Alterations in the renal microenvironment during the development of lupus nephritis

*Longitudinal studies in a murine model of systemic lupus erythematosus*

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A dissertation for the degree of Philosophiae Doctor

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2. LIST OF PAPERS


IV. Alterations in Wnt pathway activity in serum and kidneys during lupus development. Tveita A, Rekvig OP. Arthritis & Rheumatism. 2010. Accepted for publication

APPENDIX II

3. ABBREVIATIONS

ANA: Anti-nuclear autoantibody
ADAM: A disintegrin and metalloproteinase
APC: Antigen-presenting cell
B/W: Mice of the (New Zealand Black x New Zealand White)F1 progeny
DAMP: Damage-associated molecular pattern
DIF: Direct immunofluorescence
DKK: Dickkopf
Dnase: Deoxyribonuclease
dsDNA: Double-stranded DNA
ECM: Extracellular matrix
EDS: Electron-dense structure
GBM: Glomerular basement membrane
GSK3b: Glycogen synthase kinase 3b
HSPG: Heparan sulphate proteoglycans
IC: Immune complex
ISR: International Society of Nephrology
JNK: c-Jun N-terminal kinase
LN: Lupus nephritis
LRP: LDL receptor-resembling protein
MHC: Major histocompatibility complex
MMP: Matrix metalloproteinase
mRNA: Messenger RNA
PAMP: Pathogen-associated molecular pattern
RES: Reticulo-endothelial system
RPS: Renal Pathology Society
SDS: Sodium dodecyl sulphate
sFRP: Secreted frizzled protein
SLE: Systemic lupus erythematosus
TdT: Terminal deoxynucleotidyl transferase
TIMP: Tissue inhibitor of metalloproteinases
TLR: Toll-like receptor
TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling
UUO: Unilateral ureteral obstruction
WIF-1: Wnt-inhibitory factor 1
4. INTRODUCTION

4.1 Historical perspectives

The history of systemic lupus erythematosus (SLE) dates back to medieval times, with accounts of women with a characteristic symmetrical erythematous rash of the cheeks and nasal bridge, resembling a wolf's (Latin: lupus) bite. The first detailed descriptions of the disease are found in the writings of medical pioneers of the late 1800s, including Móric Kaposi and Sir William Osler, as reviewed in [1]. In the more than 100 years that have passed since Osler’s papers describing the many facets of this complex disease, SLE has been a subject of great fascination and frustration amongst medical researchers and clinicians worldwide.

It was not until 1948 that Dr. Hargraves, a physician at the Mayo Clinic, made the first observations identifying SLE as an autoimmune disease [2]. In the late 50s, it was discovered that lupus patients had high levels of circulating autoantibodies against nuclear structures (anti-nuclear autoantibodies; ANA), and diagnostic immunofluorescence assays were developed for the demonstration of ANAs. Closer examinations of the binding targets of ANAs revealed a particular affinity for double-stranded DNA (dsDNA) and histone peptides. Although ANAs are found in several systemic autoimmune disorders, anti-dsDNA autoantibodies displayed a very high specificity for SLE. Since then, the basis for the emergence of autoreactivity against double-stranded DNA (dsDNA) has been one of the main topics in SLE research.

One of the most common manifestations of SLE and a major contributor to the morbidity and mortality of the disease, lupus nephritis (LN) is the end-organ disease that has been most extensively investigated. The most apparent feature of the renal disease is the development of glomerulonephritis, associated with the deposition of autoantibodies within glomerular membranes.
4.2 Epidemiology and classification of SLE

4.2.1 General features

SLE is a disease of the young, with 85% of patients developing the disease before the age of 55 [3], of which 20% are children below 16 years of age [4]. Estimates of the prevalence of SLE vary greatly, ranging from 20 to 150 per 100 000 [5,6], higher among Afro-Americans and Asians. The incidence has risen threefold during the last 40 years, owing to improved detection of mild disease [7]. A sex hormone influence on pathogenesis is assumed based on the 7:1 to 15:1 female-to-male ratio of SLE [8]. In further support of this view, in children the female-to-male ratio is 3:1 [9].

Susceptibility for SLE development is assumed to have a genetic basis, however concordance rate estimates in monozygotic twins vary greatly (14-69%) [10]. These figures are similar to those found for other autoimmune diseases. Several SLE predisposing loci have been identified, of which the major histocompatibility complex (MHC) locus is the most prominent. Apart from very rare single gene mutations that cause lupus-like diseases (reviewed in [11]), no single gene polymorphism has been identified that by itself causes high SLE susceptibility.

4.2.2 Diagnostic and prognostic markers in SLE

SLE can manifest itself by a wide variety of symptoms, affecting virtually any organ, and the pattern of symptoms may vary considerably over time. The most commonly involved tissues include skin, the musculoskeletal and hematological system, kidneys, the cardiovascular system and the central nervous system.

The diagnosis of SLE can be challenging, especially in patients presenting with symptoms from only one organ or with ill-defined systemic complaints. A classification scoring system has been developed that includes the most commonly
observed clinical and serological disease manifestations. This system was developed by the American College of Rheumatology (ACR) for the purpose of classification in clinical studies, and not as a diagnostic tool, but is still widely utilized by clinicians as an aid in establishing the diagnosis of SLE [12]. The 11 ACR criteria in their latest, revised form [13] are listed in Appendix 1. For a definitive diagnosis of SLE, 4 or more criteria should be fulfilled. Given the dynamic nature of SLE manifestations, not all criteria need to be present at the same time.

4.3 Pathophysiology of SLE

4.3.1 Emergence and maintenance of autoimmunity

The models to explain SLE pathogenesis are as varied as they are many, which seems to be the case for several of the autoimmune diseases. Classically, they include i) infectious processes in which cross-reactivity against self antigens is responsible for autoimmune reactions [14], ii) disturbances in lymphocyte regulation that generates a state of immunological hyper-reactivity, iii) environmental effects [15], and iv) genetic disturbances in various aspects of tissue homeostasis. During the last decade, research has been largely focused on the latter type of models, which will therefore be the focus of the following discussion and for the analysis of the present results.

The mechanisms underlying the appearance of circulating autoantibodies against nuclear structures, and dsDNA in particular, is by many considered the key to understanding SLE. Indeed, studies have shown that the presence of circulating DNA/anti-dsDNA immune complexes maintained by repeated injections can mimic SLE manifestations such as glomerular immune complex deposition and the development of proteinuria [16]. Of note, renal disease is reversed upon terminating the injections. Similarly, injection of monoclonal autoantibodies isolated from SLE
patients and B/W mice cause reversible proteinuria, most likely by binding to their antigen targets forming immune complexes, either within the circulation or locally within the kidneys [17,18,19].

The dominance of nuclear autoantigens and the presence of chromatin-associated antigens in immune complex deposits in SLE has led to the hypothesis that deficiencies in the clearance of dying cells serves as a source of autoreactivity. Normal cell death through apoptosis is a well-orchestrated process in which cellular antigens are rapidly degraded within intact membranes. The release of chromatin into the extracellular milieu triggers inflammation through the exposure of so-called danger signals [20,21,22]. These are endogenous structures that are exposed within the extracellular milieu in the setting of various kind of cellular damage, and include a growing number of substances including high-mobility-group box 1, heat shock proteins and uric acid, as reviewed in [23]. Release of danger signals can occur during necrotic cell death, which disrupts cellular membranes and causes leakage of intracellular debris. During apoptosis, however, efficient degradation of chromatin by endonucleases is considered one of the hallmarks of the early stages of cell death.

Apoptotic cells are efficiently cleared by phagocytosis by tissue macrophages and to various extents by other cell types. If phagocytosis is inhibited, retained apoptotic cell fragments will degrade, undergo secondary necrotic transformation, and trigger the release of pro-inflammatory factors including chromatin [24]. Previous studies have found clearance of apoptotic cells to be impaired in SLE patients [25,26,27,28]. Similarly, patients with genetic deficiencies of the complement factor C1q which is involved in the uptake of apoptotic cells by phagocytosing cells commonly develop SLE [29]. Blood from SLE patients have increased levels of apoptotic leukocytes [30,31]. Taken together, these data suggests the occurrence of an
apoptotic cell clearance deficiency in SLE. Under circumstances of increased apoptotic cell loads, such a deficiency would provide a source of secondary necrotic cells, trigging inflammation and the development and maintenance of autoimmunity [32]. Such a model might explain the tendency of various types of tissue damage, including trauma, infections [33] or ultraviolet light [34] to cause “disease flares” [35] in SLE.

