TGF	Tissue Growth Factor
Th	T helper
TLR	Toll Like Receptor
TMA	Thrombotic Microangiopathy
TNF	Tumour Necrosis Factor
Treg	Regulatory T-cell
TWEAK	Tumour Necrosis Factor-like Weak Inducer of Apoptosis
Urine P/C	Urine protein/creatinine ratio
VCAM	Vascular Cell Adhesion Molecule
WHO	World Health Organization

1 SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

1.1 BACKGROUND

Systemic Lupus Erythematosus (SLE) is a chronic inflammatory autoimmune disease that can affect almost any organ. SLE is often considered as the prototype autoimmune disease as almost all components of the immune system contribute to the typical autoimmune findings and tissue pathology (1). The disease is characterized by production of autoantibodies to nuclear components, a diverse set of clinical manifestations and an unpredictable course.

SLE is predominantly a disease of young women with a peak incidence between the ages of 15-40 and a female: male ratio of 6-10:1. Clinical manifestations differ between individuals, with disease severity ranging from very mild to fulminant disease, and many organs may be involved. The organs most frequently affected are joints, skin, kidneys, serous membranes, the hematopoietic system, blood vessels and the central nervous system. General symptoms such as malaise, fatigue and fever are all common. Treatment recommendations depend on disease severity and comprise symptomatic, immunosuppressive and supportive therapy. Despite significant improved prognosis over the past decades, the mortality still exceeds that of the general population (2).

Production of autoantibodies against nuclear antigens (ANA) is the hallmark of SLE and is detected in > 95 % of the patients (3). Anti-double stranded DNA (anti-dsDNA) are among the most specific antibodies, present in 50-70% of lupus patients at some point of the disease, and tend to reflect disease activity in many cases (4). The etiopathogenesis for SLE, although not fully understood, comprise genetic, environmental and hormonal factors.

1.2 EPIDEMIOLOGY

1.2.1 Incidence and prevalence

SLE is a rather rare disease although incidence varies among different populations and also according to the time period studied as well as to changes in diagnostic criteria. The incidence in USA has been estimated to 2-7.6/100,000 persons per year (5). In Sweden, an annual incidence of 4.8 cases per 100,000 persons per year has been reported (6).

The prevalence of SLE differs but generally ranges from 20 to 70 per 100,000, and is reported to be 2 to 4 times more frequent among non-white populations around the world (7).

1.2.2 Mortality

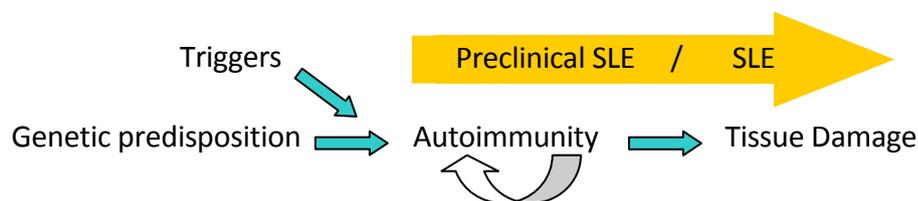
The prognosis for lupus patients has improved dramatically from an estimated 5-year survival of less than 50 % in the 1950s to a 5-year survival of 95 % and a 10-year survival of over 90 % today (8, 9). A bimodal pattern of mortality was described already 35 years ago. Deaths early in the course of the disease have been associated

with active lupus and severe infections whereas deaths late in the course of the disease are mainly due to complications from cardiovascular disease (10). The risk for deaths, primarily related to lupus activity has decreased over time, while the risk for deaths due to cardiovascular disease has not declined. The mortality rate in SLE is still higher than in the general population (11). However, a recent study from Brazil demonstrated that unlike in developed countries, renal failure and infectious diseases are still the most frequent causes of death (12).

Several risk factors for a worse prognosis have been identified including young age at diagnosis, African-American ethnicity, poor socioeconomic status and certain disease manifestations, in particular lupus nephritis (LN). Male gender has repeatedly been reported to be associated with poor prognosis, however data is conflicting and some studies found females to have worse outcome (8, 9, 11, 13).

1.3 ETHIOLOGY AND PATHOGENIC FACTORS

In recent years, advances in genetics and new insights of the molecular mechanisms that mediate immune system activation have identified key mechanisms in the pathogenesis in SLE. Although still not fully understood, the etiopathogenesis comprises genetic susceptibility, hormonal influence and environmental triggers. These factors act on the immune system, resulting in multiple abnormal immune responses and the development of autoimmunity. Autoantibodies and cytokines amplify the immune system activation, leading to a vicious circle which generate inflammation and tissue damage (1).



1.3.1 Genetics

The genetic contribution to the development of lupus is supported by observations of familial aggregation and by the increased concordance among twins (> 20 % among monozygotic and 2-5 % among dizygotic twins) (14, 15). In rare cases, SLE is associated with deficiency of a single gene (e.g.. the complement components C1q, C2 and C4) but more commonly, the combined effects of variations in a number of genes contribute to disease susceptibility.

Genetic variation as a part of the cause for lupus was first demonstrated in the 1970s with associations in the human leukocyte antigen (HLA) region. In recent years, genome wide association studies (GWAS), including large amounts of DNA samples from lupus patients and controls, have identified a substantial number of genes that predispose to lupus. Most of these genes are involved in key pathways for activation or regulation of immune responses (16, 17).

1.3.2 Hormones

A long-recognized observation is the marked female predominance in SLE, particularly among younger adults of which more than 90 % of patients are female (18). This suggests an important role for female hormones, but a protective role for male hormones or effects from genes on the X chromosome are also possible. The latter is supported by the fact that individuals with an extra X chromosome (Klinefelter's syndrome, XXY) have increased risk for development of SLE which has also been demonstrated in mouse models (19, 20).

Hormones may influence the immune system in many ways. In general, estrogens are believed to enhance at least the humoral immunity whereas androgens and progesterone may act as immuno-suppressants. Estrogen receptors are expressed on most immune cells and have been implicated in multiple immune responses (13, 21, 22). Oral contraceptives and hormonal replacement therapy have for long been avoided in lupus. However, studies on the safety of treatment with exogenous estrogens have shown conflicting results and it has been suggested that they can be used with caution in subgroups of patients with stable and non-severe disease (23).

1.3.3 Environmental factors

Ultraviolet radiation is the most obvious environmental factor that has been linked to SLE, and exposition to sunlight has been shown to trigger lupus flares. Other factors that have been implicated are cigarette smoking, infections (particularly Epstein-Barr virus, EBV), exposure to crystalline silica and dietary factors (24-26).

Certain medications can also induce a variant of SLE, drug-induced lupus (27). Drugs identified are, among many others, procainamid, hydralazin, sulphasalazine, antiviral agents and more recently TNF blockers (28).

Vitamin D is increasingly recognized to be involved in multiple autoimmune diseases, including SLE. Vitamin D has multiple effects on the immune system of which many are opposite to the immunological aberrations in patients with SLE. Vitamin D insufficiency is prevalent in patients with SLE and levels have been shown to correlate to disease activity. It has been suggested that vitamin D deficiency may be an environmental trigger for SLE. However low levels of vitamin D in SLE patients can be related to multiple factors such as avoidance of sunshine, renal insufficiency and an effect of medications and it is not clear whether low vitamin D has causative effects or is a result of the disease (29, 30).

1.4 IMMUNOPATHOLOGY

1.4.1 Overview of the immune system

The physiological function of the immune system is defense against foreign substances, particularly infectious microbes. A fundamental property of a healthy immune system is self-tolerance, i.e. to be unresponsive to self-antigens. However, dysfunction of the

complex system that preserves self-tolerance can occur, self-antigens are then recognized as foreign and immune responses that are normally protective leads to inflammation, tissue injury and the development of autoimmune disease. The immune system comprises different lines of defense; the first-line of defense is called the innate immune system and the later the adaptive immune system.

The innate immune system provides an early, first-line of defense against foreign microbes. Innate immune responses are fast but non-specific and the reactions are essentially the same in repeated infections, i.e. does not confer immunologic memory. The components of innate immunity are 1) the physiological and chemical barriers of epithelial surfaces (skin and mucous membranes); 2) phagocytic cells (macrophages, dendritic cells and neutrophils) and natural killer (NK) cells; 3) the complement system and 4) inflammatory mediators such as cytokines.

Innate immunity is activated mainly through the recognition of structures that are shared by multiple microbes, pathogen-associated molecular patterns (PAMPs), by pattern recognition receptors (PRRs), and does not distinguish fine differences between foreign substances. PRRs can also recognize endogenous damage-associated molecular patterns (DAMPs). Toll-like receptors (TLRS) are the most studied PRRs, which upon stimulation initiate multiple immune responses.

When activated, phagocytic cells release multiple inflammatory mediators (e.g. prostaglandins, NO and several cytokines) and the immune responses are amplified by recruitment of more immune cells, activation of the complement system and production of several molecules (e.g C-reactive protein CRP), all promoting rapid defense. The innate immune system also activates the adaptive immune system (31- 33) .

The adaptive immunity, also referred to as the acquired immunity, can recognize and react to a large number of microbes and molecules (antigens). The adaptive immunity is highly specific and can distinguish between even closely related molecules. Adaptive immunity is characterized by immunologic memory, i.e. repeated exposure to the same antigen leads to a more vigorous response. Adaptive immune responses require that antigens are captured and displayed by antigen-presenting cells (APCs), the most specialized are dendritic cells (DCs) which are regarded the major link between innate and adaptive immunity (31).

Adaptive immune responses comprise humoral immunity (the principal defense against extracellular pathogens) which is mediated by B-lymphocytes and antibodies, and cell-mediated immunity (defense against intracellular pathogens), mainly mediated by T-lymphocytes.

T-cells recognize peptide fragments displayed on APCs by specific receptors (that are structurally related to antibodies) expressed on the cell membrane, the T-cell receptor (TCR). T-cells are divided into two main subgroups, T-helper ($CD4^+$) and cytotoxic ($CD8^+$) cells. $CD8^+$ T-cells induce cell death of infected cells.

T-helper (Th) cells produce large amount of cytokines and, according to the profile of produced cytokines, are further divided into functional subsets i.e. Th-1, Th-2 and Th-17 cells. Th-1 cells produce primarily interferon (IFN)- γ and promote cell-mediated responses mainly by activating macrophages. Th-2 cells produce primarily IL-4 and are involved in humoral responses, including defense against parasites. Th-17 cells, a more

recently identified subset, produce primarily IL-17 and promote recruitment of neutrophils to inflammatory sites and are involved in defense against fungi (31, 34, 35). Specific forms of CD4⁺ cells, the regulatory T-cells (Tregs), have regulatory and immunosuppressive functions, mainly by suppressing self-reactive T-cells, and play a crucial role in maintaining peripheral tolerance.

B-cells are the only cells capable of producing antibodies. B-cells are derived from the bone marrow where they mature sequentially to immature B-cells that enter the blood and then migrate to secondary lymphoid organs. Some antigens can activate B-cells independently of T-cells, but the activation of B-cells to produce high affinity antibodies is dependent on help from CD4⁺T-cells. Upon activation, immature B-lymphocytes proliferate and differentiate via plasmablasts into antibody-producing plasma cells or to memory B-cells. Selection against self-reactive responses occurs at many stages during B-cell maturation. In addition to antibody production, B-cells are also involved in immune regulation by their ability to act as APCs and by cytokine production (32, 36).

1.4.2 The immune system in SLE

The immune dysregulation in SLE is characterized by multiple abnormalities involving both the innate and adaptive immunity, and affecting both humoral and cell-mediated immune responses. The complexity of the immune aberrations in SLE allows only a brief overview to be included in this text.

Impaired clearance of remnants from dead and dying cells is considered a key event for loss of cell-tolerance and, thus, for the pathogenesis of SLE (1, 37-39). Accelerated apoptosis or failure to clear apoptotic material may increase the amount of nuclear antigens presented to T-lymphocytes by APCs, leading to activation of self reactive B-cells and the generation of nuclear autoantibodies, a hallmark of SLE (Figure 1).

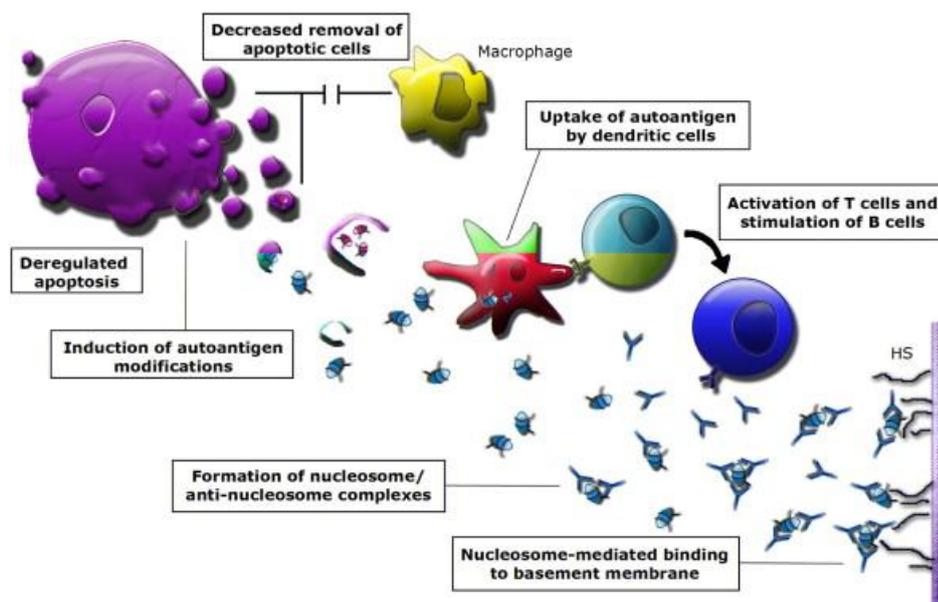


Figure 1 Hypothesis for the pathogenesis of SLE. Deregulated apoptosis and/or insufficient removal of apoptotic cells lead to the release of (modified) nuclear antigens into the circulation. This leads to the activation of APCs, a T cell-mediated autoimmune response, and the formation of pathogenic ICs. Adapted from Munoz et al (42) with permission from the publisher.

Dendritic cells (DCs) play a central role in the aberrant immune responses in SLE; they present self-antigen that activates auto-reactive T-cells, promote B-cell proliferation and release cytokines. Importantly, in response to nucleic acid-containing immune-complexes (ICs), plasmacytoid DCs (pDCs) produce large amounts of interferon (IFN)- α , a key cytokine in lupus. RNA or DNA containing ICs induce IFN- α production in pDCs by signaling mediated by TLRs (40, 41).

Another mechanism for activation of pDCs by endogenous DNA in SLE involves neutrophil extracellular traps (NETs). NETs arise from the death of neutrophils (NETosis) and contain nucleic-acid-complexes that can induce IFN- α production in pDCs (40).

1.4.2.1 The complement system

The complement system consists of a large number of proteins that interact in a regulated manner; the activation of one component activates the next in a cascade reaction. Three major pathways, the classical-, the mannose-binding lectin- and the alternative pathway activate the complement system. All pathways lead to formation of enzymatic complexes (C3 and C5 convertases), release of anaphylatoxins that attract white blood cells and formation of the membrane-attack complex (MAC). The classical pathway is mainly initiated by interaction of C1q with antibodies in ICs. The many biological functions of complement include opsonization to facilitate phagocytosis, clearance of ICs and dying cells, recruitment of inflammatory cells and cytolysis. The complement system has important protective functions but can also, when inappropriately activated, cause tissue damage (31, 43).

Support for complement involvement in the pathogenesis of SLE originates from observations of decreased serum levels of complement components in patients with active disease and findings of complement deposits in affected organs, such as skin and kidneys. Several functions of the complement system have been implicated in SLE, of which a major role has been addressed the clearance of ICs and apoptotic cells.

However the role for complement in SLE is dual; on one hand complements facilitate the clearance of ICs and apoptotic material, and thereby protect against autoimmunity, but on the other hand contribute to tissue damage when activated by ICs (44, 45).

Inherited deficiencies of complement components such as C1, C2 and C4 strongly predispose to the development of SLE (44). More than 90 % of individuals with genetic C1q deficiency develop SLE, which thus is the strongest genetic risk factor known for the disease. C1q may have several implications in lupus pathogenesis and was demonstrated to inhibit IC-induced production of IFN- α by pDCs (46).

Low serum concentrations of complement components such as C1q, C3 and C4 are typical findings in SLE, mainly during flares but sometimes also in quiescent disease.

1.4.2.2 Autoantibodies

Antibodies against self-antigens are the hallmark for SLE and may be present many years before clinical signs of the disease (47, 48). Antinuclear antibodies (ANA) are the

most characteristic and are present in > 95 % of lupus patients. However, ANAs are not specific and can be detected in a variety of autoimmune and infectious conditions and also in healthy individuals, especially among elderly (3).

Antibodies against double stranded DNA (anti-dsDNA) are most extensively studied and are considered to be involved in the pathogenesis (49). Anti-DNA are highly specific for lupus; present in 50-70 % of patients but in less than 0.5 % of healthy individuals or patients with other autoimmune diseases (50).

Antibodies may induce tissue damage by formation of ICs leading to complement activation and subsequent influx of inflammatory mediators and also by direct complement-mediated lysis or Fc-receptor-mediated responses.

Among the large number of autoantibodies known, only a limited number are used in present clinical practise. Some are associated to specific disease manifestations; such as associations of anti-DNA-, anti-nucleosome-, anti-Sm- and anti-C1q antibodies to nephritis, of anti- Ro to skin disease and fetal heart problems and of anti-phospholipid antibodies to thrombosis and pregnancy loss (4, 17).

1.4.2.3 Lymphocyte abnormalities

When the tolerance is broken, auto-reactive T-cells and B-cells participate in the amplification and perpetuation of the autoimmune and inflammatory responses.

1.4.2.3.1 T-cells

T-cells play a central role in the pathogenesis of lupus; they regulate B-cell responses, produce cytokines and infiltrate target tissues, thus contributing to organ damage. The differentiation of CD4⁺ T-cells into effector T-cells depends on the cytokine milieu and co-stimulation of APCs. Briefly, IL-12 promotes differentiation to Th1, whereas IL-4 promotes development of Th2-cells. Th-1 induced cytokines are elevated in SLE and are considered to play a central role (51).

Th-17 cells differentiate from naive T-cells under the influence of IL-6, IL-1 and TGF- β and are dependant on IL-23 for their maintenance (34, 52). Th -17 cells produce mainly IL-17 but also other cytokines such as IL-21, IL-22 and IFN- γ . Differentiation to Th-17 cells occurs in a reciprocal manner with the development of Tregs, determined mainly by the presence of IL-6 (53).

