PhD thesis

Identification of novel microRNA-gene circuits in Human Lupus Nephritis:

Evidence for modulation of Kallikrein genes by miR-422a

Ανίχνευση νέων microRNA-Γονιδίων Στόχων στη Νεφρίτιδα του Λύκου:

Ενδείξεις Ρύθμισης των Γονιδίων της Καλλικρεΐνης από το miR-422a

Eleni Krasoudaki, MD

Laboratory of Autoimmunity and Inflammation

Department of Rheumatology, Clinical Immunology and Allergy

University of Crete School of Medicine

Heraklion, 2018

Table of Contents

1. Abstract

Systemic lupus erythematosus (SLE) is a challenging autoimmune disease with manifestations derived from the involvement of multiple organs including the kidneys, the joints, the nervous system as well as hematopoietic organs. Lupus nephritis (LN) is one of the most severe manifestations of SLE and a major risk factor for morbidity and mortality. The frequency is particularly high in juvenile-onset SLE and in patients of African ancestry. Ten percent of patients with LN will develop end stage renal disease (ESRD). The main pathological feature in LN is immune complex formation and deposition in the kidney, which results in intraglomerular inflammation with recruitment of leucocytes and activation and proliferation of resident renal cells. Gene expression studies in LN have revealed gene networks that involve not only immune cell infiltration but also extracellular matrix formation and renal tissue repair and fibrosis.

Micro-RNAs (miRNAs) come from a large family of small non-coding RNA sequences, which are about 22 nucleotides in length. They constitute critical posttranscriptional regulators of gene expression and act either as translational inhibitors of gene expression or by degrading mRNA transcripts since they bind to complementary sequences in the 3' untranslated region (3'UTR) of target mRNAs. Multiple studies have shown that a cluster of key miRs are highly expressed in the kidney and play a critical role in renal development and function. They also affect the diabetic or polycystic kidney, the kidney with IgA, acute injury or renal cell carcinoma.

The role of miRNAs as transcriptional modulators in the pathogenesis of SLE has recently emerged and their regulatory effects on DNA methylation pathway, type I IFN pathway, estrogen and regulatory T-cells are becoming clear. MiRNA expression has been studied in peripheral blood mononuclear cells (PBMCs), CD4 T cells, kidney biopsy tissue and Epstein-Barr virus transformed B-cell lines from patients with SLE.

In this study, we investigated the role of miRNAs in human LN. To this end, we analysed the miRNA profile in kidney biopsy samples of patients with LN by TaqMan based array aiming to identify novel genes involved in disease pathogenesis. We identified important miRNA-gene pairs and constructed gene networks that are potentially involved in LN pathogenesis. We focused on miR-422a, which is the most upregulated miRNA that correlates with active

histological lesions and its target Kallikrein-4 (KLK-4), a serine esterase with putative renoprotective properties.

Compared to normal tissue a 24-miRNA signature defines human LN with 9 miRNAs up-regulated and 15 miRNAs down-regulated, and miR-422a exhibits the highest up-regulation (17.2-fold). These mi-RNAs with altered expression are predicted to target genes involved in inflammatory signaling pathways, membrane transporters and cell homeostasis such as TGF-β, protein kinase A, ERK/MAPK, NF-κB, HNF4A, Wnt/β-catenin signaling. Bioinformatic analysis predicted that miR-422a has a binding site in the 3'UTR of KLK4 gene, a member of the kallikrein family of serine proteases. This was validated by overexpression of miR-422a, which suppressed by 65% KLK4 luciferase activity and by 82% KLK4 mRNA levels in Human embryonic kidney - 293 cells (HEK-293).

In order to monitor miR-422a/KLK4 expression during LN progression, we used NZB/W F1 lupus mice. We found that at early stages miR-422a was 4.1-fold up-regulated, while KLK4 mRNA levels were 3.4-fold down-regulated while at later stages miR-422a was 9.4-fold up-regulated and KLK4 mRNA levels were 7.6-fold down-regulated. In unaffected NZW kidney and human control specimens, KLK4 protein was strongly expressed, mainly in the cytoplasm of renal tubular as well as mesangial cells. In contrast, KLK4 showed absent or faint or moderate expression in renal parenchymal cells of proteinuric NZB/W F1 and most patients with active LN. Together, these data implicate miRNAs and KLK4, a secreted serine esterase with angiogenic and extracellular matrix remodeling properties, in the pathogenesis of immune-mediated LN.