4.3.2 Production, elimination and deposition of immune complexes

Immune complexes are generated by the interaction of antibodies and soluble forms of the corresponding antigen. Such complexes may form within the circulation or locally within the tissues. The size and solubility of immune complexes is determined by the molar ratio of antigen to antibodies as well as the size and number of binding sites on the antigen [36]. Immune complexes are predominantly cleared by the reticulo-endothelial system (RES) of liver and spleen by Fc-receptor binding and subsequent phagocytosis [36,37]. When the levels of circulating immune complexes is increased, immune complexes can also be found deposited in the extracellular matrix of several organs, including the glomerulus [38], lungs [39], skin [40] and blood vessels [41]. Under normal circumstances, such immune complexes are rapidly and effectively cleared, as evidenced by experimental injection of large amounts of immune complexes [42], but retainment can trigger local inflammatory disease, such as is the case in vasculitides and glomerulonephritides. The dynamics of immune complex formation and its deposition within tissues are topics of great interest in SLE research, as they are considered a key mediator of end-organ disease.
4.3.3 Animal models for the study of SLE

SLE is not strictly a human disease, and is known to occur spontaneously in animals, especially in dogs, where the incidence is considerable [43]. For practical reasons, mice have become the preferred subject of studies of SLE. The most extensively studied model of spontaneously developing SLE-like disease is the F1 crossbreed progeny of the highly inbred mice New Zealand Black (NZB) and New Zealand White, referred to as (NZBxNZW)F1. Whereas the parental strains can develop proteinuria and evidence of autoimmune disease at old age, their offspring consistently develop an autoimmune phenotype with anti-dsDNA autoantibodies, progressive lupus-like glomerulonephritis, splenomegaly and arthritis occurring at around 20-35 weeks of age, and eventually they die from end-stage renal failure or cardiovascular disease [44,45].

4.4 Lupus nephritis

4.4.1 General features of lupus nephritis

In the early 1950s lupus nephritis was the main contributor to early death in SLE patients, with an estimated 5-year survival rate of 25-40% following the diagnosis of nephritis [46]. Over the ensuing 35-40 years, advances in clinical medicine including cytostatic therapy, immunosuppressants, antihypertensive drugs and new antibiotics, as well as the introduction of dialysis and renal transplantation have resulted in a drop in 5-year mortality from LN to <10% [47]. Still, aggressive LN subtypes require high-intensity immunosuppressive regimens and contribute considerably to the growing number of renal transplant recipients and patients requiring chronic dialysis, so the burden of disease and quality-of-life effects of lupus nephritis in SLE patients is still considerable.
Estimates of occurrence of the spectrum of renal disease in SLE patients vary from 35% to over 90% [48,49]. This likely reflects the great diversity in the clinical manifestations and disease severity of lupus nephritis. Whereas in most patients LN is limited to asymptomatic, self-limiting low-grade proteinuria (proteinuria is found in about 42% of SLE patients [50]), others progress towards end-stage renal failure in a matter of weeks to months, and respond poorly even to high-intensity immunosuppressive therapy.

There are several challenges in dealing with lupus nephritis. Firstly, clinical symptoms as well as laboratory findings have low predictive value in identifying patients at risk of developing severe renal disease. Monitoring of anti-dsDNA and other groups of autoantibodies has been proposed to serve as indicators of renal disease activity [51,52,53,54], but in general, their sensitivity and specificity are unsatisfactory, and their utility in clinical practice remains controversial. Evaluation of LN therefore generally requires histopathological examination of renal biopsies to establish the nature and extent of renal pathology, although the prognostic information provided by a kidney biopsy is limited by the fact that transformation of one morphological pattern to another occurs in 15 to 40% of patients during serial follow-up [55,56,57]. The requirement for an invasive study such as a renal biopsy as part of initial and follow-up disease monitoring is largely impractical and carries considerable procedural risk, so there is a great interest in identifying useable markers of disease progression in LN. A number of assays in both serum and urine have been evaluated, but so far, none of these have found practical application.
4.4.2 Classification of lupus nephritis

Several systems of classification have been developed to categorize the complex and overlapping manifestations of lupus nephritis, taking into account both histopathological appearance, patterns of immune complex deposition as well as clinical presentation, progression and prognosis.

The most common clinically used classification system today is that of the International Society of Nephrology and Renal Pathology Society (ISR/RPS) working group on the classification of lupus nephritis, which is periodically revised by a consensus meeting, the latest revision undertaken in 2003 [58] (Table 1). This system divides LN into six major classes, with the dominating pattern of cellular proliferation and matrix expansion being the defining characteristic. Also, the pattern of glomerular immune complex deposition, as visible by direct immunofluorescence (DIF) and electron microscopy, differ between the classes. This classification system has proven valuable as a prognostic index, but is limited by a considerable overlap and unpredictable changes in histological patterns in the individual patient over time.

**Table 1 - Abbreviated International Society of Nephrology/Renal Pathology Society (ISN/RPS) classification of lupus nephritis (2003)**

<table>
<thead>
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<th>Class</th>
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<tr>
<td>Class I</td>
<td>Minimal mesangial lupus nephritis</td>
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<tr>
<td>Class II</td>
<td>Mesangial proliferative lupus nephritis</td>
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<tr>
<td>Class III</td>
<td>Focal lupus nephritis</td>
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<tr>
<td>Class IV</td>
<td>Diffuse segmental (IV-S) or global (IV-G) lupus nephritis</td>
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<tr>
<td>Class V</td>
<td>Membranous lupus nephritis</td>
</tr>
<tr>
<td>Class VI</td>
<td>Advanced sclerosing lupus nephritis</td>
</tr>
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</table>

Generally speaking, class I- and II changes are considered mild renal disease, and rarely progress to renal failure in humans, whereas classes III and IV are the subtypes classically associated with rapidly progressive glomerular damage. Class V has a lower rate of progression to end-stage renal disease, but is associated with an increased occurrence of thromboembolic diseases. Class VI is characterized widespread glomerulosclerosis in the absence of active inflammatory activity, and represents the end stage healing process following burned-out chronic or acute forms of nephritis.

4.4.3 Pathogenesis of lupus nephritis
Lupus nephritis is characterized by the appearance of immune complex deposits along glomerular membranes and within the mesangium. These immune complexes typically consist of DNA-anti-dsDNA aggregates, but other antigens are localized to these regions as well, including nucleosomal chromatin, histones, complement factor C1q, Sm, SSA/SSB, ubiquitin and ribosomal proteins [59]. The site of origin of these immune complexes is a matter of controversy. The proposed mechanisms for their appearance include in situ formation by binding of circulating autoantibodies to membrane-bound antigens or “trapping” of circulating immune complexes either passively or by affinity of the immune complex antigens against extracellular membrane components, as discussed in [60,61]. Regardless of the mechanism of their appearance, it is clear that the pathological effects of immune complexes are largely affected by their localization within the glomerulus. This is reflected in the extensive classification system needed to encompass the various faces of lupus nephritis.

Mesangial and subendothelial immune complex deposits are in contact with the vascular compartment, allowing for complement activation and consequent
chemokine release and influx of inflammatory cells. This leads to cellular proliferation with generation of active urinary sediments, proteinuria and often-rapid progression towards end-stage renal failure. In contrast, in patients with membranoproliferative disease (Class III/IV), biopsies show evidence of subepithelial immune complex deposits separated from the vasculature by the glomerular basement membrane (GBM). This prevents the release of chemoattractant proteins into the circulation. Patients with such deposits therefore present with proteinuria without signs of active sediments, and biopsies show histological signs of membranous nephropathy, with a diffusely thickened GBM but little evidence of cellular proliferation or inflammatory cell infiltrates (Class V).