SLE-patients have a disturbed balance among Th-cell subsets cells, as well as decreased or impaired Tregs, and recent reports suggest a central role for Th-17 cells. Increased numbers of IL-17 producing cells in peripheral blood and increased levels of IL-17 have been reported in both SLE-patients and lupus-prone mice (54-57).

Lupus patients have increased numbers of CD3⁺CD4⁻CD8⁻ T-cells (double negative T-cells, DNT) in peripheral blood and in T-cells infiltrates in target organs (kidneys), which produce cytokines such as IL-17 and IFN- γ (56, 58).

Additionally, follicular T-helper cells (T_{FH}), a recently described CD4⁺ subset induced by IL-6 and IL-21 and are important for B-cell help, are suggested to be involved in SLE pathogenesis (59).

1.4.2.3.2 B-cells

Loss of B-cell tolerance with auto-reactive B-cells that produce an array of antibodies is a central feature in SLE. Except their maturation into antibody-producing plasma cells, B-cells are involved in the pathogenesis of lupus by acting as APCs, thus regulating T-cell activity, and by cytokine production.

In SLE, B-cell lymphopenia, with altered frequencies of B-cell subsets in peripheral blood has repeatedly been reported; the absolute number of naive CD19+B-cells and memory B-cells are reduced, whereas circulating plasmablasts are expanded, especially at active disease (60). Later studies have reported a number of abnormalities among B-cell populations in peripheral blood of SLE-patients, comprising different stages of B-cell maturation including increased number of plasmacells (61, 62).

The causes for overactive B-cells are not fully understood, and could be due to defects in tolerance checkpoints as well as to stimulatory effects of an overactive inflammatory environment in SLE where e.g. high levels of B-cell-activating factor (BAFF) and high IFN- α levels are present.

1.4.2.4 Cytokines

Cytokines are proteins that function as signal molecules for communication between cells. Several cytokines, originating from both innate and adaptive immune cells, contribute to immune dysfunction and mediate inflammation and tissue damage in SLE.

Cytokines that have been implicated include IFN- α , IL-6, IL-1, tumour necrosis factor (TNF)- α , IFN- γ , IL-12, IL-18, IL-10, transforming growth factor (TGF)- β , IL-2, BAFF and more recently IL-17, IL-21 and IL-23 (63, 64).

IFN- α is mainly produced by plasmacytoid dendritic cells (pDCs) and its most prominent function is to mediate early immune responses to viral infections. IFN- α is a central cytokine in lupus. SLE patients have increased serum levels of IFN- α that have been demonstrated to correlate to both disease activity and severity. A majority of patients have an increased expression of IFN- α regulated genes in peripheral blood mononuclear cells (the interferon signature) (65).

IFN- α is involved in multiple innate and adaptive immune responses of importance in lupus. Together with many other effects, IFN- α promotes maturation and activation of DCs and stimulates the differentiation of B-cells to antibody producing plasma cells. The formation of nucleic acid-containing ICs induces further IFN- α production in pDCs, thus forming a vicious circle. Indeed, IFN- α treatment of non-autoimmune diseases may lead to autoantibody production and a lupus-like disease (65, 66).

IL-6 is produced by many cell types including monocytes, endothelial cells and T- and B- lymphocytes. It has a range of biological actions on various target cells, including the induction of acute-phase proteins, activation of macrophages and differentiation of B-cells and T-cells (towards a Th-17 phenotype) (64). IL-6 is elevated in serum of lupus patients and was demonstrated to correlate to disease activity (67).

Increasing evidence support a major role for **IL-17** and IL-17-producing cells in the pathogenesis of many autoimmune diseases including SLE. IL-17 (i.e. IL-17A) is the main cytokine from CD4⁺Th-17 cells but can also be produced by other cells such as CD4⁻CD8⁻ double-negative T (DNT) cells, T-cell receptor (TCR) $\gamma\delta$ -cells, NK-cells and neutrophils.

IL-17 has strong proinflammatory effects; IL-17 induces other cytokines such as IL-6, IL-1, TNF- α and IL-21 and promotes recruitment of inflammatory cells (neutrophils and monocytes) to the inflamed tissue and facilitates T-cell infiltration (66, 68, 69). IL-17, acting in synergy with BAFF also promotes proliferation of B-cells and their differentiation into antibody-producing plasmacells (70).

SLE-patients have increased serum levels of IL-17, which correlate to disease activity (55, 57), as well as an increased proportion of IL-17 producing cells (as described above in the section on T-cells). IL-17 producing T-cells have been detected in inflammatory infiltrates in LN, of which a majority were DNT cells (58).

IL-23 is produced by APCs and induces expansion and/or stabilisation of Th-17 cells (52) thus forming the **IL-23/IL-17 axis**. SLE patients have high serum levels of IL-23 (55, 57). An expansion of T-cells expressing both high IL-17 and IL-23-receptor was demonstrated in lupus-prone mice (71).

1.4.2.5 HMGB1

High-mobility group box 1 protein (HMGB1) is a nuclear DNA-binding protein found in all mammalian cell nuclei. Extracellular HMGB1 has been identified as a proinflammatory mediator and has been proposed to contribute to the pathogenesis of multiple inflammatory and autoimmune diseases, including SLE (72, 73).

HMGB1 can be actively secreted from activated immune cells such as macrophages, DCs and endothelial cells or passively released from any injured or necrotic cells. When translocated from the nucleus to the extracellular milieu, HMGB1 acts as an "alarmin" or a DAMP, a danger signal that can activate the immune system and has been demonstrated as a key factor in necrosis-induced inflammation (74). HMGB1 can induce other cytokines such as TNF- α , IL-1 and IL-6 (75), and has additionally multiple effects on the immune system (such as differentiation and proliferation of immune cells, angiogenesis and chemotaxis) and can act on its own or by forming complexes with cytokines or other molecules (eg IL-1, nukleosomes and DNA) (76).

Elevated serum levels of HMGB1 have been demonstrated in SLE and correlate to disease activity in some studies (77-81). In cutaneous lupus, increased amounts of cytoplasmatic and extracellular HMGB1 have been detected, the most pronounced expression found in clinically active skin lesions (82).

An association to renal disease has also been suggested. Recently, high serum levels of HMGB1 in SLE were shown to associate to the presence of renal involvement (79). High serum levels as well as a pronounced expression of HMGB1 in renal tissue have been demonstrated in LN-patients, (80), and was also recently found in the urine from patient with active LN (83).

Several biological properties of HMGB1 may be of importance in lupus. Inappropriate apoptosis is regarded a key event in the pathogenesis of SLE (84) (discussed earlier in this text). In primary apoptosis, HMGB1 is tightly attached to chromatin and there is almost no release of HMGB1. However, if apoptotic cells are not properly cleared, they may undergo secondary necrosis leading to release of HMGB1 in complex with nucleosomes (85). Circulating HMGB1-nucleosome complexes have been detected in serum from SLE-patients (86). HMGB1-nucleosome complexes from apoptotic cells can induce the production of proinflammatory cytokines from macrophages and DCs (IL-6, IL-1, IL-10 and TNF- α), maturation of DCs as well as anti-dsDNA production *in vitro*. In addition, when these complexes were incubated with anti-dsDNA antibodies they induced IFN- α production from pDCs (86). Another study also reported that IC-induced secretion of IFN- α by pDCs was dependent on the presence of HMGB1 (87). Altogether this indicates a role for HMGB1 in breaking tolerance against nuclear antigens.

Recently it was also shown that NETs, released during NETosis, contain HMGB1 (88).

Antibodies against HMGB1 have also been detected in SLE-patients and may correlate to disease activity (79, 89, 90). Importantly, serum components as well as the presence of antibodies can interfere with methods for detection of HMGB1 (i.e. ELISAs) (89).

1.5 CLINICAL FEATURES

SLE is a highly heterogeneous disorder. The organs most frequently affected are joints (arthritis/arthritis), skin (photosensitivity and rash), kidneys (glomerulonephritis), serous membranes (pleuritis, pericarditis and peritonitis), blood and blood vessels (anaemia, leucopenia, thrombocytopenia, vasculitis and thrombo-occlusive manifestations) and multiple neurologic- and neuropsychiatric manifestations. Three main patterns of SLE disease activity have been described: relapsing-remitting, chronic active and long quiescent disease (91). The chronic active form was the most frequent and it was suggested that significant morbidity in lupus is derived from the persistent disease activity.

1.6 CLASSIFICATION CRITERIA

Criteria for classification of SLE were first published in 1971 and revised by Tan *et al* in 1982 (92) (Table 1). The 1982 American College of Rheumatology (ACR) classification criteria for SLE were developed to provide precise definitions of SLE, mainly for research purpose, and reflect the major clinical features and laboratory findings. They include 11 (9 clinical and 2 immunological) criteria, of which at least 4 must be present for diagnosis of SLE. A modification was made 1997, in which positive LE cells were deleted and positive antiphospholipid (APL) antibodies were added to the criteria (93), however the 1982 criteria by Tan *et al* have to date been the most commonly used.

Table 1. The 1982 ACR classification criteria for SLE

	Criterion	Definition
1	Malar rash	Fixed erythema, flat or raised, over the malar eminences
2	Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur
3	Photosensitivity	Exposure to ultraviolet light causes rash
4	Oral ulcers	Includes oral and nasopharyngeal, observed by a physician
5	Arthritis	Non-erosive arthritis, involving two or more peripheral joints, characterized by tenderness, swelling or effusion
6	Serositis	Pleuritis or pericarditis
7	Renal disorder	Proteinuria > 0.5 g or 3+ on dipstick or cellular casts
8	Neurologic disorder	Seizures or psychosis without other causes
9	Hematologic disorder	Hemolytic anemia or leucopenia (<4 x10 ⁹ /l) or lymphopenia (<1.5 x10 ⁹ /l) or thrombocytopenia (<1 x10 ⁹ /l) in the absence of offending drugs
10	Immunologic disorder	Positive LE cell preparation or anti-dsDNA or anti-Sm or false positive VLDR
11	Anti-nuclear antibody	An abnormal titer of ANA by immunofluorescence or an equivalent or assay at any point in time in the absence of drugs known to induce ANAs.

Recently, new classification criteria were proposed by the Systemic Lupus International Collaborating Clinics (SLICC) group (94). A set of 17 criteria (11 clinical and 6 immunological) was formulated in order to be more clinical relevant and comprise new immunological knowledge. At least 4 criteria, including at least one clinical and one immunologic criterion, must be met for the diagnosis of SLE according to the SLICC criteria. However in case of biopsy proven LN, the presence of ANA or anti-dsDNA is sufficient for diagnosis. Whether these new criteria will be generally used for research and/or clinical purpose is yet not known.

1.7 ESTIMATIONS OF DISEASE ACTIVITY AND DAMAGE

Several tools for assessment of disease activity in SLE have been developed. One of the most commonly used are SLEDAI (SLE Disease Activity Index), a global score reflecting the last 10 days (95).

The BILAG- (British Isles Lupus Assessment Group) index focuses on changes in disease activity over time and is based on the clinician's intention to treat (96, 97). BILAG is an ordinal scale that grades disease activity in nine organ systems (i.e. the constitutional, mucocutaneous, neuropsychiatric, musculoskeletal, cardio-respiratory, gastrointestinal, ophthalmic, renal and haematology domains). Disease activity is graded into five levels; Grade A= very active, B= moderate active, C= mild activity, D= no activity but previously affected and E= no current or previous disease activity.

The SLICC/ACR damage index reflects cumulative organ damage (98) and is also useful for predicting long-term prognosis. Renal damage has been demonstrated to be the most important predictor of mortality within the SLICC-index (99).

2 LUPUS NEPHRITIS (LN)

LN is one of the most common and most severe manifestations of SLE, affecting up to 60 % of patients at some point of the disease. The highest frequencies of renal involvement are found in juvenile onset-lupus patients (50-80%) as compared to less than 30 % in late-onset lupus (>50 years). In the SLE cohort at Karolinska University hospital, the prevalence of LN was found to be 42 % (Svenungsson, personal communication). Most patients develop nephritis early in their disease (within 5 years from diagnosis), especially among children and adolescents in whom renal disease is often a presenting feature of SLE. However, nephritis may occur any time during the course of the disease (100, 101).

A recent study on LN patients followed between 1975 and 2005 found that although the overall mortality has decreased, it remained stable over the last decade and the risk for end stage renal disease (ESRD) remained constant over the last 30 years (102). In addition, morbidity and mortality is higher among patients with LN compared to SLE patients overall (9, 11).

Although the treatment of LN has improved, not all patients respond to standard immunosuppressive treatment, 35 % have at least one renal relapse and 5-20 % develop ESRD after 10 years (103). In addition, treatment-related toxicity remains a concern.

2.1 CLINICAL FEATURES

The most characteristic feature of LN is proteinuria but the presentation of renal disease is variable, ranging from no symptoms (detected by renal biopsy, known as “silent nephritis”), to mild proteinuria, hematuria or active urinary sediment and to more severe proteinuria (nephrotic range > 3.5 g/day) or acute nephritic syndrome with progression to acute renal failure. Seldom, patients present with isolated renal insufficiency and hypertension as the initial manifestation (104).

In many cases, extra-renal manifestations (such as fatigue, fever or arthralgia) dominate the clinical presentation.

2.2 DEFINITION

The criteria for diagnosis of renal disorder according to the ACR-criteria (92) include the presence of: 1. Persistent proteinuria >0.5 g/day (or 3+ dipstick reaction for albumin) *or* 2. Cellular casts (red blood cell, hemoglobin, granular, tubular or mixed). In the recent proposed SLICC criteria renal disorder is defined as Urine protein-to-creatinine ratio (or 24-hour urine protein) representing 500 mg protein/24 hours or red blood cell casts (94).

2.3 PATHOLOGICAL MECHANISMS

Despite intense investigation, the pathogenesis and mechanisms underlying renal injury are not completely understood and, as for SLE in general, involves almost all components of the immune system.

Glomerular immune complexes (ICs) are considered the primary mediators for LN and initiate multiple immune responses in both innate and adaptive immune cells. Moreover, renal infiltration by T-cells, macrophages and dendritic cells and an array of cytokines contribute to the progression of renal damage.

2.3.1 Autoantibodies and immune-complex formation

The presence of antibodies is required for development of LN, of which anti-dsDNA (and anti-nucleosome) antibodies have been most closely linked to the pathogenesis. A pathogenic role for anti-dsDNA is suggested by the correlation of serum antibody levels with nephritis, rising titres often associated to increased disease activity and the presence of anti-dsDNA antibodies in glomerular immune deposits in humans and mice with active nephritis (45).

In addition, autoantibodies with multiple specificities were reported in a study on post-mortem renal tissue and include DNA, chromatin, histone, SSA, SSB, C1q and Sm, all of which may be of importance (105).

There are three proposed mechanisms for formation of glomerular ICs, all of which may contribute:

- 1) deposition of preformed circulating ICs
- 2) cross-reactivity with *in situ* renal antigens (such as laminin and α -actinin)
- 3) direct binding to nucleosomes on the glomerular basement membrane (either circulating nucleosomes that deposit in the kidney due to inappropriate apoptosis, or nucleosomes originating from injured glomerular cells).

Most evidence supports the two latter mechanisms (45, 106). ICs activate immune pathways by activation of Fc-receptors and TLRs and/or by activating the complement cascade, leading to the release of cytokines and influx of inflammatory cells. Inflammatory cells as well as intrinsic glomerular cells contribute to and mediate tissue damage in LN (106).

Despite the role for anti-dsDNA antibodies, not all anti-dsDNA seem to be pathogenic. Not all patients with persistently high anti-dsDNA levels develop LN and only some anti-dsDNA antibodies deposit in the kidney and cause nephritis when passively transferred to mice (107-108). Recent evidence support the findings that glomerular deposition (and pathogenicity) of anti-dsDNA in LN is mediated by their binding to nucleosomes deposited on the glomerular basement membrane. Anti-nucleosome antibodies have been reported in up to 87 % of SLE-patients, have in some studies been shown to associate to renal disease and have been proposed as better markers for SLE than anti-dsDNA (109). A recent longitudinal study in LN-patients found that both anti-dsDNA and anti-nucleosome antibodies associated to renal disease activity (108).

Antibodies to C1q (anti-C1q) are detected in around 30–60 % SLE-patients and have in many studies been associated with renal involvement (110, 111). Anti-C1q antibodies are deposited in glomeruli, and have been shown to induce renal disease when bound to

C1q-containing ICs in the glomerulus (112). High titres of anti-C1q have been reported in association with high activity index on renal biopsies in active LN (113).

2.3.2 Cellular abnormalities and cytokines

In LN kidney biopsies the mononuclear cell infiltrates are mainly found in a periglomerular and peritubular interstitial distribution, and consist primarily of CD3 + T-cells, but some B-cells and macrophages are also found. Within the glomerulus, the monocytes/macrophages constitute the major cell type. Several studies have demonstrated a correlation between the amount of tubulointerstitial inflammation and the risk of renal failure (41,114).

T-cells are important in LN. They contribute to tissue injury by activation of antibody-producing B cells, recruitment of inflammatory cells and production of cytokines. Infiltrating T cells, including CD4⁺, CD8⁺ and IL17-producing CD4⁺CD8⁻ double-negative T cells express a wide array of cytokines (58, 115).

A number of studies have demonstrated IL-17 producing cells in the renal tissue from LN patients and in lupus mouse models (57-58, 71). DNT-cells infiltrate the kidneys of LN patients, produce high amounts of IL-17 and IFN- γ and have been suggested to be the major source for IL-17 in LN (58).

Chen *et al* found high serum levels of IL-6, IL-17 and IL-23 in LN patients as well as increased expression of these cytokines in glomeruli. Of interest, the amount of IL-17 and IL-23 correlated to renal activity index and was most pronounced among patients with LN class IV-G (57). In the study by Zhang *et al* "IL-23-treated lymphocytes" induced DNT cell proliferation, and when mice were injected with these lymphocytes, they developed nephritis (71). Another study found that IL-23 receptor deficiency prevented the development of nephritis in lupus prone mice (116).

Thus, increasing evidence supports a role for IL-17 producing cells in LN (117) but also in other non-lupus inflammatory renal diseases (118).

High serum levels and increased renal expressions also of Th-1 cytokines (mainly IL-18, IL-12 and IFN- γ) have repeatedly been reported in studies on LN (51). It is believed that Th1, Th 2 and Th-17- related cytokines may all be involved in different stages of the pathogenesis.