2. Περίληψη

Ο Συστηματικός Ερυθηματώδης Λύκος (ΣΕΛ) αποτελεί ένα συστηματικό νόσημα αυτοάνοσης αιτιολογίας με προσβολή πολλών οργάνων όπως οι νεφροί, οι αρθρώσεις, το νευρικό σύστημα και τα αιμοποιητικά όργανα. Η νεφρίτιδα του λύκου αποτελεί από τις πιο σοβαρές εκδηλώσεις της νόσου με σημαντικά ποσοστά νοσηρότητας και θνητότητας. Εμφανίζεται κυρίως σε νεαρές ηλικίες και σε ασθενείς Αφρικανικής καταγωγής. Δέκα τοις εκατό των ασθενών θα εμφανίσουν νεφρική νόσο τελικού σταδίου. Τα κύρια ιστοπαθολογικά χαρακτηριστικά της νόσου αφορούν την εναπόθεση ανοσοσυμπλεγμάτων στο σπείραμα, που οδηγεί σε φλεγμονή με ενεργοποίηση λευκοκυττάρων και κυττάρων του σπειράματος. Γονιδιακές μελέτες έχουν αποκαλύψει δίκτυα που εμπλέκουν τόσο το ανοσοποιητικό σύστημα όσο και τη δημιουργία εξωκυττάριας θεμέλιας ουσίας και τη συμμετοχή των «τοπικών» κυττάρων στη παθογένεια της νόσου.

Τα miRNAs αποτελούν μια νέα ομάδα εξελικτικά καλά διατηρημένων μικρών μορίων RNA που δεν κωδικοποιούν για πρωτεΐνες και έχουν μήκος 22 νουκλεοτιδίων. Θεωρούνται σημαντικοί αρνητικοί ρυθμιστές της έκφρασης γονιδίων σε μετα-μεταφραστικό επίπεδο. Ανάλογα με τον βαθμό συμπληρωματικότητας, τα miRNAs προσδένονται στα mRNAs-στόχους τους και είτε προκαλούν την αποικοδόμηση τους, είτε αναστέλλουν την μετάφραση τους σε πρωτεϊνικά μόρια. Πολλές μελέτες έχουν δείξει ότι εκφράζονται στο νεφρό και εμπλέκονται τόσο στην ανάπτυξη και τη φυσιολογική λειτουργία του, όσο και σε παθολογικές καταστάσεις όπως η διαβητική νεφροπάθεια, η πολυκυστική νόσος, η IgA νεφροπάθεια, η οξεία νεφρική βλάβη ή οι κακοήθεις όγκοι του νεφρού.

Ο ρόλος των miRNAs ως μεταγραφικοί ρυθμιστές στη παθογένειά του ΣΕΛ έχει μελετηθεί εκτενώς και έχει βρεθεί ότι εμπλέκονται σε μονοπάτια μεθυλίωσης του DNA, IFN-I, οιστρογόνων και ρυθμιστικών Τ-λεμφοκυττάρων. Η έκφραση τους έχει επίσης μελετηθεί σε μονοκύτταρα περιφερικού αίματος, CD4 Τ-λεμφοκυττάρων, βιοψίες νεφρού καθώς και Β-κυτταρικές σειρές επιμολυσμένες με EBV.

Με στόχο την ανεύρεση νέων γονιδίων που συμβάλλουν στην νεφρίτιδα στο ΣΕΛ, στην παρούσα εργασία μελετούμε την έκφραση και λειτουργία των miRNAs στο νεφρικό ιστό ασθενών με νεφρίτιδα του ΣΕΛ με την χρήση της τεχνολογίας των μικροσυστοιχείων (microarrays). Ταυτοποιήσαμε έναν αριθμό miRNAs – γονιδίων στόχων με διαφορετική

έκφραση και κατασκευάσαμε δίκτυα γονιδίων που πιθανόν εμπλέκονται στη παθογένεια της νόσου. Το miR-422a βρέθηκε να έχει τη μεγαλύτερη έκφραση, να συσχετίζεται με ενεργείς ιστολογικές βλάβες στο σπείραμα και να ελέγχει την έκφραση της Καλλικρεϊνης 4 (KLK-4), μίας σερίνης εστεράσης με πιθανή προστατευτική δράση στο νεφρό.