4.4.4 Glomerular extracellular membranes and matrices in lupus nephritis

Apparent expansion and structural changes in the glomerular extracellular membranes can be seen even in patients with mild forms of lupus nephritis (Class I/II). The key importance of the site(s) of immune complex deposition for the clinical manifestations of lupus nephritis has led to an interest in understanding the interactions between immune complexes and the GBM. Moreover, the structural changes occurring within this highly specialized barrier are thought to be important in the formation of a dysfunctional glomerular filtration system and the development of proteinuria.

The term GBM refers to the extracellular matrix (ECM) interface that separates the endothelial cells of the glomerular capillary lumen and the podocytes that line the visceral side of the capsular space (Bowman’s space), as illustrated in Figure 1. In addition to the GBM, the glomerulus contains a more extensive mesangial extracellular matrix that branches out throughout the interstitial space of the
glomerular corpuscle. Not a part of the glomerular filtration barrier, this membrane is less specialized than the GBM, but is nonetheless a common location for immune complex deposits. The term “glomerular extracellular membranes” is therefore used herein to include both the GBM and the mesangial matrix.

The glomerular extracellular membranes are composed of a number of proteins, including collagen, heparan sulphate proteoglycans (HSPG), laminins, and fibronectin [62,63]. These form a complex meshwork that serves a number of purposes including the provision of mechanical strength of tissue compartments, sequestration of signaling substances and matrix-cell interactions involved in anchoring as well as cellular growth and differentiation [64].
The GBM is characterized by the presence of particular subsets of collagen IV molecules which are thought to be important for its functions in ultrafiltration [65]. Histological and immunohistochemical analyses have indicated that the collagen IV matrix expands during the development of lupus nephritis, but it is unknown whether this reflects increased accumulation of collagen or structural changes that alter the appearance of the membrane. Previous experiments in our group have shown a considerable affinity of chromatin particles to extracellular matrix proteins, including collagen IV [66], suggesting that ECM structure may influence the tendency for immune complexes to accumulate within glomerular membranes. It would seem possible that increased availability of collagen IV and alterations in the structural integrity of the collagen matrix could contribute to immune complex deposition during disease progression.

Collagen IV is secreted as monomers that join end-to-end, forming long fibrillar chains. Cross binding of fibrils generates a tightly woven collagen network. The collagen IV monomers are composed by three intertwining chains, of which there are six varieties, named collagen IV α1 through α6. Various triplet combinations form different collagen IV isomers, which have different properties in terms of tissue distribution and biophysical properties [67]. The biological importance of various collagen IV isoforms is well illustrated by hereditary glomerulopathies such as Alport syndrome, in which nonsense mutation of the col4a3 gene, encoding the α3 chain of collagen IV, prevents the expression of the collagen IV isoform α3α4α5 within the GBM [68]. These patients have a thickened and dysfunctional GBM and develop proteinuria progressing to end-stage renal disease [69,70].
4.4.5 Matrix metalloproteinases in extracellular membrane turnover

The proper structure and functioning of extracellular matrices relies on its constant renewal, and homeostasis is achieved by balancing synthesis of membrane components and their destruction by various secreted proteases. The enzymes responsible for membrane degradation include members of the serine and threonine proteinases families and the matrix metalloproteinases (MMPs). The MMP family of proteinases encompasses over 20 different divalent cation-dependent enzymes with diverse and overlapping substrate specificities ([an updated list of substrates is available at the Overall lab webpage][71]). Of these, MMP-2 and -9 (referred to as the gelatinases) are notable for their affinity for collagen IV, and has therefore been a focus of the current work. A schematic overview of their structure is shown in **Figure 2**. The gelatinases show wide tissue distribution, and serve a number of biological functions.

*From Tveita et al., Arthritis Research & Therapy 2008 10:229*
A more comprehensive review of the structure and functions of the gelatinases and a discussion of their relevance to various renal pathologies including lupus nephritis [72] has been included as an appendix (Appendix 2).

Within the glomerulus, MMP-2 appears to be the predominant gelatinase in healthy individuals, showing constitutive expression in both mesangial cells and podocytes [73]. MMP-9 expression, on the other hand, appears to be induced by various pro-inflammatory signaling cascades under circumstances of tissue injury, especially in mesangial cells. MMP-9 is also expressed in activated infiltrating macrophages/monocytes in inflammatory states [74,75]. MMP enzyme activity is negatively regulated by tissue inhibitors of metalloproteinases (TIMP) 1-4. The MMP/TIMP ratio is therefore essential in regulating net MMP activity in vivo, although this view is somewhat simplistic, as reviewed in [72].

Gene expression analyses have indicated increased expression of MMP-2 and MMP-9 in both human and murine lupus nephritis [76,77], but similar increases in expression of several of the TIMPs complicates the picture [78,79,80], and the net effects on gelatinase activity remains uncertain. Also, the tissue distribution of MMP and TIMP expression within the kidney in lupus nephritis is undetermined.

4.5 Wnt signaling

Amongst the cellular signaling pathways known to affect the synthesis and turnover of extracellular matrices, Wnt-signaling has recently been found to play a role in the development of proteinuria [81] and renal fibrosis [82] in several animal models. Emerging evidence suggest that activation of Wnt signaling may induce synthesis of both matrix metalloproteinases [83] and various ECM constituents, including collagens and fibronectin [82]. These data makes renal Wnt signaling a potentially
important element in understanding the alterations in the glomerular extracellular milieu occurring as part of the pathogenesis of glomerulonephritis.

The Wnt pathway is a signaling pathway found to be highly conserved within a wide number of organisms. Originally identified to be involved in embryogenesis, it has recently been discovered that Wnt signaling also influences adult tissue homeostasis, and it has been implicated in several human diseases, especially malignant diseases, where it seems to be important in regulating cancer growth and metastasis.

With growing insight into the complexity of Wnt signaling, it is apparent that the Wnt pathway branches out to at least three downstream cascades. The following sections will focus entirely on what is referred to as the canonical Wnt/beta-catenin pathway, as this has been the topic of studies in the present work. Suffice it to mention, therefore, that Wnt signaling also includes the non-canonical Jun kinase and Wnt/Ca$^{2+}$ pathways [84]. Furthermore, there is widespread crosstalk between all branches of Wnt signaling and other pathways, an insight that is continuously expanding.

The Wnt/beta-catenin pathway is activated through binding of one of the many secreted Wnt proteins (Wnt1-11 and Wnt16) to transmembrane receptors of the Frizzled or Lipoprotein receptor-related protein (LRP) families. Activation of these receptors induces dephosphorylation of the transcription factor beta-catenin [85]. This enables beta-catenin to translocate to the nucleus, allowing it to form a complex with T-cell factor (TCF) to activate the transcription of a number of Wnt target genes. The activity of the Wnt pathway is modulated by a multiprotein complex consisting of glycogen synthase kinase 3 (GSK3b), Axin and the tumor suppressor gene adenomatous polyposis coli (APC). This complex phosphorylates beta-catenin, targeting it for proteosomal destruction, as illustrated in Figure 3.
In addition, Wnt signaling activity can be modulated by secreted Wnt antagonists, which include the Dickkopf (Dkk) family of proteins, secreted Frizzled proteins (sFRPs), Wnt inhibitory factor (WIF-1) and Soggy [85]. The DKK proteins bind to LPR5/6, preventing interaction with Wnt/Frizzled complexes, and promote internalization of the LPR receptors. Dkk-1 serves as a direct inhibitor of the Wnt/beta-catenin pathway [86], whereas the effects of Dkk-2/-3 are less clear. The sFRPs and WIF-1 work by binding to Wnt proteins, preventing their binding to Frizzled receptors. sFRPs can also form inhibitory complexes with Frizzled receptors (reviewed in [85]).