B-cells contribute to LN mainly by producing autoantibodies. Studies in lupus mouse models demonstrated infiltrating plasmacells in renal tissue and their numbers correlated with serum anti-dsDNA titers. These plasmacells secrete antibodies with various antigen specificities and may contribute to an *in situ* IC-production. (119). Plasmacells as well as germinal center-like structures with T-cell-B-cell aggregates found in kidneys from LN patients also suggest *in situ* secretion of antibodies in humans. Mice with B-cells unable to secrete antibodies can still develop mild nephritis, indicating that B-cell functions, such as antigen presentation and cytokine production also contribute to the pathogenesis (106).

Neutrophils, macrophages and DCs infiltrate the kidney and contribute to injury. Neutrophils are sources of NETs that are present in ICs deposited in kidneys from LN-patients (120), which may contribute to kidney injury through the release of autoantigens, activation of pDCs and production of IFN- α (88). DCs and macrophages produce inflammatory cytokines and recruit additional inflammatory cells and have been found in renal tissue from LN-patients (106).

2.4 DIAGNOSIS

2.4.1 Laboratory measurements

Clinically, LN is evaluated by urine analysis (dip-slide procedure and urine sediment), 24-hour urine protein-excretion or spot urine protein to creatinine (urine P/C) ratio, serum creatinine, serum albumin, anti-DNA titers and serum complement levels. Typical findings in patients with active LN include albuminuria, leucocyturia, hematuria and granular-, red blood-cell- or hyaline casts as well as rising levels of anti-dsDNA, low complement levels and low serum albumin in many cases (100).

2.4.1.1 Proteinuria

The standard (and most accurate) method for quantifying of urine protein is obtained by 24-h urine collection. However this is an impractical method and inadequate collections are common which have lead to the more frequent use of spot urine measurements (121). Good correlations between the spot urine P/C ratio and 24-h urine protein have been demonstrated across a wide range of proteinuria in LN, thus supporting its use in screening and monitoring in LN-patients (122).

A review of the ACR criteria for definition of LN recommended that spot urine P/C > 0.5 may be replaced for the 24 h protein measurement (123). However, it may be optimal to assess the exact amount of proteinuria to confirm the validity of the spot P/C method in individual patients using a standard 24-h collection (100).

2.4.1.2 Estimation of renal function

Renal function is most often assessed by the level of serum creatinine as a surrogate marker for glomerular filtration rate (GFR), although factors such as age, body composition, gender, ethnicity and diet can influence serum creatinine levels and therefore its accuracy. Methods measuring clearance of exogenous markers (such as iohexol) are the “gold standard” for defining GFR but these methods are often too difficult and expensive for routine clinical practice (124).

A number of equations including creatinine and demographic data have been formulated to better estimate GFR than creatinine alone, of which one of the most used is the Modification of Diet in Renal Disease (MDRD) formula (125, 126). Estimated GFR (eGFR) by MDRD is calculated by information of creatinine, gender, age and race (i.e. black or not) and provides accurate estimates of GFR when < 60 ml/min/1.73 m³, but is less reliable in patients with GFR>60 (127, 128). Serum cystatin C may provide a better filtration marker than creatinine, especially at higher levels of GFR (127).

2.4.2 The role of renal biopsy

A renal biopsy is the “gold standard” for diagnosis of LN, is necessary for classification and is the basis for treatment strategy decisions (129-131). The biopsy findings are of importance to determine activity and chronicity for the disease and may also provide prognostic information. In addition, it is important to rule out non-lupus renal diseases that may mimic LN (132).

Several studies have demonstrated that clinical and laboratory findings cannot accurately predict histopathology and thus, the threshold for performing a biopsy on suspicion of renal involvement should be low. Recent recommendations propose that a renal biopsy should be performed in case of persistent proteinuria > 0.5 g/day especially with hematuria and/or cellular casts, and should also be considered in cases of persisting isolated hematuria or leucocyturia (after exclusion of other causes, such as infection) or in occurrence of unexplained renal insufficiency with normal urinary findings (130).

The biopsy should ideally include at least 10 glomeruli and should be examined with light microscopy, immunofluorescence (IFL) and, if possible, electron microscopy (EM) in order to diagnose and classify the renal disease. Immunofluorescence evaluation for immunoglobulin (Ig) and complement (C) deposits normally includes IgG, IgA, IgM, C3 and C1q.

2.5 HISTOLOGICAL CLASSIFICATION

LN comprises several patterns of renal disease, including glomerular, tubulointerstitial and vascular pathology. Specifically, the term LN should be used for immune complex-mediated renal injury (133). Although all compartments of the kidney can be affected in LN, the classifications of LN have all been based on the glomerular lesions.

The first classification for the different forms of LN was formulated 1974 and later revised 1982 and 1995 (134) (Table 2).

The 1982 World Health Organization (WHO) morphologic classification of lupus nephritis includes classification of mesangial, proliferative and membranous LN and separates the focal proliferative segmental lesions of LN class III from the global diffuse form (class IV).

The 1995 revised WHO classification and the most recent International Society of Nephrology/ Renal Pathology Society (ISN/RPS) classification separates proliferative LN class III and IV depending on how many glomeruli are affected (< 50 % in class III and >50 % in class IV) (135).

The ISN/RPS classification further subdivides class IV LN depending on if the affected glomeruli have mainly segmental (class IV-S) vs. global lesions (IV-G) (Table 3).

Findings of acute or chronic lesions or both are indicated as A, C or A/C. The presence of tubular atrophy, interstitial inflammation and fibrosis, and arteriosclerosis or other vascular lesions should be indicated and graded (mild, moderate, severe) (135).

The ISN/RPS classification system is now the most used and is generally recommended (130, 131). The inter-observer reproducibility compared to the WHO classification have

improved (136). Major changes from the WHO classification include the elimination of the normal biopsy category (WHO I) and the subcategories of membranous Class V, clear distinction between the classes based upon the amount of glomeruli affected and the subdivision of LN class IV into IV-S and IV-G. In addition, sclerotic glomeruli owing to scarred LN are taken into account when assessing the percentage of glomeruli affected (136).

The subdivision of LN class IV S and G has been controversial; it was introduced due to findings that biopsies with predominantly segmental lesions had more fibrinoid necrosis but less immune deposits compared to global lesions (suggesting other pathogenetic mechanisms), and was associated with poor prognosis (137). Several later studies could not identify a significantly worse outcome in IV-S than IV-G (133, 138). However, many studies have proposed that the segmental lesions reflect pathogenetic mechanisms that are distinct from the global lesions, and may instead have similar mechanisms as anti-neutrophil cytoplasmic antibody (ANCA)-positive vasculitis associated glomerulonephritis (137, 139, 140).

Table 2. The 1995 World Health Organization Morphologic Classification of Lupus Nephritis (134)

Class	Definition
Class I	Normal glomeruli (a) normal (by all techniques) (b) normal by light microscopy, but deposits by electron or immunofluorescence microscopy
Class II	Pure mesangial alterations (mesangiopathy) (a) mesangial widening and/or mild hypercellularity (b) moderate hypercellularity
Class III	Focal segmental mesangiocapillary proliferative glomerulonephritis (<50 % glomeruli) associated with mild or moderate mesangial alterations and/or segmental subendothelial deposits (a) with 'active' necrotizing lesions (b) with 'active' and sclerosing lesions (c) with sclerosing lesions
Class IV	Diffuse proliferative severe mesangial/mesangiocapillary proliferative glomerulonephritis with extensive subendothelial deposits Mesangial deposits always present and frequently subepithelial deposits (a) with segmental lesions (b) with 'active' necrotizing lesions (c) with 'active' and sclerosing lesions (d) with sclerosing lesions
Class V	Diffuse membranous glomerulonephritis (a) pure membranous glomerulonephritis (b) associated with lesions of category II (a or b)
Class VI	Advanced sclerosing glomerulonephritis

Table 3. International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification of lupus nephritis (135)

Class	Definition
Class I	Minimal mesangial lupus nephritis Normal glomeruli by light microscopy, but mesangial immune deposits by immunofluorescence
Class II	Mesangial proliferative lupus nephritis Purely mesangial hypercellularity of any degree or mesangial matrix expansion by light microscopy, with mesangial immune deposits A few isolated subepithelial or subendothelial deposits may be visible by immunofluorescence or electron microscopy, but not by light microscopy
Class III Class III (A) Class III (A/C) Class III (C)	Focal lupus nephritis Active or inactive focal, segmental or global endo- or extracapillary GN involving <50% of all glomeruli, typically with focal subendothelial immune deposits, with or without mesangial alterations Active lesions: focal proliferative LN Active and chronic lesions: focal proliferative and sclerosing LN Chronic inactive lesions with glomerular scars: focal sclerosing LN
Class IV Class IV-S (A) Class IV-G (A) Class IV-S (C) Class IV-G (A/C) Class IV-S (C) Class IV-G (C)	Diffuse lupus nephritis Active or inactive diffuse, segmental or global endo- or extracapillary GN involving >50% of all glomeruli, typically with diffuse subendothelial immune deposits, with or without mesangial alterations. This class is divided into diffuse segmental (IV-S) LN when >50% of the involved glomeruli have segmental lesions, and diffuse global (IV-G) LN when >50% of the involved glomeruli have global lesions. Segmental is defined as a glomerular lesion that involves less than half of the glomerular tuft. This class includes cases with diffuse wire loop deposits but with little or no glomerular proliferation Active lesions: diffuse segmental proliferative LN Active lesions: diffuse global proliferative LN Active and chronic lesions: diffuse segmental proliferative and sclerosing LN Active and chronic lesions: diffuse global proliferative and sclerosing LN Chronic inactive lesions with scars: diffuse segmental sclerosing LN Chronic inactive lesions with scars: diffuse global sclerosing LN
Class V	Membranous lupus nephritis Global or segmental subepithelial immune deposits or their morphologic sequelae by light microscopy and by immunofluorescence or electron microscopy, with or without mesangial alterations Class V LN may occur in combination with class III or IV in which case both will be diagnosed Class V LN may show advanced sclerosis
Class VI	Advanced sclerotic lupus nephritis >90% of glomeruli globally sclerosed without residual activity

Indicate and grade (mild, moderate, severe) tubular atrophy, interstitial inflammation and fibrosis, severity of arteriosclerosis or other vascular lesions. GN=Glomerulonephritis, LN= lupus nephritis

2.5.1.1 Activity and chronicity

A scoring system with quantitative information regarding acute and chronic lesions are often added to the WHO and ISN/RPS classifications, of which the most commonly used is the index system described by Austin *et al* (141) (Table 4).

The histopathological findings, including information on active and chronic lesions, may provide prognostic information. High chronicity items score have been reported to be predictive of poor renal outcome (142). In a large study by Contreras *et al*, both high activity and chronicity scores were associated to poor prognosis (143)

Table 4. Activity and chronicity indices

Activity index	Chronicity index
Glomerular abnormalities 1. Cellular proliferation 2. Fibrinoid necrosis or karyorrhexis ^x 3. Cellular crescents ^x 4. Hyaline thrombi or wire loops 5. Leukocyte infiltration	1. Glomerular sclerosis 2. Fibrous crescents
Tubulointerstitial abnormalities 1. Mononuclear cell infiltration	1. Interstitial fibrosis 2. Tubular atrophy

Each item is graded as 0, 1, 2 or 3 (absent, mild, moderate, severe). ^x Fibrinoid necrosis and cellular crescents are weighted by factor of 2. Maximum score is 24 for activity and 12 for chronicity index.

2.5.2 Mesangial LN (class I and II)

In class I and II, IgG, IgM, IgA C3 and/or C1q staining (detected by IFL), and immune deposits (EM), are located in the mesangium only. Although small subepithelial or subendothelial deposits may be identified by IFL/EM, no endocapillary proliferation, necrosis, or crescents should be present. The tubulointerstitial and vascular compartments are generally preserved.

Clinically, most patients with class I-II lesions present with asymptomatic proteinuria (subnephrotic range) and/or asymptomatic hematuria with normal renal function, and these classes are rarely associated with renal dysfunction (144).

2.5.3 Proliferative LN (class III and IV)

Proliferative LN (PN) comprises the proliferative glomerular lesions seen in class III and IV LN, most often accompanied by tubulointerstitial and vascular lesions. Although it has been suggested that different pathological mechanisms are involved in class III vs. class IV LN, they generally share clinical features, natural histories and responses to therapy (145). The typical findings are subendothelial immune deposits, with or without mesangial alterations, and a variety of acute and/or chronic lesions. The lesions may involve < 50 % of all glomeruli as in class III or >50 % as in class IV, and

may be global (IV-G) or segmental (IV-S), involving > 50 % or < 50 % of the glomerular tuft respectively).

In PN, endocapillar proliferation, with thickening of glomerular capillary wall and increased number of endothelial- and mesangial cells and infiltrating monocytes causes narrowing of the glomerular capillary lumina. Acute lesions include crescents, necrosis, subendothelial deposits seen by light microscopy (wireloops) and hyaline thrombi, whereas the chronic lesions comprise glomerular sclerosis and fibrous crescents. Typical IFL pattern includes varying amounts of IgG, IgM, IgA C3 and C1q in the mesangium and in the peripheral capillary loops, representing subendothelial and possible subepithelial deposits. Especially in class IV, active interstitial inflammation and vascular lesions are often present (144).

Clinically, PN is the most common severe form of LN, which typically presents with hematuria, active urinary sediment, varying degrees of proteinuria and sometimes with impairment of renal function.

2.5.4 Membranous LN (class V)

Membranous LN (MN) is defined by subepithelial immune deposits (with or without mesangial deposits and proliferation) in > 50 % of the glomeruli. The glomerular basement membranes may appear normal in early stages, but is often diffusely thickened and in advanced forms, double contours of the capillary wall may be seen due to intramembranous deposits. IFL reveals Ig, C3 and C1q staining of the capillary walls and typical subepithelial immune deposits are seen on EM. Immune deposits may also be seen directly by light microscopy as small spikes (144). The target of injury for the ICs in MN is thought to be the podocytes although less is known regarding the pathogenesis as compared to PN.

The clinical presentation of MN is characterized by moderate-severe proteinuria (often in the nephrotic range), usually normal renal function and frequently normal levels of complement and negative anti-dsDNA antibodies (146).

2.5.5 Vascular lesions

Several different vascular lesions may be present in LN. Thrombotic microangiopathy (TMA) may accompany all classes of LN and can involve capillaries, arterioles and arteries. Findings of TMA include fibrin thrombi, intima oedema and mesangiolysis and are often associated with the presence of anti-phospholipid antibodies.

Vascular immune deposits are common findings on IFL or EM, particularly in class IV. Seldom, massive intimal immune deposits cause non-inflammatory narrowing or occlusion of vascular lumens in small arteries or arterioles, referred to as lupus vasculopathy. Very rarely, a transmural necrotizing vasculitis with leukocyte infiltration of the vessel walls is found.

TMA, lupus vasculopathy and lupus vasculitis can be seen with or without immune complex-mediated glomerulonephritis and are all associated with an increased risk of poor renal outcome (133, 144).

SLE-patients with ANCA seropositivity and renal biopsy findings with features of both LN (classes II-V) and ANCA-associated necrotizing and crescent glomerulonephritis

have been reported. In these cases, there was extensive necrosis and crescent formation but only few subendothelial immune deposits (147).

2.6 TREATMENT

The type of treatment for LN depends on the severity of renal disorder. Patients with class I and II LN generally have a good prognosis and rarely require immunosuppressive treatment.

2.6.1 Treatment of class III and IV LN

The goals for LN treatment are 1) to achieve prompt renal remission; 2) to avoid renal flares; 3) to preserve renal function; and to fulfill treatment with minimal toxicity (100).

The treatment usually consists of a period of 3-6 months of intensive therapy with the aim to induce a fast and clinically meaningful response: the *induction phase*. Induction therapy normally involves the use of intravenous (iv) and/or oral corticosteroids (CSs) in combination with either iv cyclophosphamide (CYC) or oral mycophenolate mophetil (MMF). This is followed by a period of less intensive therapy with the aim to keep the patient free from disease activity: the *maintenance phase*. Maintenance therapy usually involves oral CSs at low dosages in combination with MMF or azathioprine (AZA) to maintain response (129).

2.6.1.1 Induction treatment

An early response to induction treatment has been shown to predict good long-term outcome in LN-patients (148). Overall, CYC and MMF in combination with CSs are considered equivalent for induction treatment (131, 149), but factors such as ethnicity, age, severity of clinical presentation, co-morbidities, pregnancy plans and doubts about compliance should be taken into account for therapy decisions (150).

High dose iv CYC according to the National Institute of Health (NIH) regimen consists of CYC, given as monthly iv pulses for 6 months, with a start dose of 0.75-1 g/m² and with increasing doses to reach a white blood cell count 1.5-4 x 10⁹/l. This treatment strategy was proven superior to oral or iv CSs alone in preserving renal function and, when followed by a maintenance therapy with quarterly high dose iv CYC, in preventing renal flares (151, 152). An important finding from the NIH-trials was that the superiority of CYC vs. CSs in avoiding ESRD was observed first after 5 years (and was significant after 10 years). Thus, a long-term follow-up is needed to show significant differences between different treatment strategies.

The NIH-regimen was the standard of care for many years, although associated with many side effects such as risk for severe infections and premature ovarian failure (103). Later studies found that the adding of quarterly high dose CYC for two more years after induction was associated with significantly more side-effects and not more effective than using AZA or MMF for maintenance therapy (153) The prolonged CYC treatment is not generally recommended although the NIH-regimen (with 6 monthly pulses) may still be considered in some patients with severe disease (131).

Low dose iv CYC regimen according to the Euro-Lupus Nephritis Trial (ELNT) was developed in order to minimize the toxicity of the drug. The Euro-lupus regimen consists of iv CYC, given as a fixed dose of 500 mg every 2 weeks for three months (total six pulses) followed by maintenance therapy with AZA. A controlled randomized trial on biopsy proven PN comparing this low dose CYC regimen with the NIH-protocol found no difference in efficacy between the two regimens after a median of 41 months, but severe infections were less common in the low dose group (154). Long-term follow-up of the ELNT found no differences in renal outcome after 10 years between the low vs. the high CYC dose regimens (148). The Euro-lupus regimen has largely replaced the NIH-protocol for LN-induction treatment and is today generally recommended (130, 131).