Σε σύγκριση με τον υγιή ιστό, στους νεφρούς των ασθενών με νεφρίτιδα ΣΕΛ παρατηρήθηκε μεταβολή της έκφρασης σε 24 miRNAs (αυξημένη έκφραση σε 9, μειωμένη έκφραση σε 15 miRNAs). Το miR-422a παρουσίασε τη μεγαλύτερη αύξηση (17.2 φορές). Τα προβλεπόμενα γονίδια – στόχοι εμπλέκονται σε σηματοδοτικά μονοπάτια φλεγμονής, μεταφορέων μεμβράνης , κυτταρικής ομοιόστασης όπως TGF-β, protein kinase A, ERK/MAPK, NF-κB, HNF4A, Wnt/β-catenin. Η βιοπληροφορική ανάλυση έδειξε ότι το miR-422a συνδέεται στην 3'UTR περιοχή του γονιδίου της KLK4 και καταστέλλει την έκφρασή της. Σε πειράματα διαμόλυνσης των κυττάρων HEK293, η υπερέκφραση του miR-422a κατέστειλε κατά 65% τη λουσιφεράση του γονιδίου της KLK4 και κατά 82% τα επίπεδα του mRNA της.

Μελετήθηκε η έκφραση των miR-422a/KLK4 κατά την εξέλιξη της νεφρίτιδας του λύκου σε ποντίκια NZB/W F1. Στα πρώιμα στάδια νεφρίτιδας ΣΕΛ (ποντικοί NZBx NZW F1 ηλικίας 2 μηνών) παρατηρήθηκε αύξηση του miR-422a κατά 4.1 φορές και μείωση του KLK4 mRNA κατά 3.4 φορές. Οι παραπάνω μεταβολές ενισχύθηκαν σε προχωρημένο στάδιο της νόσου (ποντικοί ηλικίας 6 μηνών) με αύξηση του miR-422a κατά 9.4 φορές και μείωση του KLK4 mRNA κατά 7.6 φορές σε σύγκριση με νεφρικό ιστό από υγιείς ποντικούς. Σε υγιείς μάρτυρες (ανθρώπινο ή δείγμα από ποντίκι) η KLK4 εκφραζόταν στο κυτταρόπλασμα τόσο των σωληναριακών όσο και των μεσαγγειακών κυττάρων. Αντιθέτως, δεν παρατηρήθηκε έκφραση της στα παρεγχυματικά κύτταρα NZB/W F1 ή ασθενών με πρωτεΐνουρία. Συνολικά, τα παραπάνω δεδομένα αναδεικνύουν το πιθανό ρόλο των miRNAs και της KLK4 στη παθογένεια της νεφρίτιδας του λύκου.

3. Abbreviations

AKI	Acute kidney injury
CKD	Chronic kidney disease
ESRD	End stage renal disease
HEK-293	Human embryonic kidney - 293 cells
KLK-4	Kallikrein-related peptidase 4
LN	Lupus nephritis
LncRNAs	Long noncoding RNAs
MiRNA	MicroRNA
PBMCs	Peripheral blood mononuclear cells
pre-miRNA	Precursor miRNA (micro ribonucleic acid)
pri-miRNA	Primary miRNA
3'UTR	3' untranslated region
SLE	Systemic lupus erythematosus
NETs	Neutrophil Extracellular Traps

4. Introduction

4.1. Systemic lupus erythematosus (SLE)

Systemic lupus erythematosus (SLE) is a challenging autoimmune disease with manifestations derived from the involvement of multiple organs including the kidneys, the joints, the nervous system as well as the hematopoietic organs [1]. The incidence of SLE seems to be increasing, most probably because of the identification of milder cases and the improved survival of the patients. It mainly affects women of childbearing age, with a male-to-female ratio of about 9:1 and the prevalence is reported substantially higher in individuals of African-American or African-Caribbean descent [2]. The diagnosis of lupus is based on particular clinical and serological profiles while prognosis and disease severity vary significantly among patients with SLE [3].

Lupus is a multifactorial disease with evidence of genetic susceptibility and environmental effects. Dysfunction in both innate and adaptive immunity manifest by disturbances in apoptotic cell clearance, cytokines, B-cell immunity and T-cell signaling [4]. Deficiencies in components of the complement cascade as well as multiple disease susceptibility loci are well known to predispose to the development of SLE. Excessive apoptotic debris and defects in their clearance have an important pathogenic effect. Studies have also shown intense polyclonal B-cell activation as well as T-cell dysfunction to be implicated in disease pathogenesis [5-7].