In unstimulated cells, beta-catenin is rapidly degraded through the proteosomonal apparatus. In adult human tissues, constitutive Wnt signaling activity is generally low, with the notable exception of tissue stem cells. In recent years,
however, it has been found that Wnt activity is increased during the course of several diseases. The growing list of diseases in which Wnt signaling has been implicated includes many types of cancer, neurologic diseases, bone, kidney and skin diseases, as reviewed in [87]. The involvement of the Wnt pathway in renal diseases was first discovered with the finding that mRNA expression of the Wnt agonist Wnt4a was highly increased in experimental unilateral urethral obstruction (UUO) [88], a rat model of obstructive uropathy. In the same model, suppression of Wnt signaling by injection of recombinant sFRP4 [89] or Dkk-1 [82] appeared to attenuate progression of renal fibrosis. Similarly, modulation of glomerular Wnt signaling has been found to affect the progression of various forms of glomerulopathies. Studies have shown that glomerular damage and subsequent development of proteinuria induced by adriamycin could be strongly attenuated by suppression of beta-catenin expression [81]. In contrast, diabetic nephropathy in rats is associated with a Dkk-1-mediated reduction in Wnt pathway activity, and preserving Wnt signaling yielded favorable results in this model [90]. These conflicting results demonstrate that the effects of renal Wnt signaling are not clear-cut. One possible explanation could be the different nature of these diseases; the former representing an acute inflammatory glomerulopathy, whereas the latter constitutes a chronic process dominated by fibrosis rather than inflammation. Similar differences are apparent when analyzing the consequences of altered matrix metalloproteinase activity in various glomerulopathies, as discussed in [72].

From the perspective of SLE, another interesting aspect of Wnt signaling is its apparent involvement in apoptotic signaling. It appears that Wnt activation has anti-apoptotic effects, at least in vitro, by increasing the threshold for induction of apoptosis by various stimuli [91]. Conversely, Dkk-1 serves as a pro-apoptotic factor
It remains a matter of controversy whether Dkk-1 can itself induce apoptosis. Work in immortalized cell lines and various cancer cell lines has found Dkk-1 to lower the threshold for apoptosis induced by other stimuli, but find no direct apoptosis-inducing effect [94,95]. In contrast, over-expression of Dkk-1 appeared to have direct apoptosis-inducing effects in hepatic stellate cells [96] and in a melanoma cell line [97]. The latter study found the apoptosis-inducing effect to be mediated through activation of c-Jun N-terminal kinase (JNK) rather than through the canonical Wnt/beta-catenin pathway. Similar results were also seen in placental choriocarcinoma [98]. A recent study in SLE patients found consistently increased serum levels of Dkk-1 compared to normal healthy controls, although no significant association was found between Dkk-1 concentration and commonly used serological or clinical disease indices [99]. Although these results are inconclusive with respect to the origin or consequences of the increase in circulating levels of Dkk-1, they provide clues to a potential role of alterations in Wnt signaling during the course of SLE.

4.6 Deoxyribonucleases and autoimmune diseases

Autoantibodies against double-stranded DNA (dsDNA) is considered a hallmark of SLE, and anti-dsDNA antibodies are reported to occur in about 75% of SLE patients at some point [100]. Numerous studies report autoantibodies against DNA and DNA-containing particles (nucleosomes/chromatin) as a major constituent of glomerular immune complex deposits [101,102,103,104].

Previous studies have confirmed the presence of DNA and chromatin-associated antigens in electron-dense structures at the ultrastructural (electron microscopy) level using both antibody-based and terminal deoxynucleotidyl
transferase dUTP nick end-labeling (TUNEL) methods [105,106]. Together, these studies provide basis for the assumption that chromatin structures form an important antigen target in SLE and in LN [107,108]. The occurrence of chromatin structures deposited within the GBM is a surprising finding, as chromatin degradation by endonucleases is a key event in the orderly process of apoptotic cell death [109,110,111], as discussed in section 4.3.1. By contrast, death by necrosis causes leakage of chromatin particles through a disrupted plasma membrane, and externalized chromatin serves as a potent danger signal, causing immunological activation of nearby antigen-presenting cells and induction of a pro-inflammatory response [112]. If apoptotic cells are not efficiently cleared, leakage of chromatin particles and secondary necrotic transformation is seen [113].

Increases in circulating DNA content is a common finding in a variety of inflammatory diseases, including trauma [114], myocardial infarction [115] and cancer [116]. How such circulating DNA is removed is not known, but it is assumed that secreted deoxyribonucleases (Dnases) play an important role. SLE patients have been reported to harbor higher levels of circulating DNA than the baseline population [117,118,119]. These DNA structures have been reported to be sizes corresponding to the expected sequence length of mono- and oligonucleosomes [120], which would seem to indicate that chromatin degradation products formed during cell death accumulate in the extracellular milieu in these patients, either as a result of massive cell death or faulty chromatin disposal [121,122]. At the same time, it has been known since the 1960s that serum Dnase activity is decreased in SLE patients [123].

Deoxyribonuclease 1 (Dnase1) was the first secreted Dnase to be identified in serum. The relative contributions of various tissues in the synthesis of circulating Dnase1 is unknown, although the gastrointestinal tract, kidneys, liver and spleen are
considered major sources [124]. Dnase1 appears to be responsible for the bulk of DNA degrading capacity within the circulation [125]. However, Dnase1\(^{-/-}\) knockout mice showed evidence of residual Dnase activity that has been attributed to the enzyme Dnase1-like 3 (Dnase1L3, also known as Dnase gamma) [126]. To date, the list of known secreted endonucleases include Dnase1 as well as the Dnase1-like family (Dnase1L1 through -3). These proteins vary in tissue distribution, and their biological importance remains unclear.

Based on the above-mentioned findings of decreased serum nuclease activity in SLE patients, there has been a great interest in disturbed function of Dnase1 as a potential mediator of extracellular chromatin accumulation, and possibly the emergence of antinuclear autoreactivity. Dnase1-deficient mice were found to produce anti-dsDNA and anti-nucleosomal antibodies, glomerular immune complex deposition and development of proteinuria [127]. Furthermore, a 2001 article reported the presence of a heterozygous Dnase1 non-sense mutation in two Japanese SLE-patients displaying very high levels of anti-nucleosomal antibodies [128]. Reduced levels of Dnase1 has been observed in sera of SLE patients, with a inverse correlation between disease activity and serum Dnase1 activity [129]. The same is true for serum and urine Dnase1 activity in young (NZBxNZW)F1 mice [130].

The origin of reduced Dnase1 activity in SLE patients has been a matter of controversy. Some authors have found circulating inhibitors of Dnase1, including glomerular actin [123] and neutralizing autoantibodies [131] to be responsible, whereas others have not been able to reproduce these results [129]. Of note however, many of these studies have been limited by their reliance on single non-specific \textit{in vitro} assays of Dnase activity. Interpretations on alterations in Dnase1 activity are complicated by the currently limited knowledge of the factors controlling its
expression, and as stated above, the existence of a family of incompletely characterized Dnase1-like enzymes with similar biochemical characteristics.

Despite the focus on Dnase1 activity in the circulation of SLE patients, little is known about its expression and activity within the tissues. A recent study of apoptosis-related gene expression in kidneys of B/W mice revealed a dramatic decrease in Dnase1 mRNA expression accompanying the development of proteinuria [132]. Follow-up studies revealed a close association between renal Dnase1 mRNA levels and the development of severe renal manifestations, including glomerular capillary immune complex deposits and histopathological changes consistent with membranoproliferative lupus nephritis [133], which suggest that renal Dnase1 may contribute to the progression of particular subsets of lupus nephritis.

The association between the appearance of chromatin-associated immune complexes within the GBM and the apparent disappearance of an enzyme assumed to play a key role in eliminating extracellular chromatin offers an attractive hypothesis to explain how tissue-specific changes in gene expression could influence the pattern of immune complex deposition [134,135]. This also provides a potential link between systemic autoimmunity and the development of end-organ disease.
5. AIMS OF THE THESIS

• Investigate the mechanistic basis of apparent matrix expansion in developing lupus nephritis and its relevance to the formation of electron-dense structures (Paper I and II)

• Characterize changes in collagen IV synthesis and turnover during the course of development of lupus nephritis in (NZBxNZW)F1 mice. (Paper II)

• Identify proteases responsible for collagen degradation within the glomerulus in lupus nephritis. (Paper I and II)

• Evaluate location, expression and activity of the major renal endonuclease Dnase 1 in kidneys during the development of nephritis. (Paper III)

• Assay the regulation of the canonical Wnt pathway in lupus development, and its possible influence on apoptosis. (Paper IV)
6. MATERIAL AND METHODS

6.1 Animals and tissue collection

All experiments were approved by the Norwegian Ethical and Welfare Board for Research Animals. Female BALB/c and B/W mice were used, and urinary dipstick analyses were obtained on a weekly basis to monitor the development of proteinuria. Animals were euthanized by CO₂ suffocation at the age of 4, 8, and 20 weeks or upon development of proteinuria (3/4+ on dipstick, corresponding to >300mg/dL). Terminal blood samples collected from the heart were used to prepare sera. Kidneys were immediately extirpated, sliced, and preserved in RNAlater™, fixed in 4% paraformaldehyde or McDowell’s fixative for morphological studies by electron microscopy, snap-frozen in liquid N₂ for protein extraction, or embedded in optimal cutting temperature compound and frozen in liquid N₂ for immunofluorescence microscopy studies.