MMF for induction was initially reported to be as effective as oral CYC in the first trial on 42 LN-patients (155). The efficacy of MMF in therapy resistant LN was also demonstrated in small studies (156). In a large randomized LN trial by Ginzler *et al*, MMF (1.5-3 g/day) was superior to iv CYC (NIH) at inducing complete renal remission at 6 months (22.5 % in the MMF-group vs. 5.8 % for CYC). This study consisted of 140 patients of which > 50 % were African-Americans, known to have severe disease, and >40 % had severe proteinuria (>3.5 g/day). Serious infections were less common in the MMF group (157). Chan *et al* reported equal efficacy after 5 years for MMF (used for both induction and maintenance therapy) and a regimen of CYC for induction followed by AZA for maintenance, with less severe infections in the MMF-group (158).

However, in a multiethnic trial on 370 LN-patients, the Aspreva Lupus Management Study (ALMS), MMF and CYC (NIH) were found to be equivalent at inducing renal response at 6 months (56 % in MMF vs. 53 % for iv CYC). The primary objective of the study, which was superiority of MMF over CYC, was thus not met. There were no significant differences in adverse events between the two treatment groups (159). Today, MMF is considered to be at least equivalent to CYC, although long-term data from large trials (10-year follow-up) are not yet available (150).

2.6.1.2 Maintenance treatment

In the study by Contreras *et al* 59 patients with severe LN were included. After induction therapy with iv CYC (NIH) and CSs, the patients were randomized to receive further iv CYC every 3 months, AZA or oral MMF. Both MMF and AZA were superior in efficacy than CYC and were associated with significantly lower incidence of severe infections, sustained amenorrhea and hospitalizations (153). After that study, AZA or MMF has been generally recommended for maintenance treatment.

Recently, two trials of maintenance therapy in LN have been completed. In the MAINTAIN nephritis trial, 105 patients with PN were included. After induction treatment with 6 pulses of low dose iv CYC (Euro-lupus regimen), all patients were randomized to either AZA or MMF. Time to renal flare (the primary end point) after a mean follow-up of 48 months did not differ between the two groups. Adverse events, except for transient cytopenias, which were more frequent in the AZA group did not differ either (160). Repeat renal biopsies, however performed only in 30/105 patients

after 2 years, did not differ in terms of activity or chronicity indices comparing the AZA and the MMF-group (161).

In the maintenance phase of the ALMS trial, 227 patients who had responded to the induction therapy of either CYC or MMF were re-randomized to 3 years of treatment with either MMF or AZA. MMF was significantly superior to AZA with respect to the primary endpoint which was time to treatment failure (defined as renal flare, sustained doubling of creatinine, initiation of rescue therapy, ESRD), regardless of the initial induction therapy. The incidence of adverse events, most common infections, was similar in the two groups, although withdrawals due to adverse events were more frequent in the AZA group (162).

The patient population and study designs of the two studies differ much, and are difficult to compare, and both AZA and MMF may be considered for maintenance therapy. There are currently no clear recommendations for how long time the maintenance therapy should be continued (131), but continuous immunosuppression for at least 5 years has been shown to be beneficial to prevent renal relapse (163).

2.6.2 Treatment of class V LN

Less evidence is available about treatment for MN (LN class V). MN often occurs in combination with class III or class IV LN, and in this case the proliferative lesions should guide therapy.

The therapy of pure class V is often guided by the grade of proteinuria. In patients with nephrotic range proteinuria (> 3.5 g/day), immunosuppressive therapy is usually added by combining CSs with CYC, MMF, AZA or cyclosporine A (CsA) (150).

There are few studies on pure class V LN. One study on 42 patients with pure class V LN compared oral CSs alone with oral CSs in combination with either oral CsA or iv CYC. Both CYC and CsA had better response rates than CSs alone after 1 year, but after 3 years the relapse rates were higher with CsA (164).

A retrospective analysis of 84 patients with pure class V LN found that MMF and iv CYC (NIH-regimen) were equally effective at 24 weeks (165). The Euro-lupus CYC regimen has not been evaluated in MN and, considering the toxicity of high dose CYC, recent recommendations support the use of MMF in pure MN (130-131).

AZA is also used in MN and has been reported to be effective and well tolerated in MN (166), although no randomized controlled studies have been performed.

2.6.3 New treatments

Despite improved treatment regimens, only 30–60 % of patients with PN respond to treatment with either MMF or CYC within 6 months, 55–80 % of patients respond within 12–24 months and 10–20% do not respond at all (167). Renal relapses are common and 5–20 % of patients with LN develop ESRD within 10 years (103). Accordingly, there is a need for improved treatment strategies in LN. Increased knowledge about the pathogenesis for SLE has resulted in development of new immuno-modulatory therapies, mainly by targeting B-cells.

B cells are involved in the pathogenesis of LN through their maturation into antibody-producing plasma cells, by the production of cytokines and also by acting as antigen-presenting cells (APCs) and activating T-cells. B-cell targeted therapy is thus an attractive therapeutic approach for LN (167). Important potential targets for B-cell therapies include cell surface molecules such as CD20 (rituximab) and CD22 (epratuzumab) and maturation and growth factors such as BAFF (belimumab) (168). Blocking co-stimulatory signals between APCs and T-cells with CTLA4 (abatacept), to thereby inhibit T-cell activation, is another target pathway that currently is being investigated (41)

In addition, biologic agents targeting many different cytokine pathways (including IFN- α , TNF- α , IFN- γ and IL-6) have been or are currently studied in SLE. Trials targeting the IL-23/IL-17 pathway are underway for several autoimmune diseases, but the role of these therapies in SLE remains to be studied (169).

2.6.3.1 Rituximab

Rituximab (RTX) is an IgG mouse–human chimeric monoclonal antibody directed against the CD20 cell surface receptor, which is expressed on both immature and mature B cells, but not on hematopoietic stem cells or plasma cells. Binding of RTX to CD20 results in cell lysis by antibody-dependent cellular cytotoxicity, complement activation or induction of apoptosis. Treatment with RTX thus eliminates peripheral B cells but spares plasma cells and stem cells (167).

In later years, B-cell depletion with RTX has shown promising results in many observational studies in LN (170-177). These studies, mainly focusing on patients with refractory LN patients not responding to conventional therapy, have overall reported significant improvement regarding proteinuria, renal function and serological findings. However, the study designs have differed regarding dosage of RTX, concomitant use of CSs and other immunosuppressive treatments, and also with respect to response criteria used.

Recently, a large study comprising pooled data of 164 LN patients from different centers was published (178). Complete response (CR) was defined as normal serum creatinine with inactive urinary sediment and 24-hour urinary albumin <0.5 g, and partial response (PR) as a >50 % improvement in all renal parameters that were abnormal at baseline, with no deterioration in any parameter. When renal response was evaluated at 12 months, 67 % had achieved a CR or PR, the best response was achieved in patients with mixed PN/MN and in class III. A GFR <60 ml/min/1.73 m² and nephrotic range proteinuria at baseline were associated with poor response.

Controlled clinical trials with RTX in SLE have been unsuccessful. The Lupus Nephritis Assessment with Rituximab (LUNAR) trial included 144 patients with LN class III and IV (179). The patients were randomized to RTX (1000 mg x2 at baseline and 6 months) or placebo in addition to MMF and initial high-dose CSs. The primary end-point, superior partial or complete renal response at 12 months did not differ between the groups (57 % in the RTX group vs. 46 % in the placebo group). However, significant improvements in C3, C4, and anti-dsDNA antibody levels were observed with RTX. Rates of serious adverse events, including infections, were similar in both groups.

A recent review of the use of RTX in LN summarized the results from open studies and case reports, including the effectiveness and safety for treatment of refractory and relapsing LN and found that 65–80 % of LN patients exhibit either a CR or PR to RTX. In studies with a follow-up period of more than 12 months, the median relapse rate was 20 % (167). Except for LN, RTX is also increasingly used in other glomerular diseases such as ANCA-associated vasculitis, mixed cryoglobulinemia and idiopathic membranous nephropathy (180).

Although the LUNAR trial failed, the positive results from the many uncontrolled studies support the use of RTX for the treatment of refractory or relapsing LN, also included in recent recommendations for the management of LN (130, 131).

2.6.4 Supportive treatment

In addition to immunosuppressive treatment, the role of global care for LN-patients is increasingly recognized. Angiotensin converting enzyme (ACE) inhibitors and/or angiotensin-II receptor blockers (ARBs) should be considered in all LN-patients to reduce proteinuria and protect renal function. Blood-pressure should be tightly controlled. Regular follow-up should include control of risk factors for cardiovascular disease (eg dyslipidemia and smoking) and other co-morbidities such as osteoporosis and infections (150). The use of hydroxychloroquine is associated with improved renal outcome (181), and both anti-platelet and/or hydroxychloroquine should be considered in LN-patients with anti-phospholipid-antibodies, although evidence from controlled studies is lacking (130).

2.7 DEFINITIONS OF RESPONSE

Several sets of criteria for definition of response in LN have been published, but none have been generally utilized which is further illustrated by the differences in response criteria used in clinical trials. All sets of criteria that have been published are based on the amount (or reduction) of proteinuria in combination with measures of renal function and often other urinary findings are also included

The ACR response criteria for LN define: 1) complete response (CR): $>50\%$ reduction in proteinuria to urine P/C to <0.2 mg/mg *and* $eGFR > 90$ ml/min/1.73 m² *and* inactive urinary sediment and 2) partial response (PR): $>50\%$ reduction in urine P/C to a value of 0.2-2 *and* 25 % increase in eGFR (if abnormal at baseline) *and* inactive urinary sediment (182).

In the more recent European consensus statement, response is defined as: 1) CR: proteinuria ≤ 0.2 g/day *and* normal (GFR > 90) or stable (within 10 % of normal GFR if previously abnormal) renal function *and* inactive urinary sediment and 2) PR: proteinuria ≤ 0.5 g/day *and* normal (GFR > 90 ml/min) or stable ($< 10\%$ deterioration from if previously abnormal) renal function *and* inactive urinary sediment (129).

The recent Joint European League Against Rheumatism and European Renal Association-European Dialysis and Transplant Association (EULAR/ERA-EDTA)

recommendations for LN define: 1) CR: urine P/C < 50 mg/mmol (or proteinuria < 0.5 g/24 h) and normal or near-normal (within 10 % of normal GFR if previously abnormal) GFR and 2) PR: \geq 50% reduction in proteinuria to subnephrotic levels and normal or near-normal GFR (130).

The clinical trials have all had different primary end-points, with different response criteria, and often different time points for evaluation of response. This was highlighted in a study by Wofsy *et al*, in which the effect of abatacept in a trial in LN-patients differed substantially depending on which response criteria were used (183). The early NIH- trial of iv CYC used progression to ESRD as its primary outcome measure (151). The ELNT on high vs. low dose CYC also used treatment failure as the primary outcome measure, the definition of failure was absence of primary response, occurrence of severe renal flare or doubling of serum creatinine (154). More recently, the trials of MMF and the LUNAR trial on RTX have focused on response rates instead of failure rates, but response was defined differently in each trial (157, 159, 179).

For very long-term follow-up studies on LN a composite endpoint of death and development of ESRD, and sometimes also including sustained doubling of serum creatinine, have been used (102, 148, 184-186).

For pure MN, few response definitions have been proposed. In the study on MN by Radhakrishnan *et al*, CR was defined as urine protein < 300 mg/day after 24 weeks, and PR was as urine protein < 3.5 g/day after 24 weeks and a 50% reduction from the baseline values (165). In the study by Austin *et al*, CR was defined as proteinuria < 0.3 g/day and PR as < 2.0 g/day with a > 50 % reduction from baseline (164).

Notably, there are no generally accepted definitions of histopathological response in LN and histological data has not been included in the criteria today available.

2.8 FLARES / RELAPSES

Renal flares are common, and have been reported to occur in 27-66 % in different studies (187). Incidence of flares varies due to the populations studied, the histological classes of LN included, the treatments given and definitions of renal flare.

Before the introduction of more routine long-term maintenance therapy, up to 50 % of patients with PN relapsed after stopping immunosuppressive therapy, but more recent studies have reported a decreased incidence of flares (188). In the two recent studies on maintenance therapy, the flare rate was reported to be 19-36 % (160, 162).

In the European consensus statement three types of flares were defined (129):

- 1. Proteinuric flare:** persistent increase in proteinuria > 0.5 g/day after a complete response has been achieved or a doubling of proteinuria > 1.0 g/day, after achieving a partial response.
- 2. Nephritic flare:** increase or recurrence of active urinary sediment (increased hematuria with or without reappearance of cellular casts) with or without a concomitant increase in proteinuria. Nephritic flares are usually associated with a decline in renal function.
- 3. Severe nephritic flare:** increase or recurrence of active urinary sediment with an increase > 25 % in serum creatinine.

2.9 MONITORING OF LN

2.9.1 Routine laboratory measurements

Monitoring of LN patients includes regular control of serum creatinine, serum albumin, proteinuria, urine analyses and levels of C3/C4 and anti-dsDNA antibodies (the interval for measurement depends on whether it is an active or previous nephritis) (131).

2.9.2 Biomarkers

Although conventional laboratory markers are useful in the clinical diagnosis of LN and for discrimination to other (non-lupus) renal disease, they are often insufficient for monitoring disease activity, including detection of renal flares, distinction between active disease and chronic damage and identification of patients at higher risk for long-term complications.

Many studies have focused on identifying novel biomarkers such as autoantibodies, serum cytokines and chemokines and urinary markers that better reflect renal disease activity however none have yet replaced conventional clinical parameters in clinical practise. It has been suggested that a panel of biomarkers should be used to provide the best specificity and sensitivity. Most reports on potential biomarkers are from cross-sectional studies and there are not much data on longitudinal measurements (189-191).

2.9.2.1 Antibodies

Anti-dsDNA antibodies are the classical biomarker of LN, although the sensitivity and specificity of anti-dsDNA for detecting renal disease activity varies among studies (around 50-70 % and 50-75% respectively) (189). In a large study on 228 LN patients the sensitivity of anti-dsDNA in detecting a renal flare was 70 % and specificity 67 % but the positive predictive value for flare was only 31 % (192). Although, it was suggested that anti-nucleosome antibodies might be a superior biomarker than anti-dsDNA, they correlate with each other and have similar frequencies in active LN in most studies (108, 190). One study reported high anti-dsDNA and anti-nucleosome antibodies in active LN patients, with decreasing levels after treatment, but with no rise in antibody titers preceding renal flares (193).

Anti-C1q antibodies associate with renal involvement in SLE, they correlate to renal disease activity (with often high specificity but varying sensitivity) in many studies, (111, 190, 194), and have been proposed as a biomarker for renal disease activity (110). In the study by Moroni *et al*, anti-C1q was slightly better than anti-DNA in terms of sensitivity and specificity, however 20% of patients who developed a renal flare had normal levels of anti-C1q, while 30% of patients in remission had persistent high levels of anti-C1q (192).

Overall, studies found that anti-C1q and anti-dsDNA/anti-nucleosome antibodies performed almost equally well and that both are useful for diagnosing LN, but are insufficient for monitoring disease activity (190, 193). A combined anti-C1q and anti-dsDNA antibody testing may increase both sensitivity and specificity for prediction of renal flares (195)

Recent studies have reported high serum levels of antibodies against CRP (anti-CRP) in SLE-patients, and these were shown to correlate with both renal disease activity (as determined by BILAG) and activity indices on renal biopsies. Anti-CRP positivity was also associated to poor response to treatment (196). Positive correlations between levels of anti-CRP antibodies and renal chronicity indices were also reported in LN (197).

2.9.2.2 Cytokines

Many studies have evaluated the association of cytokine markers with disease activity and organ manifestations in SLE. Studies have differed in their designs, by testing cytokines individually or in groups, in different lupus populations and in different samples (blood, urine and tissue biopsies) (198). For LN, association to disease activity and serum and/or urinary levels have been reported for cytokines such as IL-6, TNF- α , IL-18, IL-12, TGF- β , IL-17 and IL-23, of which some were associated with more severe LN or correlated to indices on renal biopsies (57, 198-200). Urinary levels of the cytokine tumour necrosis factor-like weak inducer of apoptosis (TWEAK) has been shown to associate to active LN, and a longitudinal study found the highest levels at the time-point for renal flare (201). However, longitudinal studies with repeated measurements of cytokines in association to renal biopsy findings and treatment responses have not been performed.

2.9.2.3 Urinary markers

Urinary substances have been proposed to reflect the actual renal tissue inflammation and damage better than markers in blood. Several urine biomarkers such as chemokines, cytokines and adhesion molecules have been identified. In addition, proteomic studies have identified a number of proteins that are associated with LN (41).

Several studies have identified urinary monocyte chemoattractant protein 1 (MCP-1) to associate with renal disease activity, with increasing levels prior to renal flare, and also to correlate to histopathological findings (189).

Neutrophil gelatinase-associated lipocalin (NGAL), a protein that is unregulated in response to renal injury, has also been identified as a promising biomarker for LN-activity. Studies have shown that urinary NGAL levels correlate with measures of renal disease activity, and rising levels predicted a renal flare (121). However, like MCP-1, NGAL is not specific for LN and is also increased in several other types of kidney injuries (189).

A recent study compared three different urinary markers, all of which can be involved in the inflammatory process in LN; MCP-1, the chemokine CXCL16 and vascular cell adhesion molecule 1 (VCAM-1). In that study, VCAM was most strongly associated with clinical active renal disease and was also associated with the activity index on renal biopsies (202).

Several other urinary biomarkers have also been identified for LN. However, none is generally available in clinical routine, despite MCP-1 being identified as a potential marker almost 20 years ago (198).

2.9.3 The role of repeat renal biopsies

Arguments have been raised that repeat biopsies should be performed to define response after immunosuppressive treatment; to thereby identify patients who may need prolonged or intensified therapy, but also to avoid overtreatment (203). Again, biomarkers currently available do not accurately reflect findings in renal tissue and, despite apparent good clinical response after immunosuppressive therapy, many LN patients still may have histopathological inflammatory activity (204).