Lupus nephritis (LN) is one of the most severe manifestations of SLE and a major risk factor for morbidity and mortality [8]. The frequency is particularly high in juvenile- onset SLE and in patients of African ancestry [9, 10]. Ten percent of patients with LN will develop end stage renal disease (ESRD) [8]. The risk is higher in certain classes of LN. In class IV lupus nephritis, the risk of ESRD may be as high as 33% over 10 years [11]. The main pathological feature in LN is immune complex formation and deposition in the kidney, which results in intraglomerular inflammation with recruitment of leucocytes and activation and proliferation of resident renal cells. The location of immune complex is linked to histopathology and the intensity of the inflammatory response.

Lupus is developed in susceptible individuals exposed to certain environmental triggers. This leads to the development of autoantibodies that cause inflammation and tissue injury. The human HLA loci has been most prominently associated with SLE, while the expression of neutrophil-associated genes have been linked to LN [12-14]. IFN – related genes seem to precede neutrophil activation with the latest to contribute to LN pathogenesis even in death. When they die, neutrophils release neutrophil extracellular traps (NETs), which facilitate inflammation, endothelial damage and more IFN-a release [15, 16]. Complement is another factor to play important role in LN pathogenesis either directly by mediating kidney injury or indirectly through recruitment of leucocytes to the kidney and consequently increasing renal inflammation. Autoantibodies to complement C1q (anti-C1q) are associated with the diagnosis of LN, while anti-C3b IgG might be used as biomarker of LN flare [17].

4.2. Renal response to injury

Although the activation of immune system is essential for the development of LN, renal response to immune-complex deposits is an important factor for the progression of the disease. Gene expression studies in LN have revealed gene networks that involve not only immune cell infiltration but also extracellular matrix formation and renal tissue repair and fibrosis [18, 19].

Renal infiltration with mononuclear cells, which are dominant in the interstitium is associated with poor prognosis in SLE. These cells acquire an activated phenotype during active nephritis that reverses upon remission induction. During disease onset they are a major renal source of several pro-inflammatory cytokines and chemokines but they secrete molecules associated with tissue protection and repair that in excess may contribute to tissue degradation [20, 21]. Noticeably, macrophage gene-expression profile is different between the glomerular and interstitial compartments, confirming that distinct renal mononuclear phagocytes infiltrate the different intrarenal structures in LN [20]. After intergrossing the C57L/J *Cgnz1* locus to NZM2328 lupus-prone mice, the hybrid female mice developed acute glomerulonephritis mediated by immune complex deposits in the kidney and complement activation; nevertheless, they were resistant to development of severe proteinuria and ESRD [19]. The *Cgnz1* locus contains mitochondrial, autophagy-related and cell survival genes that might regulate clearing of deposited immune-complexes and podocyte survival. These data suggest that both

autoimmunity and end-organ damage may contribute to pathogenesis and progression of nephritis [19].

4.3. MiRNAs: emerging role in gene regulation and human disease

Micro-RNAs (miRNA) come from a large family of small non-coding RNA sequences, which are about 22 nucleotides in length. They constitute critical posttranscriptional regulators of gene expression and act either as translational inhibitors of gene expression or by degrading mRNA transcripts [22-24]. Victor Ambros and his colleagues discovered the miRNAs in 1993. Lin-4 and let7-a were the first members of the miRNA family and were identified in C. Elegans and in H.sapiens, respectively [25-27]. These regulatory miRNA are conserved among species and are implicated in virtually every biological process in multicellular organisms. In humans, they play a vital role in a number of biological functions like developmental abnormalities, carcinogenesis, autoimmunity, apoptosis and cell proliferation [28-30]. Today more than 1800 miRNAs have been identified in humans (www.mirbase.org). The function of a miRNA can be interpreted as the sum of the function of genes it regulates. Based on complementarity, miRNA target-prediction algorithms have been developed and it is assumed that each miRNA could target several hundred genes and make fine-scale adjustments to protein output [31, 32]. On the contrary, different miRNAs can control a single mRNA target and on the whole they could be used to identify new players implicated in disease pathogenesis in terms of diagnostics and therapeutics [31, 33].