6.2 Immunohistochemistry

Indirect immunofluorescence studies were performed using freshly prepared non-fixated cryosections. Negative controls included omission of primary antibody and assays using total serum IgG from the relevant species in concentrations corresponding to that of the antibody in question. For all assays, identical magnification and exposure settings were used for all slides.

6.3 RNA isolation, cDNA synthesis and realtime-PCR analysis

Upon collection, tissues were immediately placed in RNAlater™ (Ambion Inc, Austin, TX) preservation solution to prevent RNA degradation upon storage. RNA
isolation from tissues was performed using a commercially available kit (EZ1 RNA Tissue mini kit; Qiagen, Hilden, Germany). For cell culture experiments, a Trizol™-based isolation protocol (Ambion) was utilized. RNA concentration and purity was verified using spectrophotometrical methods, and cDNA synthesis was done using equal amounts of RNA for all samples. Realtime PCR was performed using the ΔΔCt method, in which the amplification threshold is normalized against that of a reference gene that is assumed to be stably expressed in all cells (a so-called housekeeping gene). The selection of housekeeping genes for the realtime PCR studies was done based on absolute quantification assays for a number of candidate genes. Commercially available, pre-designed TaqMan™ primer/probesets (Applied Biosystems) were utilized for all the assayed genes.

6.4 Gel zymography

Gel zymography is a classic method to measure enzymatic activity from a tissue sample in vitro. Protein samples are electrophoretically separated on a sodium dodecyl sulphate (SDS) polyacrylamide gel that contains a substrate for the enzyme(s) to be studied. The SDS is then washed out of the gel with 2% (v/v) Triton X-100 in water, allowing for (partial) renaturing of the proteins. Incubation of the gel under optimized conditions in the presence of required co-factors then allows for the enzymatic breakdown of the substrate. After incubation, visualization of remaining substrate allows for the identification of areas of degradation. This provides information about the molecular weight of the responsible enzymes, and their relative contribution to substrate degradation. By comparison with purified enzyme preparations, estimates of absolute enzyme concentrations can also be made. The inclusion of various inhibitors to the reaction mixture during incubation allows for
further identification of the responsible enzymes.

For gelatinase zymography, gelatin was added to the gel, and the gel developed by Coomassie blue staining, which colors areas of high protein content. For Dnase zymography, salmon sperm DNA fragments were added to the gel, and the gel developed by ethidium bromide staining, which intercalates with DNA, allowing visualization of areas containing DNA under ultraviolet transillumination. Areas of reduced ethidium bromide staining indicated the concentration and molecular weight of active nucleases within the samples.

6.5 In situ zymography

The activity of enzymes in vitro is influenced by a multitude of factors, including the presence of other peptide, electrolytes and pH. Such influences are eliminated when employing gel-based enzymatic assays. Also, information about the distribution of active enzyme within the tissue is missed. To be able to obtain some of this information, in situ enzymatic assays were utilized for both collagen-degrading and DNA-degrading activity.

For the assay of collagen-degrading activity, a previous protocol was adapted[136], in which kidney cryosections were overlaid with a fluorescently labeled gelatin substrate and incubated with a reaction mixture at 37°C in the dark. In its native form, fluorescence in this substrate is quenched, but is detectable by fluorescence microscopy upon proteolytic degradation. Thus, areas of fluorescence correspond to areas of gelatinolytic activity. By adding various inhibitory substances to the gelatin substrate, the nature of the responsible enzymes could be further pinpointed.

A similar assay was developed for the in situ detection of DNA-degrading activity in kidneys. This method utilized a terminal deoxynucleotidyl transferase (TdT) DNA
fragmentation detection kit. TdT is a DNA polymerase that adds nucleotides to the 3’ end of DNA fragments. By introducing fluorescently labeled nucleotides, incubation of sections with TdT allows for the visualization of DNA fragmentation within cells. Freshly prepared kidney cryosection were incubated with a Dnase activation buffer at 37°C. After the incubation, TdT-based labeling of DNA fragments was performed. Areas with nuclease activity were thus visualized as fluorescent dots by fluorescence microscopy, with fluorescence intensity reflecting relative nuclease activity in the sections being compared.

6.6 Radial diffusion nuclease activity assay

To take into account the effects on total Dnase activity of various inhibitory substances present in tissue samples, a previously developed radial diffusion assay was utilized for studies of DNA-degrading activity in kidney and serum samples[125]. Briefly, gels containing DNA were spotted with 1μL of the samples to be analyzed. Serum was spotted directly into wells in the gel. For protein extracts, total protein concentration was determined by bicinchoninic acid assay (BCA assay; Pierce Biotechnology, Rockford, IL), and normalized protein sample aliquots added to the gel. Upon incubation at 37°C, remaining DNA within the gels was visualized by ethidium bromide staining. By measuring the areas of loss of DNA staining and comparing these to recombinant Dnase1 standards, an index was obtained for DNA-degrading activity within the samples.
7. SUMMARY OF THE PAPERS

7.1 Paper I

Kidneys from female (NZBxNZW)F1 mice were collected at ages 4, 8 and 20 weeks and at the time of development of significant (>300 mg/d) proteinuria. Kidneys from age-matched BALB/c mice were used as controls.

By covering freshly prepared cryosections with a fluorescent collagen substrate, increased net collagenolytic activity was seen in proteinuric stage B/W kidneys. Gel zymography identified collagenolytic bands corresponding in size to MMP-2 and MMP-9. These collagen-degrading proteases were efficiently inhibited by EDTA, while serine protease inhibitors had no effect.

Real-time PCR demonstrated a dramatic increase in MMP-2 expression at the proteinuric stage, with a more modest increase for MMP-9. Immunofluorescence studies of sections showed strong glomerular staining for both MMP-2 and MMP-9 in proteinuric B/W mice. Furthermore, glomerular expression of the MMP9-stabilizing protein lipocalin-2 was also increased at proteinuria, providing an explanation for the abundance of MMP-9 in zymography studies.

Real-time PCR analysis of collagen IV expression, one of the major glomerular ECM constituents and an MMP-2/MMP-9 substrate, showed unchanged expression levels for the normal ECM isoform (α3α4α5) of this protein during disease progression. However, expression of other collagen IV alpha chains (α1, α2 and α6) was increased at the proteinuric stage, possibly indicating alternative collagen IV isoform being expressed within the proteinuric kidneys.
7.2 Paper II

Based on previous data indicative of altered collagen IV expression in proteinuric B/W kidneys, immunofluorescence studies were performed using collagen IV alpha-chain-specific antibodies. Increased staining was seen for both α1 and α2, suggesting increased expression of the collagen IV isoform α1α1α2 within the glomerular basement membrane (GBM). Also, an isoform containing α6, normally confined to Bowman’s capsule, was also detectable in proteinuric stage GBM. These results are in accordance with previous results by real-time PCR.