However, a follow-up biopsy in the evaluation of response after induction treatment is seldom a routine procedure. In the consensus statement by Gordon *et al* it was proposed that a repeat renal biopsy may provide help to distinguish active LN from chronic damage, in both cases proteinuria and rising serum creatinine may be present (129)

Only a limited number of studies on the value of repeated biopsies in LN have been performed. In the early study by Esdaile *et al*, decreased amounts of mesangial or subendothelial deposits on repeat biopsies (performed after a median of 25 months) were found to be the best predictor for a lower risk for renal impairment and mortality (205). In a study on 71 LN-patients, persistent inflammatory lesions on repeated renal biopsies after 6 months of immunosuppressive treatment were better than clinical data alone to predict long-term renal outcome, whereas findings on the baseline biopsy did not predict prognosis (206). Interestingly, in the same patient cohort, chronic lesions on the baseline biopsy (segmental scarring and interstitial fibrosis) were partially reversible in a subgroup of patients, in whom a decline in chronic lesions on the repeat biopsy was observed. These patients had a more favourable long-term renal outcome (207). High chronicity indices on repeat biopsies have also been shown to predict a poor renal outcome (208).

A recent study on second kidney biopsies performed after 12-18 months demonstrated that histopathological evidence of active disease at repeat biopsy, regardless of clinical response, was predictive of poor renal survival after a median of 8.7 years (209).

In the MAINTAIN trial comparing maintenance therapy of MMF or AZA, repeat biopsies were performed after 2 years in 30 patients and revealed no differences in activity or chronicity indices between the two treatment groups. Of note, active lesions were still present in > 50 % of the repeat biopsies (161).

2.10 PROGNOSTIC FACTORS

Mortality rates have been reported to be increased in LN patients compared to patients with non-renal SLE. In a large study, the survival rate was 88 % vs. 94 % at 10 years in SLE patients with vs. without nephritis (9). The 10-years survival of patients included in the ELNT-study was 92 % (148). The 5-year survival was shown to be 95 % for patients followed 1996-2005, as compared to 83 % during 1975-1985 (102).

The risk for ESRD at 10 years varies in different studies between 5-20 %. In the ELNT 13 % had a sustained doubling of serum creatinine, including 7% of patients with ESRD at 10 years. In the study by Croca *et al* 8 % had developed ESRD after 5 years (with no decline over a 30-year period). In the study by Contreras *at al* 23 % of patients developed doubling of creatinine or ESRD after a mean follow-up of 37 months (143).

A number of demographic, socio-economic, genetic, clinical and histological factors have been described to influence prognosis in LN. Numerous studies have reported higher incidence of nephritis and more severe disease among African-, Asian- and Hispanic Americans vs. Caucasians (101, 210).

Factors for a poor prognosis include: *demographic* factors (male gender, African ethnicity, Hispanic ethnicity, young age at diagnosis and poor socio-economic status) *serological findings* (anti-dsDNA and antiphospholipid antibodies), *histopathological factors* (crescents and fibrinoid necrosis, high chronicity and activity indices, interstitial inflammation/fibrosis and vascular lesions) and several *clinical findings* (high creatinine, nephrotic syndrome, failure of initial response to treatment, persistent hypertension and low complement). In addition delay in treatment, short time of maintenance therapy, renal flares and, importantly, poor compliance are all associated with poor outcome and many of the prognostic risk factors interplay (143, 211).

Although MN is generally reported to have better prognosis than PN, a proportion of patients still develop an impaired renal function. In a study on 103 MN patients, the renal survival without development of CKD (GFR <60 ml/min) was around 86 % after 10 years, and 7.5 % patients with pure MN had developed ESRD after 15 years. Negative prognostic factors included high creatinine, high chronicity index, failure to achieve remission and renal flares (185).

3 AIMS

Overall aim

The overall aim of the studies was to investigate clinical and histopathological findings in LN patients, both at active disease and after immunosuppressive treatment, and to evaluate the role of novel inflammatory markers in association to renal response.

Specific aims

To investigate response to treatment in LN, as well as predictors of renal response by studying both routine and novel biomarkers in association to short and long term renal outcome (**paper I-IV**).

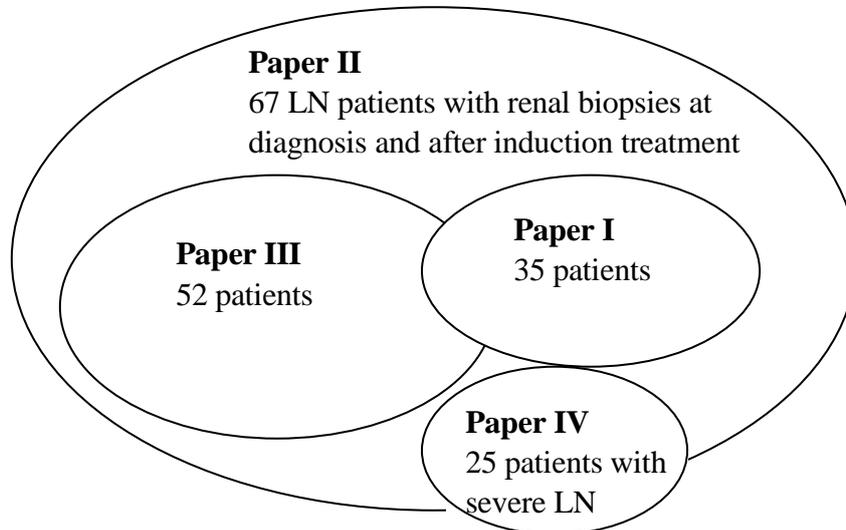
To study the role of repeated renal biopsies performed in LN patients after induction treatment. By comparing clinical and histopathological findings at the time for renal biopsies, our aim was to improve the knowledge on the role of repeated renal biopsies for the evaluation of treatment response (**paper II**) and long-term outcome in LN.

To study renal tissue expression and serum levels of inflammatory mediators (HMGB1 and cytokines) considered to be involved in the pathogenesis for LN (**paper I and III**).

To study long-term outcome in RTX-treated LN-patients in order to contribute to the knowledge on achieving and maintaining response in LN (**paper IV**).

4 PATIENTS AND METHODS

4.1 PATIENTS AND STUDY DESIGN



4.1.1 Repeated biopsy LN cohort (paper I-III)

The patient cohort that paper I, II and III are based on, consisted of 67 patients with SLE and a biopsy-proven active LN (PN, MN or mixed PN/MN), during the period of 1996-2009. All patients were followed at the rheumatology clinic, Karolinska University Hospital and, as part of clinical routine, the patients underwent a second renal biopsy after induction therapy.

At the time-point for the first renal biopsy, clinical data, blood and urinary samples were collected. Serum samples were kept frozen at -70°C for future analyses. After induction immunosuppressive therapy the patients underwent a second renal biopsy after a mean time of 8 months (median 8 months, range 5-15) and again, the clinical and laboratory data were collected. Fifty-eight of the patients were female (87 %) and 9 were male (13 %) and the mean age at first biopsy was 34 years (range 18-61).

4.1.1.1 Paper I

Thirty-five patients with active LN were included in the study.

Serum levels of HMB1 were analyzed in 20 patients with baseline PN (n=17) or mixed PN/MN (n=3). HMGB1 levels and clinical and routine laboratory data were analyzed at the time-point for first (baseline) and second renal biopsies. The induction treatment consisted of corticosteroids (CSs), median dose 40 mg/day (range 20-80) in combination with CYC (n= 14) or MMF (n=6). Serum levels of HMGB1 from 48 healthy individuals were used as a control group.

For immunohistochemical staining of HMG1 in renal tissue, additionally 15 patients from the repeated biopsy cohort were included, however in 2 of the patients the stainings were performed on renal biopsies at later renal flares. In all, tissue stainings were performed on baseline and follow-up renal biopsies from 25 LN-patients.

4.1.1.2 Paper II

All 67 patients from the repeated biopsy cohort were included. All patients had active nephritis at baseline; PN (n=48), mixed PN/MN (n=9) or MN (10).

The patients were treated with CYC (n=51), MMF (n=12), RTX (n=3) and AZA (n=1). All but one patient also received prednisolone, median initial dose 40 mg/day (range 2.5 to 80) and 46/67 (69 %) of the patients received ACE- inhibitors and/or ARBs. After second biopsies, clinical and histopathological responses were evaluated and compared.

Patients with a follow-up period of five years or more since baseline biopsy were included in a long-term evaluation. Thus, 56 patients were re-evaluated at last visit 2011 after a median follow-up of 10 years (mean 9.8 range 5-15 years) since baseline biopsies.

4.1.1.3 Paper III

Fifty-two patients from the cohort were included. All patients had an active nephritis at baseline biopsies, PN (n=40), mixed PN/MN (n=4) and MN (n=8). Serum levels of several cytokines (TNF- α , IFN- γ , IL-2, IL-4, IL-6, IL-10, IL-17, IL-23 and TGF- β) were analyzed at both biopsy occasions and in 13 healthy controls.

The patients were treated with CYC (n= 40), MMF (n=9), RTX (n=2) and AZA (n=1). The cytokine levels were analyzed in association to clinical data, routine laboratory variables and histopathological findings.

In 6 patients, immunohistochemical stainings for IL-17 and CD3+ lymphocytes were performed on renal tissue from baseline and repeated biopsies.

4.1.2 Rituximab LN cohort (paper IV)

The patient cohort consisted of 25 patients with severe LN during the period of 2001-2008. All but two patients were earlier refractory to conventional therapy including CYC and /or MMF. Twenty-one of the patients had earlier been part of the “repeated biopsy cohort” from paper II. At baseline all but one patient had a recent renal biopsy showing PN (n=15), mixed PN/MN (n=2) or MN (n=7). The patients were treated with RTX in combination with iv CYC (n=21), in combination with MMF (n=2) and as mono-therapy (n=2). The patients were followed every second to third month during the first year and every 6 months thereafter, with clinical examination and blood samplings.

In 20 patients, a repeated biopsy was performed after a mean time of 8 months (range 3-12). The patients were followed until a renal flare occurred or until retreatment with RTX was given for non-renal lupus manifestations.

4.2 LABORATORY MEASUREMENTS

4.2.1 Routine laboratory parameters

Renal evaluation included urine analyses (dipslide procedure), urine sediment and investigation of 24-hour urine-albumin excretion. Renal function was determined by serum creatinine ($\mu\text{mol/l}$) and by estimated glomerular filtration rate (eGFR) using the Modification of Diet in Renal Disease (MDRD) formula (125). In paper IV, GFR was also assessed by clearance of iohexol according to clinical routine.

4.2.2 Autoantibodies and complement

Assessments of serum IgG anti-dsDNA antibodies were made by different methods in the studies.

In **paper I and III**, anti-dsDNA antibodies were analysed by immunofluorescence microscopy using *Crithidia luciliae* as a source of antigen, cut-off titre 1:10.

In **paper II**, anti-dsDNA antibodies were analysed by a multiplex method (Bio-Rad Laboratories Inc., CA, USA) according to routine of the laboratory, cut off <5 IU/ml.

In **paper IV**, measurement of anti-dsDNA antibodies was performed by a fluorescent enzyme Immuno-assay (FEIA) method (Pharmacia, Uppsala Sweden), cut-off <15 IU/ml.

Analyses of complement component C3 (normal range 0.67-1.43 g/l) and C4 (normal range 0.12-0.32 g/l) levels were determined by nephelometry (**paper I-IV**).

Analyses of complement component C1q were performed by rocket electrophoresis using rabbit anti-C1q as the antibody. Levels of C1q were expressed as the percentage of levels of healthy blood donors (normal range 76 to 136 %) (**paper I and IV**).

4.2.3 Analysis of HMGB1 (paper I)

Serum levels of HMGB1 were analyzed from cryopreserved serum by Western blot (Amersham Biosciences) and performed at The Feinstein Institute for Medical Research, New York, USA.

4.2.4 Analysis of cytokines (paper III)

Cytokines were analyzed from cryopreserved serum that had been stored at < -70 C. TNF- α , IFN- γ , IL -2, IL-4, IL-6 and IL-10 were measured using the Cytometric Bead Array (CBA), (BD Biosciences Pharmingen, USA) technique. IL-17, IL-23 and TGF- β were measured with Enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon, UK).

4.2.5 Detection of CD 19+ B-cells (paper IV)

Circulating B cells in the peripheral blood were evaluated by detection of CD19+ B cells by flow cytometry. The limit for detection of CD19+B cells was <0.01x10⁹/L (normal 0.12-0.38).

4.3 HISTOLOGICAL ASSESSMENT

Renal biopsies were performed by percutaneous ultrasonography-guided puncture. The renal tissue obtained was evaluated by light microscopy, immunofluorescence and electron microscopy.

In paper I, III and IV, the biopsies were graded according to the 1995 WHO classification of lupus nephritis (134).

In paper II, all biopsies were reclassified to the more recent ISN/RPS classification (135). All biopsies were also scored for activity and chronicity indices (141). The same pathologist evaluated all renal indices.

4.4 IMMUNOHISTOCHEMISTRY OF RENAL TISSUE

Immunohistochemical stainings (paper I and III) were performed on formaldehyde-fixed paraffin-embedded serial 4 µm sections of renal biopsies from LN patients and control renal tissue.

4.4.1 HMGB1 staining (Paper I)

Immunohistochemical stainings of HMGB1 expression were performed using a monoclonal mouse IgG2b anti-HMGB1 (2G7) as primary antibody and a biotin-labelled horse anti-mouse antibody was used for detection. As all cell nuclei contain HMGB1 and as the 2G7 antibody predominantly detects nuclear deposition of HMGB1, antigen retrieval treatment prior to staining was omitted so that the extranuclear HMGB1 was visualized more clearly. Stainings were developed by using a DAB (diaminobenzidine)-kit.

4.4.2 IL-17 staining (Paper III)

Immunohistochemical stainings was performed using mouse anti-IL17 antibodies to detect IL-17 and rabbit anti-CD3 to detect CD3+ T-cells. Donkey anti-mouse- and donkey anti-rabbit secondary antibody were used for detection of tissue bound primary antibodies. Stainings were developed using DAB.

4.5 RESPONSE CRITERIA

4.5.1 Clinical response

In paper I, III and IV renal disease activity was estimated using the renal domain of the British Isles Lupus Assessment Group (BILAG) (96, 97).

The renal domain of BILAG index grades change in disease activity into five levels (Grade A= very active, B= moderate active, C= mild activity, D= no activity but previously affected and E= no current or previous disease activity). Only features attributable to SLE should be recorded, and judged as improved, same, worse or new (supplementary data, 97). The renal category scoring is presented in table nr 5.

Table 5. BILAG, scoring of renal domain

Category	Definition
A	Two or more present (including one of 1, 4 or 5) 1. Proteinuria Urine dipstick increased >2 levels <i>or</i> 24 h urine protein >1 g <i>or</i> urine P/C > 1 mg/mmol (that has not improved by \geq 25 %) 2. Accelerated hypertension (increased to > 170/110 mm Hg) 3. Deteriorating renal function: Creatinine >130 μ mol/l (having risen to >130 % of previous value <i>or</i> GFR having fallen to < 50 ml/min <i>or</i> GFR < 67% of previous value) 4. Active urinary sediment (pyuri > 5 WCC/hpf, hematuria >5RBC/hpf <i>or</i> red cell casts) 5. Histological evidence of active nephritis (class III, IV or V) 6. Nephrotic syndrome (
B	One of 1-3 1. One category A feature 2. Proteinuria (not fulfilling category A criteria) Urine dipstick >2+ <i>or</i> 24-h urine protein \geq 0.5 g <i>or</i> urine P/C \geq 50 mg/mmol (that has not improved by \geq 25%) 3. Creatinine >130 μ mol/l, having risen \geq 115 % but \leq 130 %)
C	One of the following 1. Proteinuria (not fulfilling category A/B criteria) Urine dipstick >1+ <i>or</i> 24 h urine protein > 0.25 g <i>or</i> urine P/C > 25mg/mmol 2. Rising blood pressure (>140/90), defined as systolic rise \geq 30 mm Hg <i>and</i> diastolic rise \geq 15 mm Hg.
D	Previously renal involvement, currently inactive
E	No previous renal involvement

The renal disease activity was estimated according to BILAG at the time-point for first and second biopsies (paper I, III and IV), and was in paper IV also assessed at every visit during the follow-up period. Renal response according to BILAG was defined somewhat differently in the studies:

In **paper I**, we only graded response vs. non-response; patients having renal BILAG C or D at follow-up were regarded as responders.

In **paper III**, an improvement of at least two grades in the renal domain of BILAG (i.e. from A to C or B to D) at follow-up was regarded as a complete response (CR), whereas an improvement of one grade as partial response (PR).

In **paper IV**, a BILAG D was required for CR and C for PR, a BILAG B was regarded poor response whereas A was non-response.
A new renal BILAG A or B was regarded a renal flare.

(The rationale for having different response criteria according to BILAG will be discussed in chapter 6.1).

In paper II, clinical renal response was defined according to the European consensus statement by Gordon *et al* (129). A CR was defined as an inactive urinary sediment, proteinuria ≤ 0.2 g/day and normal (GFR > 90 ml/min), or stable renal function (within 10 % of normal GFR if previously abnormal). PR was defined by inactive sediment, proteinuria ≤ 0.5 g/day and a normal or stable renal function ($< 10\%$ deterioration from baseline if GFR was previously abnormal). Patients not reaching criteria for CR or PR were regarded non-responders (NR).

In **paper IV**, global disease activity was also estimated by SLEDAI (95).

4.5.2 Histopathological response

In paper I and II we assessed histopathological response with different classification systems (WHO in study I and ISN/RPS in study II), the definitions for histopathological response was defined as:

In **paper I**, WHO class I or II at follow-up was regarded as good histopathological response whereas WHO III-IV and V as non-response.

In **paper II**, Class I, II or III/IV- C (ISN/RPS) at follow-up was regarded as histopathological response (HR) whereas class III /IV- A or A/C and V as non-response (HNR).

These definitions were based on the presence (or not) of persistent active nephritis lesions at repeated biopsies, and will be further discussed in 6.1.2.

4.6 STATISTICS

Descriptive statistics were used to characterize the study populations, presenting mean (+/- SD) or median (range) for continuous variables and number or percentages for categorical variables.

Comparisons of variables at baseline and follow-up were made using Wilcoxon matched pair test or paired student t-test. Comparisons of continuous variables between two groups were assessed using Mann-Whitney test or student t-test as appropriate. Comparisons between multiple groups were made using the Kruskal-Wallis test. For categorical variables the Chi-square test was used. Correlations were calculated using Spearman's rank correlation and by Fisher r-to-z test (paper IV).

Kaplan Meier survival curves were constructed for time to PR/CR in paper IV. Statistical significance was set at the level of $p < 0.05$.