Increased expression of the MMP-like protease A disintegrin and metalloproteinase domain-containing protein 15 (ADAM-15) was demonstrable in the glomeruli of proteinuric stage mice, complementing previous findings of increased MMP-2 and MMP-9 activity in these mice. *In vitro* digestion by incubation of renal cryosections with recombinant forms of MMP-2 and -9 verified that the aberrant, proteinuric stage collagen IV matrix was amenable to degradation by these MMPs.
7.3 Paper III

Previous studies have shown DNA and several DNA-associated proteins to be present in regions of immune-complex deposits within glomerular basement membranes (GBM). This suggests that deposited autoantibodies are associated with retained extracellular chromatin particles. The major secreted DNA degrading enzyme in humans and rodents is Dnase1, and serum activity of this enzyme has been reported to be decreased in serum from SLE patients. Previous real-time PCR analyses revealed a dramatic decrease in the expression of \textit{Dnase1} in proteinuric stage B/W mice [132], which appears to correlate with the development of GBM-associated immune complexes and histological changes resemblant of membranoproliferative lupus nephritis [133]. To substantiate these findings, we confirmed that these changes were paralleled by decreased Dnase1 protein levels, and that these changes translated to decreased DNA-degrading activity \textit{by in situ} and zymographic methods. In line with previous data, decreased Dnase1 expression was seen only in mice with histologically severe lupus nephritis with immune complex deposition within the GBM, whereas mice with milder nephritis and a mesangial pattern of immune complex deposition had near-normal levels of renal Dnase1. Immunofluorescence studies of biopsies from patients with lupus nephritis gave similar results, with decreased staining for Dnase1 found only in patients with membranoproliferative nephritis. Serum levels of Dnase1 activity dropped at around the same time as within the kidneys, but were similarly decreased irrespective of the pattern of immune complex deposition.
7.4 Paper IV

Based on recent data supportive of a role of the canonical Wnt/beta-catenin pathway in the regulation of renal extracellular matrix turnover, expression of this signaling pathway was assayed in kidneys of (NZBxNZW)F1 mice during development of lupus nephritis.

A strong increase in the expression of Wnt-responsive genes was seen within the kidney from around the time of appearance of anti-dsDNA autoantibodies, also reflected in increased levels of the activated form of beta-catenin. At the same time, serum from these mice had an inhibitory effect on Wnt signaling in mesangial cells \textit{in vitro}. Sera from 20-week old and proteinuric (NZBxNZW)F1 mice contained high concentrations of the Wnt inhibitor Dkk-1. In accordance with previous reports, Dkk-1 in the concentration range seen in lupus sera had a strong apoptosis-inducing effect on mesangial cells \textit{in vitro}.
8. DISCUSSION

8.1 Extracellular membrane changes in lupus nephritis

The glomerular basement membrane constitutes a highly specialized extracellular matrix that provides a physical and electrochemical filtration barrier, and its patency is ensured by a constant process of renewal. Under circumstances of increased stress and tissue damage, compensatory mechanisms are activated which alter membrane properties in more or less beneficial ways. These changes include altered kinetics of membrane synthesis/turnover, as well as fibrotic changes by activation of undifferentiated mesenchymal cells. Our interest in the collagen matrix stems from the finding that the nucleosomes that form an abundant element of glomerular immune complex deposits show high affinity towards collagen IV and laminin [66]. Alterations in the composition and abundance of this matrix could therefore be important in understanding the formation and localization of immune complexes in lupus nephritis.

Previous studies using immunohistochemical methods have indicated that the collagen IV matrix is thickened in patients with lupus nephritis. Using messenger RNA (mRNA) expression data and isoform-specific antibodies, we have found evidence demonstrating that qualitative changes in collagen IV matrix composition take place in nephritic stage B/W mice. Collagen IV α1α1α2 represents an isoform that is seen during embryonic kidney development, and is located within the mesangial matrix [137]. It seems likely that the abundance of this isoform within nephritic glomerular membranes may represent a compensatory switch in matrix synthesis due to increased matrix turnover that serves to preserve matrix patency. A similar isoform switch is seen in Alport syndrome, where a mutation prevents the synthesis of the α3α4α5 isoform that typifies the GBM [69]. Interestingly, this leads
to the development of an irregular, leaky membrane similar to that seen in patients with lupus nephritis. The occurrence of col4a6 within the GBM is surprising, as this type of collagen IV chain is normally only present within the parietal aspect of Bowman’s capsule. The only known collagen IV isoform known to include col4a6 is the $\alpha 5\alpha 5\alpha 6$, the mechanical and biochemical properties of which are largely unknown.

The relative affinity of various collagen IV isoforms for immune complex constituents including nucleosomes is unknown. It is possible that qualitative changes in the composition of glomerular extracellular membranes occurring as a consequence of tissue injury could increase the binding of chromatin-associated antigens or chromatin-containing immune complexes.

Based on their specificity for collagen IV, MMP2 and MMP9 (referred to as the gelatinases) constitute potentially important regulators of glomerular collagen IV matrix turnover. Indeed, gel zymography revealed that these proteins were responsible for the bulk of collagen IV-degrading activity in kidneys. Our studies revealed an increase in expression and in situ activity of both MMP-2 and -9 in proteinuric stage mice. Intuitively, these results may seem surprising, given the apparent expansion of the collagen matrix found in mice with lupus nephritis.

Several reports have been published showing strong glomerular collagen IV immunostaining in both mice and patients with LN. However, no quantitative determination of glomerular collagen IV content has been made, and the assumption that increased immunostaining translates to increased expression therefore remains uncertain. What is clear, however, is that the distribution of collagen IV is altered during LN development, and that collagen IV forms a part of the apparently expanded extracellular matrix in affected glomeruli.
Expression of MMP-9 is known to be induced by pro-inflammatory signaling cascades, including activation of toll-like receptor 9 (TLR-9) [138,139]. Deposited anti-dsDNA-antibody-containing immune complexes causes inflammation by a number of mechanisms, including activation of the complement system, as well as activation of phagocytosing cells through toll-like receptors, including TLR9 [140]. The observed increase in MMP activity could stem from such pro-inflammatory stimuli affecting resident cells or infiltrating leukocytes. Although a detailed assessment of the origin of glomerular MMP expression was not performed, the widespread distribution of MMP immunostaining throughout the glomerular compartment suggests that several cell types are likely involved. Indeed, both mesangial cells and podocytes have been shown to produce MMP-2 and MMP-9 in vitro [73,141,142].

MMP-2 and -9 have been implicated in the progression of several types of glomerulopathies, as part of the pathophysiological process in both sclerotic and inflammatory diseases, as reviewed in [72,143]. MMP inhibitory studies in various glomerulopathies have yielded very different results, demonstrating that MMPs serves different functions in various disease states [144]. In general, it appears that activation of MMPs has detrimental effects in the context of inflammation by causing excessive membrane degradation, whereas in fibrotic processes, MMPs limit the expansion of the membranes by compensatory deposition of endogenous and aberrant ECM components [143,145]. This is complicated by the fact that the progression of many inflammatory glomerulopathies includes an initial phase of inflammatory tissue damage, followed by a fibrotic process of regeneration that leads to glomerulosclerosis. The effects of strategies to modulate MMP activity are therefore likely to be highly dependent upon the stage of tissue injury. Nonetheless, inhibition
of gelatinase activity during the active stages of experimental inflammatory glomerulopathies has yielded significant attenuation of tissue injury [146]. These results warrant evaluation of the effects of MMP inhibitory strategies in active forms of lupus nephritis, including correlation of MMP activity to histological subsets of LN. Indeed, recent data from our group indicates a correlation between increased mRNA expression of MMP-2 and the development of membranoproliferative LN [133]. Likewise, urinary concentration of the MMP-9-stabilizing protein lipocalin-2 has recently emerged as an attractive biomarker for inflammatory renal damage [147,148].

8.2 Acquired loss of renal Dnase1 activity

Our interest in renal Dnase1 activity was founded on recent findings in our group demonstrating that renal Dnase1 mRNA expression and enzyme activity levels were significantly decreased in mice showing signs of lupus nephritis [132,133,149], and that reduced Dnase1 gene expression correlated with the appearance of GBM-associated immune complex deposits, consistent with of the pattern seen in membranoproliferative lupus nephritis [133]. Existing data has led to considerable controversy regarding the mechanism(s) underlying loss of serum Dnase1 activity in SLE patients, as discussed above. A detailed assessment of the various modes of Dnase1 regulation was therefore needed to identify the mechanism underlying the observed loss of renal Dnase1 activity. By including both gel-based, in situ and radial diffusion methods, we were able to assay Dnase activity both in crude extracts and after electrophoretical separation. Controlled pre-heating of samples to 56°C has been reported to reverse inhibition of Dnase1 by actin [123]. In our hands, such pretreatment did indeed increase total Dnase activity by radial diffusion, but did not
affect the relative difference in serum or renal Dnase activity between the groups, suggesting that *in vivo* inhibition by actin does not explain the observed difference in neither serum nor renal Dnase activity. Similarly, no differences were observed between radial diffusion and gel zymography assays, which further suggests that inhibition of serum Dnase1 by actin is not a major contributor to decreased serum or renal Dnase activity in B/W mice [150]. While not in agreement with the original findings by Frost and Lachmann in SLE patients, similar results have been reported by other groups [129].