5 RESULTS AND DISCUSSION

5.1 PAPER I

Renal expression and serum levels of high mobility group box 1 protein in lupus nephritis

High mobility Group Box Protein 1 (HMGB1) has been proposed to be involved in the pathogenesis for SLE and LN. Increased serum levels have been reported in SLE, the highest levels were found in patients with renal involvement (79) and increased HMGB1 expression have also been demonstrated in skin lesions in SLE patients (82). Urinary levels of HMGB1 were increased in patients with active LN (83). A previous study reported high serum HMGB1 levels and increased expression in renal tissue in ANCA-associated vasculitis patients with renal involvement (212). High levels of HMGB1 were also reported in patients with CKD (213).

However, data regarding serum levels of HMGB1 in association to histopathological findings or response to treatment in LN has not been previously reported and HMGB1 expression in renal tissue from LN patients has not been studied.

In this study, renal expression of HMGB1 was assessed by immunohistochemistry at baseline and follow-up biopsies in 25 patients. Serum levels of HMGB1 were analyzed in 20 patients at both biopsy occasions.

At baseline, patients had active nephritis, WHO III (n=15), IV (n= 12), III/V (n=2) and V (n=6), and all had high renal disease activity, BILAG A (n=32) or B (n=3). Follow-up biopsies showed WHO class I-II (n=14), III (n=6), IV (n=3) and V (12). Fifteen patients were regarded as clinical responders, BILAG C/D at follow-up.

Serum levels of HMGB1 were higher in LN-patients compared to controls ($p<0.0001$). Levels decreased only slightly (ns) and remained significantly high after treatment. In clinical non-responding patients (BILAG A/B after treatment), a trend towards higher levels of HMGB1 vs. responders at follow-up was found (ns). No clear association between clinical parameters (proteinuria, creatinine, complement levels or anti- dsDNA positivity) and serum HMGB1 levels was noted.

Immunostaining of renal tissue revealed an extranuclear HMGB1 expression in all the examined biopsies from LN-patients (fig1). The staining was predominantly found outlining the glomerular endothelium and in the mesangium and also, to a lesser extent expressed in vessels and tubular cells. There was no clear difference in the amount of HMGB1 comparing first and second biopsies, and no association between tissue expression and nephritis classification, treatment response or clinical parameters was shown. HMGB1-stainings of tissue from control renal biopsies were negative or, if present restricted to the cell nuclei.

In conclusion, we found increased expression of HMGB1 in renal tissue, which clearly indicates a role for HMGB1 in the inflammatory process in LN. The pronounced staining outlining the endothelium and in the mesangium suggest a co-localization for HMGB1 and immune deposits. This may be of particular interest since HMGB1 in complex with nucleosomes released from not properly cleared apoptotic cells have

been suggested to play a pathogenic role in SLE. HMGB1-nucleosome complexes have been reported to induce production of proinflammatory cytokines from macrophages and dendritic cells, maturation of DCs and anti-dsDNA production. Moreover, HMGB1 nucleosome complexes when incubated with anti-dsDNA antibodies also induced IFN- α production from pDCs (86). However, other methodologies are needed to further examine if HMGB1 and immune deposits co-localize in renal tissue from LN patients. The exact origin of the renal expression in our study is not clear, and the high serum levels as well as the increased tissue stainings may reflect both systemic inflammation and local inflammation within the kidney. The persistently high levels after induction immunosuppressive treatment possibly reflect persistent inflammatory activity.

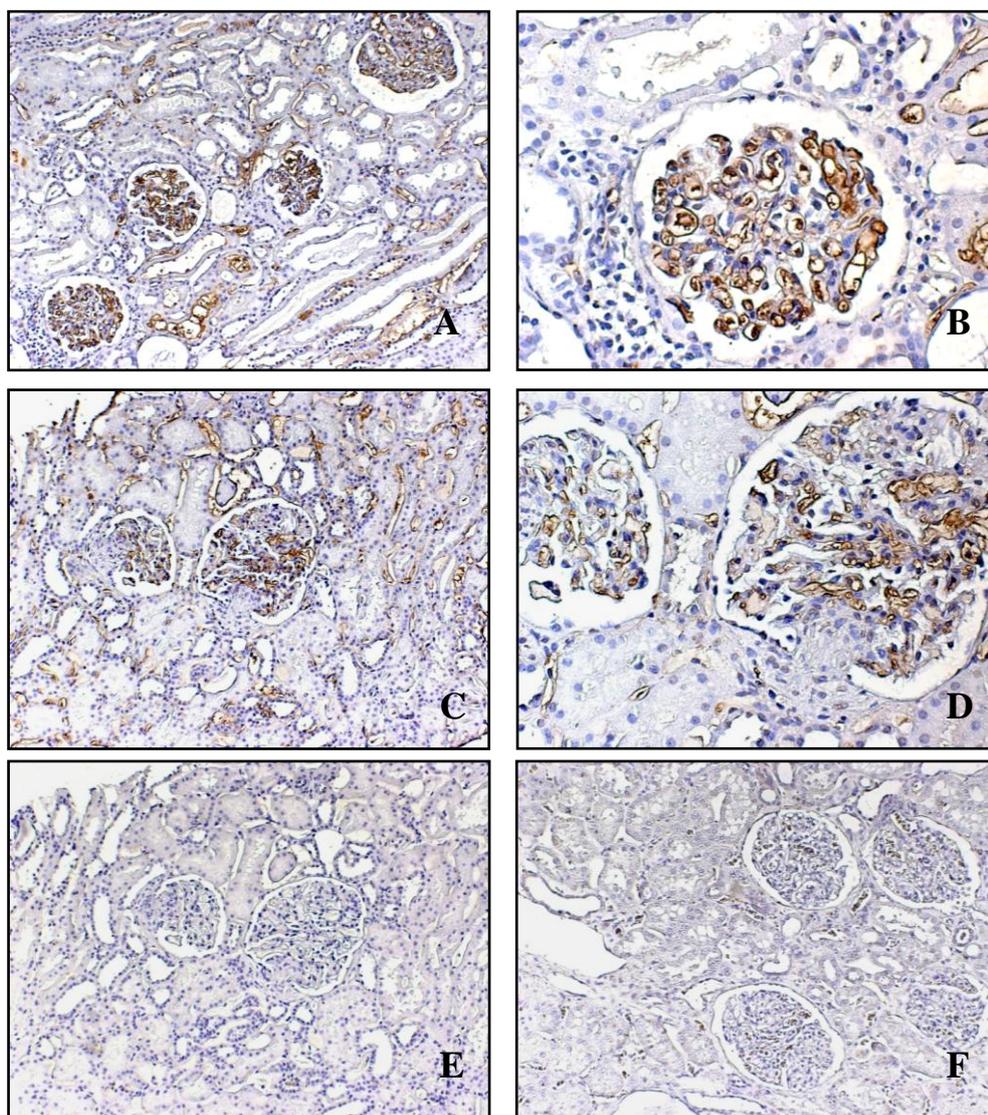


Figure 2: Expression of extranuclear HMGB1 protein in LN

Figure (A) shows a baseline biopsy from a patient (WHO class III) with increased HMGB1 staining predominantly outlining the glomerular endothelium but also expressed in the mesangium, magnified in (B). HMGB1 expression is still evident in a repeat biopsy (still WHO class III) from the same patient (C, D) although not as strong as in the baseline biopsy. In (E) a consecutive section from the same patient is stained with irrelevant isotype control antibody. (F) Shows renal tissue from healthy control with no evidence of extracellular HMGB1 expression.

5.2 PAPER II

Role of early repeated renal biopsies in lupus nephritis

Evaluation of response in LN is difficult, and despite extensive efforts, there are currently no generally utilized response criteria available. It is well known that LN patients may have inflammatory activity in the renal tissue in the setting of low-grade proteinuria, or even without clinical signs of renal involvement, and despite good clinical response to therapy (204, 214, 215). Thus, defining response based on clinical findings only may be insufficient. However, second renal biopsies after immunosuppressive therapy are seldom a routine procedure. The aim of the study was to investigate if and how repeat biopsies could contribute to the evaluation of treatment response and long-term outcome in LN.

We studied 67 LN-patients in whom repeated biopsies were performed after induction treatment, regardless of clinical response, and compared the histopathological findings to established clinical response criteria (129). Long-term renal outcome was evaluated in 56 patients.

The results were analysed in the total patient group and also subdivided in patients with PN (class III or IV) or MN (class V) at baseline biopsies. Nephritis data and laboratory findings at first and second biopsies are presented in table 6.

Table 6. Laboratory and histopathological characteristics at first and second biopsies

	First biopsy	Second biopsy	p-value
Creatinine, $\mu\text{mol/l}$	84 (44-284)	76 (45-306)	0.003
Albuminuria, g/d	1.4 (0-8.4)	0.5 (0-3.6)	<0.001
C3, g/l	0.5 (0.12-1.13)	0.79 (0.38-1.51)	<0.001
C4, g/l	0.09 (0.02-0.51)	0.13 (0.02-0.45)	<0.001
Anti-DNA ab IU/ml	165 (<5-300)	29.5 (<5-300)	<0.001
% positive(>5 IU/ml)	96	91	
Renal histology (ISN/RPS), n			
I-II	-	14	
III C	-	13	
III A or A/C	21	10	
IV C	-	1	
IV A or A/C	27	7	
III- IV/V	9	2	
V	10	19	
Vasculitis	-	1	
Activity index	5 (0-13)	2 (0-12)	<0.001
Chronicity index	1 (0-6)	1.5 (0-8)	<0.001

After induction treatment, a clinical response (CR or PR) was observed in 52 % of the patients, 17 patients had CR (25 %), 18 had PR (27 %) and 32 had NR (48 %). Of PN-patients, 17 had CR, 15 PR and 25 NR whereas no MN-patients had CR, 3 had PR and 7 NR.

Histopathological response (HR) was seen in 42 % of the patients. Of patients with baseline PN, 47 % had HR and 53 % had HNR (20/30 had persisting PN or PN/MN and 10/30 had transformed into MN). Of patients with baseline MN, only one changed nephritis class (to class III-C). There was no difference in either clinical- or histopathological response with respect to type of induction treatment given.

When comparing clinical and histopathological response we found that 29 % of the patients with CR (all PN) were HNR, and 61 % of patients with PR (15 PN and 3 MN) were HNR. In contrast, 28 % with poor clinical response (NR) had no active lesions on repeat biopsies (table 7). In table 8, laboratory findings at the time for repeat biopsy and clinical response in patients with baseline PN in HR vs. non-responders in PN

Table 7. Histopathological data at second biopsies in clinical complete-, partial and non-responders (all patients)

	CR (n=17)	PR (n=18)	NR (n=32)	p
Renal histology, n				
Class I-II	6	4	4	
Class III/IV (C)	6	3	5	
Class III/IV (A) or (A/C)	1	7	10	
Class III/IV (A)+ V	0	0	2	
Class V	4	4	11	
Activity index	1 (0-3)	2 (1-9)	2 (0-12)	0.001
Chronicity index	1 (0-6)	3 (0-8)	1.5 (0-8)	ns
Histological response				
HR, n (%)	12 (71)	7 (39)	9 (28)	
HNR, n (%)	5 (29)	11 (61)	23 (72)	

Table 8. Characteristics at repeat biopsy and in patients with baseline PN in histopathological responders vs. non-responders

	HR (n=27)	HNR (n=30)	p
Creatinine, $\mu\text{mol/l}$	74 (48-306)	76 (45-122)	ns
Albuminuria, g/d	0.2 (0-3.4)	0.6 (0.03-3.1)	0.006
Anti-DNA IU/ml	18 (<5-250)	49 (<5-300)	0.05
C3 g/l	0.74 (0.4-1.38)	0.81 (0.38-1.51)	ns
C4 g/l	0.13 (0.04-0.3)	0.13 (0.03-0.45)	ns
Hematuria, %	30	54	ns
Clinical response, n			
CR	12	5	
PR	7	8	
NR	8	17	

After the second biopsy, all but 3 patients received maintenance therapy, as decided by the treating physician. Of patients with HNR, although having clinical CR (n=5) or PR (n=11), 81 % received prolonged or intensified treatment with CYC or MMF or were switched to RTX. Of PN-patients with HNR despite CR/PR, 92 % received prolonged or intensified treatment.

At long-term follow-up, renal outcome was evaluated in 56 patients (mean age 43 years, range 26-72) with a mean time of 9.8 of years (median 10 years, range 5-15) since baseline biopsy. One of these patients had severely impaired renal function at baseline biopsy and was not included. Fourteen out of 55 patients (25 %) had an eGFR <60 ml/min, median 52 (range 31-59), thus regarded as having CKD (216), of which 3 (5 %) had developed ESRD. Except ESRD-patients, none developed a doubling of serum creatinine. Patients who had developed CKD had higher chronicity index on repeated biopsies (p=0.03) but no difference was found for activity index. Of the 3 patients who developed ESRD all had baseline PN and all were HNR with both active and chronic lesions at follow-up biopsies, class III-A/C (n=2) and class V (n=1).

In conclusion, many LN patients had persisting active nephritis lesions in renal biopsies after induction immunosuppressive treatment, despite clinical findings indicating low renal disease activity. Repeated renal biopsies thus provided information that was not captured by routine laboratory parameters. Until improved biomarkers are available for clinical use, second biopsies may be considered when evaluating treatment response in LN. Histopathological findings may also be an aid to identify patients that would benefit from intensified immunosuppressive treatment. In addition, in patients with only chronic lesions, overtreatment may be avoided.

The overall favourable long-term renal outcome may be due to a number of factors. However, our approach with repeated biopsies, and intensified or prolonged treatment given to histopathological non-responders, may at least in part have contributed.

5.3 PAPER III

IL-17 and IL-23 in lupus nephritis - association to histopathology and response to treatment

Increasing evidence supports a central role for IL-17 and IL-producing cells in the pathogenesis for LN. High serum levels of IL-17 and an increased proportion of IL-17 producing cells have been reported in SLE patients. IL-17 producing cells and increased expression of both IL-17 and IL-23 have been demonstrated in renal tissue from LN patients (55, 57, 58, 117). The role of IL-17, or IL-23, in the context of response to immunosuppressive treatment in LN has however not been studied.

In this study, serum levels of TNF- α , IFN- γ , IL-2, IL-4, IL-6, IL-10, IL-17, IL-23 and TGF- β were measured in 52 LN-patients at both baseline and repeated biopsies, and the results were analyzed in association to clinical and histopathological findings. IL-17 expression in renal tissue was assessed by immunohistochemistry in 6 patients.

At baseline, all patients had active nephritis and high renal disease activity (BILAG A/B). Nephritis- and laboratory data at first and repeat biopsies are presented in table 9. Nineteen patients (36%) were regarded as histopathological responders (WHO class I/II). Twenty-two patients were regarded as clinical CR, 20/52 as PR and 10/52 NR according to the definition used (BILAG).

Table 9 Nephritis- and laboratory data at first and second biopsies

	First biopsy	Second biopsy	p-value
Creatinine, $\mu\text{mol/l}$, median	82 (52-284)	77 (48-306)	ns
Albuminuria, g/d, median	1.45 (0-8.4)	0.5 (0-3.6)	<0.001
C3, g/l, median	0.5 (0.12-1.13)	0.74 (0.36-1.41)	<0.001
C4, g/l, median	0.11 (0.02-0.51)	0.13 (0.02-0.45)	0.002
Anti-dsDNA-ab positivity, IFL, %	84	57	
Renal histology, WHO (n), I-II	-	19	
III	15	9	
III/V	4	1	
IV	24	8	
V	8	14	
Vasculitis	1	1	
Activity index, median	5 (0-13)	2 (0-12)	<0.001
Chronicity index, median	1 (0-6)	1 (0-8)	<0.001
BILAG (renal) A	49	8	
B	3	20	
C	-	12	
D	-	12	

At baseline, most serum cytokine levels were increased compared to controls; statistically significant for IL-6, IL-10, IL-17, IL-23 ($p < 0.001$ for all) and IFN- γ ($p = 0.03$) whereas TGF- β was lower in patients vs. controls ($p < 0.001$). There was no difference in cytokine levels in patients with PN vs. MN at baseline biopsies. Overall cytokine levels decreased after treatment, significantly for IL-6 ($p < 0.001$), IL-10 ($p = 0.02$) and IL-17 ($p = 0.01$), and for IL-23 there was a trend towards lower levels at follow-up ($p = 0.06$). For TNF- α and IFN- γ there was an overall decrease in serum levels (ns), while for TGF- β an increase was documented ($p = 0.005$).

Baseline levels of IL-17 were higher in patients with a poor histopathological response (class III, IV or V) after immunosuppressive treatment, compared to patients with class I or II at follow-up ($p < 0.03$). The highest baseline levels of IL-17 were seen in patients with MN at repeat biopsy (Fig 3).

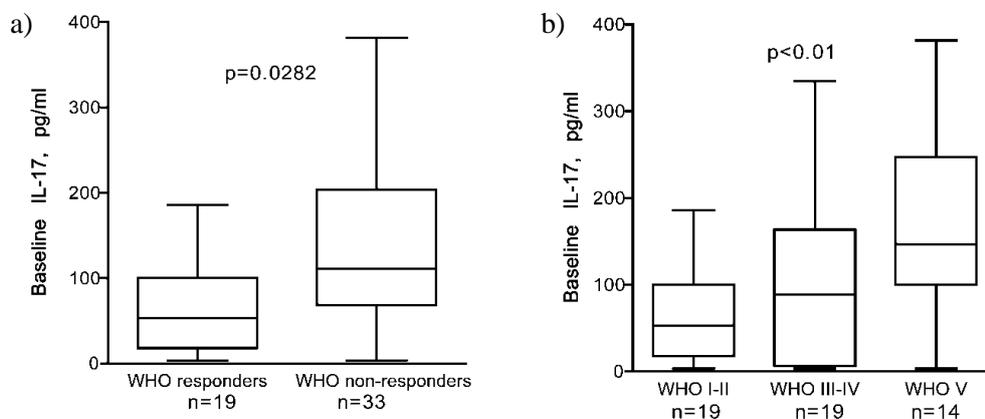


Figure 3 Baseline levels of IL-17 in a) patients with histological response vs. non-response and b) in patients with WHO class I-II, III-IV and V at second biopsies.

Patients with the highest levels of IL-17 (encompassing the upper quartile), also had high levels of IL-23, TNF- α and IFN- γ and 85 % of these patients were histopathological non-responders. Baseline levels of creatinine, proteinuria or complement levels did not differ between patients with a good vs. a poor histopathological response.

At follow-up, clinical NR-patients had higher levels of IL-23 vs. partial PR and CR. Patients with urine albumin <0.2 g/day lower levels of IL-23 ($p=0.01$) vs. patients with > 0.2 g/day. No association to clinical response was found for the other cytokines. Immunostaining revealed expression of IL-17 in renal biopsies from all LN patients (4 with class V and 2 with class IV). The staining was most pronounced in areas of CD3+ T-cell infiltrates (Fig 3).