The temporal relationship between the decrease in serum Dnase1 activity and the occurrence of anti-dsDNA autoantibodies does not favor a role for Dnase1 in the initiation of autoreactivity. Although decreased serum Dnase1 is a common finding in SLE patients, no correlation has been found between Dnase activity and disease activity indices or serological markers of disease activity [151]. However, an association was seen between low serum Dnase activity and the occurrence of active type III/IV nephropathy [152].

The data presented in paper III demonstrates a decrease in mRNA and protein level expression of Dnase1 in kidneys of mice with evidence of immune complex deposits within the GBM, whereas mice with immune complexes confined to the mesangial compartment have renal Dnase1 expression comparable to that of normal BALB/c mice. In contrast, all the proteinuric mice had similarly decreased serum Dnase1 activity, irrespective of the pattern of immune complex deposition. This discrepancy between serum and kidney Dnase1 levels suggests that the changes observed in the renal compartment are due to changes occurring within the kidney.

The mechanisms controlling the expression of Dnase1 are poorly understood. Our data suggest a uniform down-regulation of Dnase1 mRNA expression affecting
both the tubular and glomerular compartments. Identifying the means by which mRNA expression is shut down will be important in devising strategies to restore renal Dnase1 expression, and is the topic of ongoing investigations. Based on the apparent importance of Dnase1 in extracellular DNA degradation, such an intervention could potentially contribute in removing extracellular chromatin, thus preventing exposure of an immunogenic nuclear antigen, and possibly prevent deposition of glomerular immune complexes containing DNA/chromatin.

### 8.3 Wnt signaling in lupus

Current knowledge of the roles of the Wnt pathway in the post-embryonic stage in mammals is limited. The basis for our interest in this pathway was the recent discovery that activation of renal Wnt/beta-catenin signaling appears to be implicated in the development of adriamycin-induced glomerulopathy [81], and its apparent roles in the regulation of extracellular matrix homeostasis [153,154,155].

Our results revealed increased expression of Wnt target genes within the kidney despite a net Wnt inhibitory effect of serum from the same mice, at least in vitro. These findings are suggestive of local Wnt agonists serving as the predominant regulators of renal Wnt activity in vivo, and further identification of such factors is the topic of ongoing investigations in our group.

Levels of active beta-catenin were increased both in glomeruli and tubular cells, suggestive of global alterations in renal Wnt activity, although the increase in immunostaining was most pronounced within the glomerular compartment. Similarly, expression of the Wnt inhibitor Dkk-1, itself a Wnt-induced gene [156], was found to be increased in both tubuli and glomeruli. It is apparent that the increase in renal Dkk-1 expression is not sufficient to prevent a net increase in Wnt signaling during the
development of nephritis, although it might serve as a feedback mechanism to limit
activation of the Wnt pathway, as previously suggested [156]. As discussed below, it
is also possible that increased renal Dkk-1 expression could reflect activation of pro-
apoptotic signaling pathways as a result of increased cellular stress imposed by an
inflammatory response. At any rate, the significant increase in renal Dkk-1 offers a
potential explanation of the observed increase in serum Dkk-1 levels, which appears
to occur at about the same time. Evaluating the interdependence of renal Wnt
signaling and Dkk-1 levels, as well as the relationship between renal and serum levels
of Dkk-1 will likely require experimental manipulation of renal Wnt signaling in vivo.

Dkk-1 has previously been identified as an apoptosis-promoting factor in
various cell lines in vitro, although as mentioned above, controversy exists on the
matter of whether it can by itself induce apoptosis. More importantly, the in vivo
consequences of increased levels of Dkk-1 have not been assessed. Our data show an
apoptosis-inducing effect of recombinant Dkk-1 on mesangial and tubular cells when
cultured in vitro. These primary cell lines have stringent growth requirements, and it
is therefore possible that the composition of growth media and/or supplements
constitute cellular stress factors that render the cells more susceptible to apoptosis
than would be the case in vivo, thus sensitizing the cells to the effects of Dkk-1.
Nonetheless, the findings of increased levels of serum Dkk-1 in B/W mice, and the
similar increase recently reported in SLE patients [99] suggest that Dkk-1 might
indeed be a contributing factor to the increased levels of circulating apoptotic cells
seen in SLE patients [30,31]. Moreover, it has been found that Dkk-1 is induced upon
activation of pro-apoptotic pathways through p53 [157]. It is therefore also possible
that increased serum Dkk-1 is a consequence rather than a cause for increased
apoptotic cell load. Further clarification of these issues could be attained by
simultaneous monitoring of apoptotic cell load and serum Dkk-1 in vivo, and possibly by in vivo experiments utilizing the recently developed neutralizing antibodies against Dkk-1 [158].

8.4 An integrated model of immune complex deposition and progression of nephritis?

It seems clear that the inflammatory changes occurring during LN development is largely mediated by the deposition of immune complexes within glomerular membranes. T-cell mediated glomerular damage, reflected by increased T-cell influx in LN might represent an additional pathogenetic factor [159], but less is known about this cell-mediated aspect of tissue damage [160].

IC deposition triggers classical type III hypersensitivity reactions by the engagement of the complement system. Type II reactions might also be involved through autoantibodies reacting or cross-reacting with various ECM components. The characteristic ICs of lupus nephritis consist of anti-dsDNA/anti-nucleosome autoantibodies complexed with chromatin-associated antigens [161,162,163]. Although the nature and origin of such complexes remain unknown, it is tempting to speculate that they represent retained extracellular chromatin fragments released during cell death [134], which is consistent with current hypotheses of impaired apoptotic cell clearance as a pathogenetic factor in SLE [32]. This chromatin could originate from the glomerulus itself, or be deposited from the circulation [108]. Indeed, both serum and renal Dnase1 activity was found to be decreased in mice showing evidence of such deposits. Of note, however, mice with ICs confined to the mesangium showed normal renal Dnase1 activity, suggesting that serum and renal Dnase1 may affect renal disease in different ways. It is tempting to speculate that
restoring renal DNase1 activity or otherwise facilitating elimination of membrane-bound chromatin-containing ICs could serve as a therapeutic means of preventing the formation of glomerular inflammatory foci. Conceivably, this might also prevent ensuing glomerular gene expression changes, preventing remodeling of extracellular matrices and progression towards renal failure. Attempts at restoring serum Dnase1 activity in B/W mice as well as in lupus patients have been generally disappointing [164,165]. It is therefore possible that insight into the regulatory mechanisms underlying the reduction in renal Dnase1 expression would identify better, more targeted strategies to restore renal Dnase1 activity levels.

The structural and functional changes occurring within the glomerular extracellular membranes after immune complex deposition are accompanied by changes in the expression of several factors involved in ECM homeostasis. MMP activity is increased, possibly by the induction of MMP-2 and -9 by inflammatory signals and leukocyte infiltration. Immune complexes containing retained chromatin particles can also affect MMP expression through TLR-9 signaling in phagocytosing cells [139]. The increased activation of MMPs could have many effects, including disruption of normal membrane structure, leading to a dysfunctional filtration barrier. An increased turnover of matrix molecules such as collagen IV prevents cross binding of fibrils, affecting the compactness and mechanical integrity of the membrane. Such changes could also increase the permeability of the membrane allowing increased binding of immune complexes. This would generate an amplificatory system of inflammatory tissue injury progressing towards end-stage renal failure, as schematically illustrated in Figure 4. Also, proteolytic degradation might expose antigenic structures that increase the binding affinity of immune complexes and autoantibodies. As reviewed in Appendix 2 [72], it has also been proposed that
MMPs may unmask cryptic antigens that serve as targets of autoreactivity [166].

Figure 4

The collagen IV isoform switch that occurs during LN development could represent a compensatory response to increased membrane degradation, analogous to the deposition of aberrant \( \alpha_1\alpha_1\alpha_2 \) matrix within the GBM in Alport syndrome [68], as discussed in section 4.4.4. Alternatively, it is also possible that expansion of the collagen IV matrix could represent a primary element of the response to mechanical and inflammatory tissue damage, possibly as part of a tissue repair mechanism, and
that increased MMP activation represents a compensatory response.

Changes in collagen IV gene expression are likely mediated by pro-fibrotic signals. The canonical Wnt/beta-catenin pathway has recently been implicated in the pathogenesis of several renal diseases characterized by increased deposition of interstitial matrix constituents. Indeed, recent data from our group show that stimulation of Wnt-signaling by lithium chloride or over-expression of the Wnt antagonist Wnt3a induces changes in collagen IV gene expression in mesangial cells that are similar to those seen during LN development (Tveita et al., submitted).

Likewise, Wnt signaling has been reported to induce expression of matrix metalloproteinases, with MMP-2 and MMP-9 constituting potential Wnt-driven target genes [83,167]. These findings are supported by recent *in vitro* data from our lab showing that MMP-2 expression is induced by mesangial cells upon stimulation of canonical Wnt signaling (Tveita et al., submitted). The observed increase in renal canonical Wnt/beta-catenin signaling thus provides a framework to explain at least parts of the gene expression changes found to occur within the glomerulus during the development and progression of lupus nephritis. In this respect, it is interesting to note that the increase in Wnt signaling occurs in the early stages of LN development, making it a potentially attractive target for therapeutic interventions targeting glomerular remodeling processes.

### 8.5 Future directions and limitations of the studies

Like SLE in general, lupus nephritis is a very heterogeneous disease entity. The progressive and dynamic nature of lupus nephritis further complicates studies of this disease in human subjects. The development of animal models of SLE has therefore been an important feat in gaining increased insight into lupus pathophysiology. By
allowing studies through the timeline of disease development, the temporal association of various events in the pathogenesis can be determined. B/W mouse develop nephritis of various histopathological characteristics and severity, resembling human LN, and the dominating patterns of immune complex deposition vary accordingly. The validity of the present data relies on the assumption that the changes occurring in B/W kidneys are analogous to those of human kidneys. Support for such assumptions could be sought by immunofluorescence and realtime PCR studies of human biopsy material, but sound evidence of temporal associations by repeated sampling is made difficult by the invasive nature of renal biopsies. Also, the fluctuating and largely unpredictable nature of renal disease in SLE patients is a challenge in validating data from studies in mice.

Evaluating the effects of altered enzyme/signaling pathway activity in mice can be achieved by pharmacological means or by genetic modifications. For studies in B/W mice, the latter approach is made difficult by the highly inbred nature of the species used. Knockout studies in these mice are therefore a lengthy and expensive approach. Pharmacological inhibition is easier, but still there is a great variability in the age at which disease development occurs, which requires considerable number of animals and sound effects of the treatment for the provision of robust data.

In summary, follow-up data should include verification by studies on human material, interventional studies by pharmacological manipulation of relevant factors, and possibly utilization of animal models that permit the use of gene knockout strategies.
9. CONCLUDING REMARKS

During the last two decades, our understanding of the pathophysiology of lupus nephritis has improved considerably. This includes increased knowledge of the changes occurring within the glomerular extracellular milieu, and the interaction between immune complexes and components of this microenvironment. We now know that glomerular immune complexes are largely localized to distinct regions of the extracellular membranes containing undigested chromatin particles, with chromatin particles functioning as bridging structures between anti-chromatin autoantibodies and intrinsic membrane structures like collagen IV and laminins [106,107,162]. The finding that renal Dnase1 activity decreases around the time of onset of severe nephritis implicates impaired chromatin digestion as a potential explanation for the glomerular deposition of chromatin. At the same time, secreted metalloproteinase activity increases within the glomerulus, mediating increased breakdown of glomerular membranes. The finding that development of lupus nephritis is associated with increased activation of the Wnt pathway potentially offers a mechanism to explain changes occurring in glomerular extracellular matrix turnover. It also identifies an accessible target for pharmacological manipulation of renal gene expression, which could potentially affect disease progression, as has been seen in experimental models of other renal diseases [81,168].

As a whole, these data represent interconnected pieces of a puzzle that offers insight into how a systemic autoimmune condition translates into end-organ disease. By broadening our understanding into the molecular events underlying such a transition, solving this puzzle hopefully will contribute to the development of targeted therapies for lupus nephritis.
10. REFERENCES


71. The Overall lab web page. [http://wwwclipubcca].


PAPER II
PAPER III
PAPER IV
APPENDIX I
 Appendix 1
1982 revised systemic lupus erythematosus classification criteria

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Malar rash</td>
<td>Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds</td>
</tr>
<tr>
<td>2. Discoid rash</td>
<td>Erythematous raised patches with adherent keratotic scaling and follicular plugging: atrophic scarring may occur in older lesions</td>
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<tr>
<td>3. Photosensitivity</td>
<td>Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation</td>
</tr>
<tr>
<td>4. Oral ulcers</td>
<td>Oral or nasopharyngeal ulceration, usually painless, observed by a physician</td>
</tr>
<tr>
<td>5. Arthritis</td>
<td>Nonerosive arthritis involving $\geq 2$ peripheral joints, characterized by tenderness, swelling, or effusion</td>
</tr>
<tr>
<td>6. Serositis</td>
<td>(A) Pleuritis: convincing history of pleuritic pain or rub heard by a physician or evidence of pleural effusion</td>
</tr>
<tr>
<td></td>
<td>or</td>
</tr>
<tr>
<td></td>
<td>(B) Pericarditis: documented by ECG or rub or evidence of pericardial effusion</td>
</tr>
<tr>
<td>7. Renal disorder</td>
<td>(A) Persistent proteinuria $&gt;0.5$ g/d or $&gt;3+$ if quantitation not performed</td>
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<tr>
<td></td>
<td>or</td>
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<tr>
<td></td>
<td>B) Cellular casts: may be red blood cell, hemoglobin, granular, tubular, or mixed</td>
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<tr>
<td>8. Neurologic disorder</td>
<td>(A) Seizures: in the absence of offending drugs or known metabolic derangements (eg, uremia, ketoacidosis, or electrolyte imbalance)</td>
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<td></td>
<td>or</td>
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<tr>
<td></td>
<td>(B) Psychosis: in the absence of offending drugs or known metabolic derangements (eg, uremia, ketoacidosis, or electrolyte imbalance)</td>
</tr>
<tr>
<td>9. Hematologic disorder</td>
<td>(A) Hemolytic anemia: with reticulocytosis</td>
</tr>
<tr>
<td>Criterion</td>
<td>Definition</td>
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</tr>
<tr>
<td>or</td>
<td>(B) Leukopenia: &lt;4000/mm$^3$ total on ≥2 occasions or</td>
</tr>
<tr>
<td>or</td>
<td>(C) Lymphopenia: &lt;1500/mm$^3$ on ≥2 occasions or</td>
</tr>
<tr>
<td>or</td>
<td>(D) Thrombocytopenia: &lt;100,000/mm$^3$ in the absence of offending drugs</td>
</tr>
</tbody>
</table>

| 10. Immunologic disorder | (A) Positive antiphospholipid antibody or |
| or | (B) Anti-DNA: antibody to native DNA in abnormal titer or |
| or | (C) Anti-Sm: presence of antibody to Sm nuclear antigen or |
| or | (D) False positive serologic test for syphilis known to be positive for at least 6 months and confirmed by *T. pallidum* immobilization or fluorescent treponemal antibody absorption test |

| 11. Antinuclear antibody | An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with “drug-induced lupus” syndrome |

The proposed classification is based on 11 criteria. For the purpose of identifying patients in clinical studies, a person shall be said to have systemic lupus erythematosus if any 4 or more of the 11 criteria are present, serially or simultaneously, during any interval of observation. Adapted from *Petri M. Rheum Dis Clin North Am. 2005 May;31(2):245-54.*
APPENDIX II