In conclusion, increased baseline levels of IL-17 were associated with a poor histopathological response after immunosuppressive therapy in LN and thus, high levels of IL-17 may predict a more severe, or therapy resistant disease phenotype. IL-17 was also expressed in T-cells infiltrates in renal tissue. High levels of IL-23 were associated with a poor clinical outcome. Our findings support a role for the IL-23/IL-17 axis in LN and indicate that this cytokine pathway may be evaluated as biomarkers for renal disease activity and for predicting response to therapy. The findings may also point to possible targets for new treatment strategies in LN.

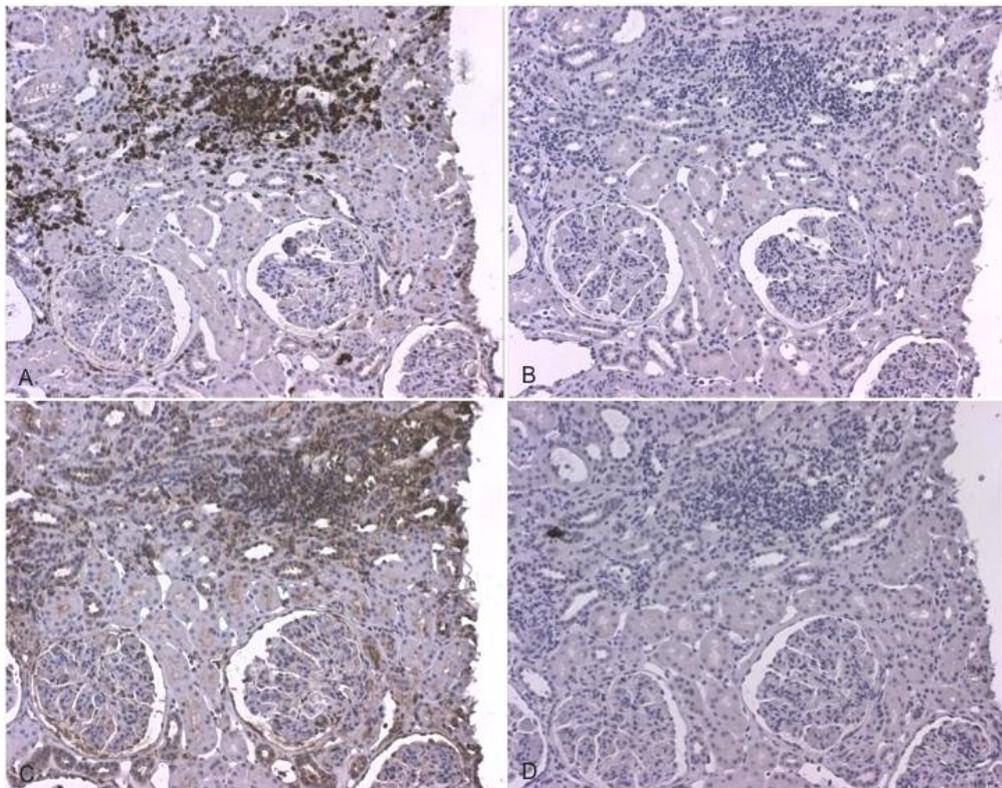


Figure 4: Immunostaining of IL-17 in renal tissue

The figure demonstrates a renal biopsy from a patient with LN WHO class V. (A) shows an inflammatory infiltrate with T cells as demonstrated by a positive CD3-staining, and in (B) the same infiltrate from a consecutive section is stained with irrelevant isotype control antibody. In (C), IL-17 staining is demonstrated, predominantly found in the inflammatory infiltrate shown in A, and (D) demonstrates staining with the corresponding isotype control antibody

The combined findings of high IL-6 and low IL-2 (detected only in 23 % of the patients) in this study is also interesting since it has been suggested that the cytokine milieu in SLE patients, with low levels of IL-2 and high IL-6 and IL-21, favors the development of Th17 cells (69).

5.4 PAPER IV

Long-term follow-up in Lupus nephritis patients treated with rituximab- clinical and histopathological response

In later years, B-cell depletion therapy with rituximab (RTX) has increasingly been used in various autoimmune diseases, including SLE. A number of uncontrolled studies have reported promising results for RTX in the treatment of mainly severe and refractory LN. A recent large study comprising pooled data of 164 LN patients reported a CR or PR in 67% of the patients after 12 months (178). Controlled clinical trials with RTX in LN have however been unsuccessful (217).

Data on long-term outcome of RTX-treatment in LN is to date limited, but small studies with follow-up periods of 2 years or more have reported a sustained response in most cases (175, 177, 218). Another study have reported poor outcome in 8 patients with refractory LN, of which 3 developed renal insufficiency after 2 years (219). We thus aimed to study long-term renal outcome and predictors of response in a larger group of LN patients treated with RTX due to inadequate response to conventional therapy.

This study included 25 patients with severe LN, of which all but two were earlier refractory to conventional therapy. The patients were treated with RTX (described chapter 4.1.2) and were then examined every second to third month during the first year and every 6 month thereafter. The patients were followed until a renal flare occurred or until retreatment with RTX was given for other reasons. In 20 patients a repeat biopsy was performed after a mean of 8 months.

At baseline, all had an active nephritis (proven by a recent renal biopsy in all but one patient), WHO class III or IV (n=15), III-IV/V (n= 2) and V (n= 7), and all had high renal disease activity (BILAG A). The median renal activity index was 4.5 (range 1-10) and the chronicity index was 3 (0-8). Repeat biopsies showed WHO class I-II (n=7), III- IV (n= 4), IV/V (n=1), V (n=8) and one renal vasculitis. Overall, the activity index decreased ($p<0.001$) with no deterioration in the chronicity index vs. baseline biopsies. The proteinuria decreased significantly, most prominently during the first 6 months of follow-up, however an additional reduction in proteinuria was seen up to 36 months (figure 5).

All patients had an initial total depletion of CD19⁺ B-cells in peripheral blood. B-cells were then detectable after a mean time of 8 months (range 2-26 months).

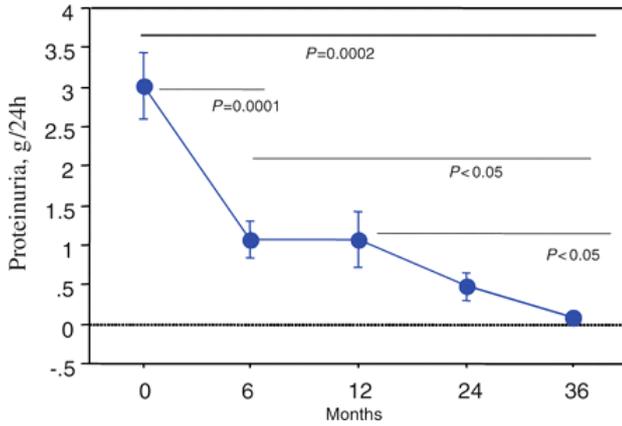


Figure 5. Reduction of proteinuria over 0-36 months

A PR was seen in 22 patients after a median of 12 months (6-36 months) and a CR was seen in 16 patients after a median of 24 months (figure 6). Only one patient was considered non-responder and 2 were poor responders. Patients achieving a CR during the first year had significantly longer B-cell depletion vs. patients with PR and those who achieved a CR later (fig 7).

A renal flare occurred in 6 patients after a mean time of 29 months (10-64), of which 4 patients had a baseline PN and 2 MN.

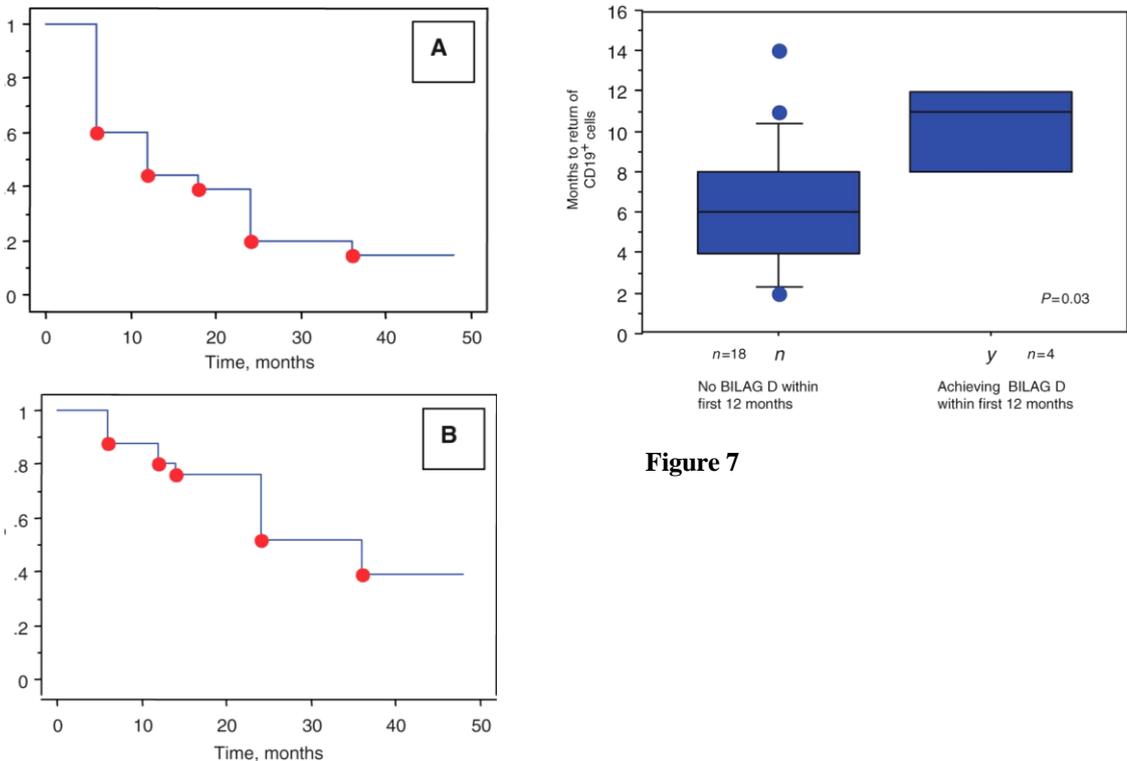


Figure 7

Figure 6. (A), time to PR (BILAG C) and (B), time to CR (BILAG D)

In conclusion, a majority of the patients achieved a renal response after treatment with RTX during the first year. Additionally patients continued to improve after the first year of follow-up, and after 2 years, 50 % had achieved a CR. A faster response was noted in patients with low baseline IgM as well as in patients with a longer time of B-cell depletion. The finding that many patients achieved a CR between 12 and 24 months suggests that future clinical trials on B-cell depletion therapy should include a longer follow-up to detect differences in response between therapies.

Although a histological improvement was seen in most patients, with an overall decrease in activity index, a proportion of patients had persisting active lesions on repeat biopsies. However, the patient cohort consisted of a group of patients with severe LN who were previously refractory to treatment, and in this context the histopathological response was regarded as favourable. Importantly, no increase in the chronicity index was documented.

Altogether this study suggests that RTX may provide a long-time improvement in refractory LN, with equal efficacy for both MN and PN.

6 GENERAL DISCUSSION

6.1 METHODOLOGICAL CONSIDERATIONS

6.1.1 Patient cohort and methods

This thesis is based on observational studies that reflect “real life” care of LN patients. This is of course a limitation, since several factors are involved in the care of patients with LN and may have impact on the results. Therapy decisions are made by taking a number of variables into account. For each individual patient, factors such as histopathological findings on the renal biopsy, age, ethnicity, comorbidities, doubts about compliance, pregnancy plans and fear of side effects, as well as severity of clinical presentation are all involved in treatment decision-making. The therapy decisions were all made by the treating clinician and the “un-controlled nature” of my studies has probably had impact on the results.

However, the above-mentioned limitations of the studies may also be their strength. All the patients included had active renal disease, including both PN and MN and with varying grade of severity, thus reflecting a broad and representative spectrum of the disease. Many patients had severe renal disease, a patient group that is often excluded from controlled clinical trials.

The conceivably most important strength of the studies is however the number of patients included in the repeated biopsy cohort. This study is unique in that repeated biopsies were performed after induction therapy in consecutive patients, regardless of clinical response. Furthermore, the patient cohort is one of the largest for studies on second biopsies in LN. Hill *et al* also reported results from a large group of LN patients (n=71), with biopsies performed after induction treatment. However the focus of that study was the evaluation of the prognostic value of a new morphological index for renal biopsies (206).

Having repeated biopsies available gave us unique possibilities to evaluate both clinical and histopathological response to immunosuppressive treatment. In addition, both conventional and novel biomarkers could be analysed in association to renal histopathological findings at different stages of disease activity, an often asked for tool when evaluating novel biomarkers in LN (189).

The results of the long-term evaluation of LN patients in **paper II** may have been affected by multiple factors. The renal out-come may have been influenced by different dosages of prednisolone given, co-morbidities compliance problems and pregnancies and also by the fact that treatment strategies have changed over time during the study with lower dosages of CYC now used, more use of MMF, prolonged periods of maintenance therapies, increasing use of ACE-inhibitors and more patients on background treatment with antimalarials, which all may have impact on renal outcome (150). Since 2001, many patients have also received RTX at later renal flares at the time for follow-up.

The long-term evaluation in **paper II** is based upon the last visit by the end of year 2011 in patients with at least 5 years of follow-up since the baseline biopsy. A more complete evaluation could have included a once-every-year follow-up design with regular evaluation of renal function in order to further define the time-point and possible predictors for loss of renal function. Another limitation in paper II is that we do not have exact measure of GFR at long-time follow-up. We here used estimated GFR using the MDRD formula. Although this is a commonly used method in CKD populations it is less reliable in patients with GFR>60 ml/min (128), also described specifically in LN patients (220). Seven out of eleven patients 7/11 (64 %) that were regarded as having CKD had an eGFR between 50-60 ml/min and there is a possibility that we underestimated the renal function by using the MDRD formula.

A limitation in **paper III** is that other, non-renal, organ manifestations or over-all disease activity were not taken into consideration when analysing the cytokine levels in association to renal disease activity. However, the high baseline levels associating with poor renal response and the findings of IL-17 expression in renal tissue from LN patients indicate that this cytokine pathway is of importance in severe LN.

A limitation in many studies on LN is the small number of patients included. In **paper I**, serum levels of HMGB1 were only available in 20 patients. However, in this study a comparatively large number of tissue stainings were performed, which all revealed increased expression of HMGB1 in renal tissue and the combined findings clearly supports a role for HMGB1 in LN.

In **paper IV**, a majority of patients responded to treatment. However, the relatively small number of patients, and with only a few renal flares, made it difficult to draw firm conclusions on predictors of flares.

6.1.2 Renal response

The definition of clinical response used in the studies differed somewhat. In **paper I, III** and **IV** renal disease activity and response were evaluated according to the renal domain of the BILAG index (97), while response in **paper II** were evaluated according to recent European consensus statement definitions (129).

In **paper I**, the main focus was to investigate serum levels of HMGB1 in association to renal disease activity and the HMGB1 expression in renal tissue. Serum levels were only available in 20 patients and the limitations in patient number made us subdivide renal outcome in two groups only, responders (BILAG C/D) or non-responders (BILAG A/B).

In **paper III**, serum cytokine levels were analysed in a larger group of patients, and thus we found it more appropriate (and clinically relevant) to divide the responders into three groups CR, PR and NR. As the BILAG index is based upon the clinician's intention to treat, depending on change in disease activity, we here regarded an improvement of at least two grades in renal BILAG (i.e. from A to C or B to D) at follow-up as a CR whereas an improvement of one grade as PR.

In **paper IV**, the main focus of the study was to investigate the time-point for achieving and maintaining a renal response following RTX therapy. In this study, having repeated and tight evaluation of the patients, it became even more important to distinguish changes in disease activity. Thus we here evaluated the patients also in terms of non-response (persisting BILAG A) and poor response (BILAG B) in addition to partial (PR) and complete (CR) response. Although we did not compare RTX with any other therapy in this study, the here used strategy for evaluation of treatment response may be a proper design for future clinical trials comparing the efficacy of different treatments.

In **paper II**, we compared histopathological findings and clinical response to treatment. We here defined clinical response according to established criteria (129). According to these criteria, the threshold for CR in regard of proteinuria is comparatively strict (≤ 0.2 g/day), which was regarded appropriate as we aimed to evaluate the presence of inflammatory lesions also in the setting of very low clinical disease activity. Since the renal BILAG domain also comprise histopathological findings, this index was not found appropriate to use as the main focus was to compare histological and clinical disease activity.

Of note, in **paper IV**, when comparing the consensus response criteria by Gordon *et al* (129) with BILAG, we found a full agreement between PR/CR as defined by the consensus statement and achieving BILAG C/D.

There are currently no definitions available or evaluated for a histopathological response in LN, and histopathological response has to date not been included in clinical trials of LN. We have chosen to define the presence of persistent active nephritis in repeat renal biopsies as a histological non-response. This was defined as WHO class III, IV or V in paper III (134), or class III or IV A-A/C or V (ISN/RPS) in paper II (135) or a renal vasculitis at repeated biopsies.

When comparing these two classification systems (WHO and ISN/RPS) there was a full agreement regarding response for all but one patient (who was considered a non-responder according to WHO but a responder, class III C, according to the ISN/RPS classification).

It is debatable if the presence or development of only chronic lesions should be regarded a response, or a treatment failure. However we choose to regard only patients with active persistent lesions as non-responders since these patients still may benefit for more intense immunosuppressive treatment.

Considering the more favourable prognosis of MN vs. PN it is also arguable if transforming from class III or IV to V is a non-response. However, a proportion of patients with MN at repeat biopsies in our cohort had relapsing disease, of which some developed PN at relapses.

As additional data, we also evaluated histopathological response as a $>50\%$ reduction in activity indices between baseline and repeat biopsies in paper III (not included in the manuscript), a response was found in 36/66 (54%) in all patients of which 33 with PN and 3 with MN (the patient with a vasculitis pattern on follow-up biopsy was not included because activity and chronicity indices were not estimated in that patient).

However, the reproducibility of the activity and chronicity index scoring is limited and is subject to inter-observers differences. No clear cut-off limits for these indices that are useful to predict prognosis have been yet determined.

6.2 FINDINGS AND IMPLICATIONS

6.2.1 Persistent inflammation after treatment

The overall finding of the studies is the persistent inflammation and immune dysregulation in LN patients, despite intense treatment. Of note, the ongoing inflammatory activity in renal tissue was not captured by routine laboratory measurements in many cases and may thus be overseen if repeat biopsies are not performed.

In **paper I**, we found increased levels of HMGB1 in serum as well as an increased expression in renal tissue, both at baseline and after immunosuppressive treatment. This is in contrast with findings in patients with ANCA-associated vasculitis (AAV) with renal involvement (212) and shows clear differences in immune regulation and response to therapy in the different renal diseases. The high baseline serum levels of HMGB1 observed decreased significantly after treatment in AAV and, in contrast to our findings in LN, a more distinct histopathological as well as clinical response was demonstrated.

In **paper II**, a persisting active proliferative nephritis (class III-IV+/- V A or A/C) was seen in 30 % of the patients at second biopsies. Overall, including the patients with class V, as much as 58 % of the patients had a persisting active nephritis at repeat biopsies. There is a possibility that the persisting renal inflammation seen could be due to the fact that the repeated biopsies were performed too early after induction treatment (after a mean time of 8 months), and that a further histopathological improvement might occur after a longer period of time. On the other hand, the persisting renal inflammation could be explained by the fact that current therapies used in LN are insufficient in a subset of patients. The latter explanation is in line with overall findings in studies on LN, although different time-points for evaluation and different response criteria were used, in which all reported on a subgroup of non-responders and a proportion of patients who proceeded to ESRD (101, 150, 167).

The findings in **paper III** support the proposal that currently used treatments may be insufficient, and do not suppress the immune dysregulation and inflammatory processes in all LN-patients. High levels of IL-17 at baseline biopsies were seen in patients who had a poor response to therapy. Importantly, in the subgroup of patients with high baseline IL-17 levels in combination with persistently high or even increasing levels at follow-up; all had an active nephritis at repeated biopsies. This indicates that patients with high IL-17, many of whom also had high levels of TNF- α , IFN- γ and IL-23, may represent a group of severe LN patients with a strong T-cell component, in whom current treatments are insufficient or inadequate. Of note, the patient with the by far highest IL-17 level at the time of follow-up, did not respond to either CYC or to later given MMF or RTX and proceeded to ESRD in 5 years (personal communication).

When analysing findings in patients in whom both serum levels of HMGB1 and cytokines were available, we found correlations between HMGB1 and IL-17, TNF- α and IFN- γ ($r= 0.70$, $r= 0.62$ and $r= 0.68$ respectively, $p < 0.05$) at the time for second biopsies (unpublished data). No correlations were found between HMGB1 and the other cytokines measured.

In **paper IV**, patients with severe relapsing LN were treated with RTX, of whom a majority achieved a beneficial response during the follow-up period. The findings support the current opinion that RTX should be considered for treatment of refractory LN (101, 221, 222), which is also included in recent recommendations for the management of LN (130, 131).

However, 24 % of the patients had a renal flare during the follow-up, which again point to the chronic ongoing or relapsing inflammatory nature of the disease. There is to date no evidence on if RTX should be used alone or in combination with other immunosuppressive therapies in order to prevent flares, or if and when repeated RTX infusions should be given. Other B-cell targeting therapies may also be relevant in LN. Belimumab, a monoclonal antibody that prevents the actions of BAFF, and thereby inhibits B-cell differentiation and survival has been successful for treatment of non-renal SLE, however not studied in LN (223).

We reported a longer B-cell depletion time to be associated with a shorter time to achieve CR, also reported by others (224). However, all benefits of RTX in LN may not be related to the humoral effects, but can also be related to effects on T cells following B-cell depletion (225). It is also probable that B-cell depletion is not efficient in all patients and other targets than B-cells may be more important in some patients.

6.2.2 The role of repeated renal biopsies

The aim in **paper II** was to investigate if repeat biopsies contribute to the evaluation of treatment response and long-term outcome in LN. We found that many LN patients had persisting active nephritis lesions in renal biopsies after immunosuppressive treatment, despite clinical findings indicating low renal disease activity. In contrast, no active inflammatory lesions on repeat biopsies were present in a group of patients with clinically poor response to therapy. Routine laboratory parameters did not reliably predict the histological findings. Despite using response criteria that require comparatively low grade proteinuria (≤ 0.2 g/day) for CR, 5/17 (29%) of patients with a clinical CR had persisting active nephritis at follow up.

Thus, until improved biomarkers are available for clinical use, we propose that second biopsies may be considered when evaluating treatment response in LN. Histological findings may identify patients who would benefit from intensified immunosuppressive treatment and, in patients with only chronic lesions, overtreatment may be avoided.

However, an important issue to consider is the potential risk for complications associated with a renal biopsy. The most serious complication is bleeding which may require blood transfusions or even invasive procedures. A recent retrospective study on 219 LN-patients investigated renal biopsy complications. Bleeding requiring blood

transfusions or arterial embolisation (n=2) were reported in 2.7 % of patients. Low platelet levels were the only predictor found for biopsy complications (226). Of 255 renal biopsies performed in LN patients 1995- 2012 at the Karolinska University Hospital (rheumatology unit), 7 patients had bleedings noted by ultrasonography (2.7 %). Two additional patients had blood-clots in the urinary bladder (without bleeding on ultrasound). Thus, a total of 9 bleeding complications were noted, but with no need for any invasive procedure or nephrectomy. In all, three patients required prolonged hospitalization due to bleeding complications (Gunnarsson, personal communication and unpublished data).

According to a comment by Bihl *et al*, major complications, including the requirement for blood transfusion or invasive intervention, have been reported in 0–6.4% of renal biopsies. Predictors of complications have included anemia and high creatinine (132).

Contraindications such as bleeding diathesis, solitary kidney and bilaterally small kidneys must be considered before a renal biopsy and, although the overall low risk for complications is low, all potential benefits or risks associated with a renal biopsy should always be considered for every individual.

The next question is *at what time-point* a repeat biopsy should be performed. As discussed earlier, there is a possibility that histological improvement may occur after a longer period of time. However, in patients having repeat biopsies performed after 1 year, we found no difference regarding renal response compared to patients with early biopsies (data not included in the manuscript). In addition, previous studies on repeat renal biopsies performed after longer time periods all report on persisting active nephritis lesions in a proportion of patients (161, 186, 205, 208). As an early clinical improvement has been shown predictive for a good long-term outcome (148), it is likely that an early histological response may also be important for the long-term outcome.

No strict guidelines are available on the use of repeat biopsy to guide treatment decision-making in patients with LN, and it is not known whether serial biopsies are superior as a guide to appropriate therapy compared to clinical data alone. Given the problems to define clinical response criteria for LN and in the light of unsuccessful clinical trials (i.e. LUNAR), it has been suggested that second renal biopsies should be considered in the study design of future trials (221).

6.2.3 Biomarkers

Although a renal biopsy may provide detailed information on renal disease activity, biopsies cannot always be performed whenever a renal flare is suspected. There is a need for improved non-invasive biomarkers for early detection of renal flares, prediction of response to therapy, distinction between active disease and chronic damage and for identification of patients with a poor prognosis (191).

6.2.3.1 HMGB1

In **paper II** we report on high serum levels of HMGB, as well as an increased expression in renal tissue at both active disease and after treatment. Although there was

a trend towards lower levels at follow-up, the levels remained significantly high as compared to controls. Many patients also had active nephritis at follow-up biopsies, which suggest that the persistent high levels may reflect ongoing renal inflammation. There was a trend towards higher levels among patients who developed WHO class V nephritis, but we found no clear differences in serum levels comparing patients with or without a persisting active nephritis at follow-up. Although previously suggested as a biomarker for disease activity in SLE (79, 83, 227), we could in this study not fully determine if HMGB1 could be evaluated as a biomarker and longitudinal studies with repeated measures are required to further elucidate this issue.

6.2.3.2 IL-17

In **paper III** we found high baseline levels of IL-17 in patients with a poor histological response, and patients with persisting or increasing levels of IL-17 all had active nephritis at follow-up. Our results thus indicate that IL-17 not only is involved in the inflammatory process in LN, but also may be a valuable biomarker for renal disease activity. Of note, neither baseline levels of C3, C4, proteinuria, creatinine or anti-dsDNA predicted poor response, nor did any of the other cytokines measured.

Biomarkers that may predict renal response in LN are still needed to be defined. A large study on the patients included in the ALMS-trial (159) has investigated predictors of renal response, including baseline demographic, clinical, laboratory and histological characteristics (228). In that study, low baseline levels of C4, longer time since LN diagnosis and low baseline eGFR were all predictive of a poor renal response at 6 months. An early (8 weeks) normalization of complement and 25 % reduction in proteinuria were predictive of a good clinical response. However, no data on histological response was available in that study.

It has been proposed that novel biomarkers should not be compared to already existing insufficient biomarkers (such as proteinuria) but instead to renal biopsy findings (189). Thus, our findings of high IL-17 in association to histopathological response may clearly be of interest, and IL-17 may be evaluated as a novel biomarker for predicting renal response, or severity of the disease.

A recent review proposed that the ideal biomarker needs to be accurate, show good sensitivity and specificity, be relatively non-invasive and be reliably reproducible. Biomarkers should also demonstrate evidence in large, longitudinal, prospective studies and be cost-effective (121). Whether IL-17 will fulfil these criteria is to date unclear but deserves more investigation.

6.2.3.3 Antibodies

We found increased anti-dsDNA antibodies in all four papers with decreasing levels after treatment, clearly supporting their well-known role in LN. Although specific for diagnosis of SLE, their sensitivity and specificity for disease activity may be limited, thus questioning their role as biomarkers (192, 193). In **paper II**, anti-dsDNA antibodies were not fully reliable as biomarkers for renal response. However, in the

group of patients with baseline PN, histopathological non-responders overall had higher levels of anti-dsDNA at follow-up biopsies. In addition, anti-dsDNA correlated to the renal activity index at second biopsies. Thus, although not fully reliable, anti-dsDNA antibodies seem to be valuable biomarkers in many cases, but they probably should be combined with other markers to be more sensitive and specific in predicting renal disease activity. In our study, as much as 91 % of the patients were still anti-dsDNA positive after treatment and anti- dsDNA could thus not be used as biomarkers for renal response.

Anti-C1q antibodies have been reported to be superior over anti-dsDNA in some studies (110, 192). We have analyzed anti-C1q-levels in 57 of the patients from the repeated biopsy LN cohort and found increased levels of anti-C1q in LN-patients vs. controls, and levels decreased significantly after treatment. However, we found no correlation to renal biopsy activity index and a substantial proportion of patients were anti-C1q negative (48%) after immunosuppressive treatment despite tissue findings of active renal disease. No difference in either baseline or follow-up levels was noted in patients with poor vs. good histological response. Thus, they were not sensitive as biomarkers for renal disease activity in the current study (unpublished data).

6.2.4 Response

An interesting question in this context is of course: what is most important, histopathological or clinical response in LN? Early clinical response has been shown to be of importance for long-term prognosis in LN (148). Persistent inflammatory lesions on repeated renal biopsies have also been demonstrated to predict prognosis (205-207, 209). Given the discordant findings on clinical and histopathological response in **paper II**, it is likely that both clinical and biopsy findings may contribute to determine prognosis. Accordingly, combined clinical and histological findings may be the most accurate way to evaluate response to LN treatment.

The consensus criteria for clinical response in **paper II** (and **IV**), are well defined and easy to use and are comparatively strict in respect of the low-grade proteinuria required for CR (129). Although the BILAG index performed well for defining response in **paper IV**, it was developed as a disease activity index, based upon the physicians intention to treat, and not primarily as response criteria (96, 97).

How to define histological response has not been established, and the definitions used in **paper I** and **III** are not generally agreed upon. However, the reversal of active lesions should be an important component of a histological response definition. The presence of specific important histological findings on repeat biopsies (such as glomerular crescents, but also vascular and interstitial lesions), should also be taken into consideration.

To define response in MN is difficult, both regarding clinical and histological response. Most patients have persisting proteinuria, and specific serological markers for MN are lacking. In **paper II**, no MN patient achieved a CR and only one transformed in nephritis class (to class III C) at repeat biopsy and was thus regarded as HR.

In **paper IV**, of 7 patients with pure MN at baseline, only one changed nephritis class (to class II) after RTX treatment. In paper IV, 4/7 MN patients achieved a CR, of which all but one occurred after 12 months, indicating that a longer period of time may be needed to define response in MN. In a study on MN patients treated with RTX, electron-microscopy revealed resorption of subepithelial immune deposits on repeated biopsies (229), suggesting that electron microscopy evaluation on the amount of immune deposits may be an alternative tool to determine histological response in MN.

6.2.5 Long term outcome and prognosis

In **paper II**, evaluating long-term renal outcome in 56 patients with a median of 10 years since baseline biopsy, 25 % had developed CKD (eGFR<60 ml/min). Of these, 3 (5%) had ESRD. In patients defined as having CKD (although not ESRD), the median eGFR was 52 ml/min (31-59) as estimated by MDRD and, none had developed doubling of creatinine. The overall renal outcome of the patients was comparatively good (102, 143, 148, 209).

The limitations of the long-term evaluation have been discussed (chapter 6.1.1) and several factors have possibly influenced the renal outcome. However, our approach with repeated biopsies, and taking both clinical and renal histological findings into account for treatment decisions, may at least partially have contributed to the overall good renal outcome. One possible factor that may have influenced the outcome is that the patient cohort mainly consisted of Caucasians, known to have less severe disease in general. Another factor that may have had impact on the long-term results is that many patients received RTX later in the course of the disease. A beneficial effect of RTX *per se* and tight control when participating in a study (**paper IV**), including frequent clinical and laboratory evaluation, control of blood pressure and other co-morbidities may both have contributed to the favourable outcome.

Although only a limited number of patients developed ESRD, 25 % of the patients had CKD according to the definition here used, which clearly points to the need for improved treatment strategies in a proportion of patients. It is well known that impaired renal function in the general population is associated with cardiovascular disease (CVD) and increased mortality (230), also shown in SLE (231). In SLE patients, increased Cystatin C as a marker of renal impairment was demonstrated to be one of the strongest risk factors for death due to CVD (232).

6.2.6 Future perspectives

One of the main focuses of this thesis is on response to treatment in LN. In the future, response criteria that can be generally used will hopefully be established. One proposal is that such criteria should include both clinical and histological response criteria, and both short and long term evaluation. However, the ultimate goal for the future would be to find a biomarker that accurately reflects the inflammation in renal tissue findings and thus reduce the need for biopsying.

A proportion of patients do not respond to standard immunosuppressive treatment, renal relapses and side effects of current therapies are still a concern and long term outcome remains unsatisfactory (150,233), as was also shown in my studies. Thus, there is definitely a need for improved and more targeted treatment strategies in LN.

The increased knowledge about the pathogenesis for SLE and the successful use of biologics in other autoimmune diseases has encouraged the development of biologic agents targeting different immune pathways in SLE, of which many currently are studied. It is important that trials on new treatments in LN will use standardized and generally accepted response criteria. The disappointing results of recent trials on LN, i.e. the LUNAR study (217) have high-lightened the need for improved response definitions and end-points in LN-trials (183, 221, 225).

Future studies on RTX in LN have been suggested to focus on therapy resistant cases (221), and the role of combination with other therapies needs to be studied. Other B-cell therapies, such as blocking of BAFF may turn out to be effective as maintenance therapy in LN but needs to be studied. Another approach for B-cell depletion would be anti-CD19, thus also targeting plasmacells, not yet studied in LN (234).

Future studies on the role of HMGB1 in LN are of great interest. Immunostaining of renal tissue revealed an extranuclear HMGB1 expression in all the examined biopsies from LN-patients in our study. However, it is not clear if the HMGB1 detected in renal tissue was bound to a specific receptor, or if it was in complex with nucleosomes or ICs deposited within the kidney. Furthermore, the source of the increased HMGB1 is unknown, it could be derived from increased circulating apoptotic cells or from injured or activated residual renal cells (235). It would be of great interest to study the precise localisation, and the eventual co-localisation with immune deposits, in renal tissue from LN patients.

Another idée is to study urine levels of HMGB1 longitudinally, possibly in association to renal histological findings in order to further investigate if HMGB1 could be a valuable biomarker for disease activity in LN. HMGB1-blocking therapies have been tested in animal models of sepsis and arthritis with promising results, but it is uncertain whether it will be used in humans in the future (236) .

Future studies on IL-17 in LN, including immuno-stainings of renal tissue, as well as repeated serum analyses, before and after immunosuppressive treatments are underway in our group. Our aim is to study the IL-17 expression in renal tissue in association to histological response and its role as a biomarker.

Recent studies, and also our findings on IL-17 in LN, indicate that the IL-23/IL-17 pathway may be a possible target for future therapies, but remains to be studied (63, 237, 238). Interestingly, it has been proposed Th17 cells may have a specific role in some but not all SLE patients. A genetically defined subgroup of SLE patients, or patients with specific disease manifestations, would thus respond to anti-IL-17 therapy (239, 240).

7 CONCLUSIONS

LN patients may have persistent inflammation and signs of immune dysregulation after induction immunosuppressive treatment, in many cases also in the setting of clinically low disease activity.

Repeated renal biopsies revealed persistent active nephritis in a proportion of patients with clinical quiescent disease. Histopathological findings on repeat biopsies added information that was not captured by currently used biomarkers.

Thus, until improved biomarkers are available, second biopsies may be a valuable complement to clinical variables for treatment evaluation in LN.

HMGB1 is increasingly recognized to be an important player in the complex pathogenesis for SLE. The increased expression of HMGB1 in renal tissue from LN patients, as well as the increased serum HMGB1 levels at both active disease and after treatment in our study strongly support a role for HMGB1 in the inflammatory process in LN and deserve further studies.

Having repeated biopsies available gave us unique possibilities not only to evaluate histological response to treatment, but also to analyse both conventional and novel biomarkers in association to renal histopathological findings. High serum levels of IL-17 were predictive of poor histological response to treatment. IL-17 may be evaluated as a biomarker for treatment response.

A majority of patients with previously refractory or relapsing LN achieved a CR when treated with RTX. Most patients achieved CR after the first year, and an earlier response was associated with a longer time of B-cell depletion. Despite lack of formal evidence, we conclude that RTX could be considered in LN-patients not responding to standard therapy.

The patients had overall favourable long-term renal outcome, which may be due to a number of factors. However, repeated biopsies helped to identify patients with persisting active nephritis that influenced treatment in many cases. This approach may at least in part have contributed to the results.

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