MiRNAs differ from long noncoding RNAs (lncRNAs) that are defined as transcript RNA molecules, longer than 200 nucleotides, without the capacity of coding proteins and reside within the intergenic stretches or overlapping antisense transcripts of protein coding genes [34]. Anomalous expressions of lncRNAs are associated with various human diseases and it is hypothesized that lncRNAs, in combination with mRNAs, are also involved in the in the pathogenesis of autoimmune and inflammatory diseases, involving the development of SLE as well [35-37].

4.3.1. MiRNA biogenesis

The biogenesis of single-stranded miRNAs occurs through a multi-step process that involves several different enzymes in the cell nucleus and the cytoplasm. The genes encoding miRNAs are much longer than the processed, mature miRNA molecules and approximately half of them are contained within the introns of protein-coding genes or in the exons of untranslated genes [38, 39]. Most of the miRNA genes are transcribed by the RNA polymerase II (Pol II) as long primary transcripts termed primary miRNAs (pri-miRNAs), which can be several hundred or thousand nucleotides long **(Figure 4.1)**. Pri-miRNAs are capped and polyadenylated and contain a local stem–loop structure that encodes miRNA sequences in the arm of the stem [40].

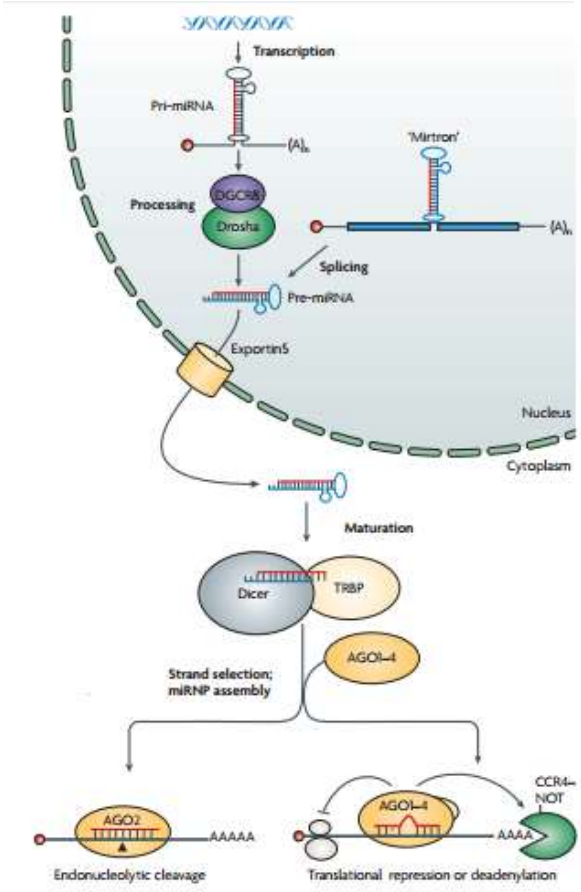


Figure 4.1. MicroRNA biogenesis

This stem–loop structure is cleaved in the nucleus by a complex consisting of the dsRNA-specific ribonuclease Drosha and its binding factor DiGeorge syndrome critical region gene 8 (DGCR8) in a process known as 'cropping'[41, 42]. Other cofactors such as p72, p68, FUS

and hnRNPA1 regulate efficiency and specificity of cleavage or act as scaffold proteins for complex formation, while other alter biogenesis of the entire miRNAome or demonstrate activity against a defined miR [43]. Drosha and cofactors generate either the 5’ or 3’ end of the mature miRNA, depending on which strand of the pre-miRNA is selected by RNA-induced silencing complex (RISC) [44].

Precursor miRNA (pre-miRNA) which is approximately 70 nt RNAs with 1–4 nt 3’ overhangs, 25–30 bp stems and relatively small loops, is released and then is actively transported to the cytoplasm via a mechanism that involves exportin- 5 (Exp5), the role of which can be complemented by alternative mechanisms as well [45, 46]. Some spliced-out introns in mammals and in other species as well, correspond precisely to pre-miRNAs (mirtrons) and bypass the requirement for Drosha–DGCR8 cropping [47].

Pre-miRNAs are subsequently cleaved by the cytoplasmic RNase III Dicer into ~22-nt miRNA duplexes [48]. One strand is then degraded while the other strand is selected to function as mature miRNA and is incorporated into the RISC. The strand with lower stability in base pairing at the 5’ and 3’ end of the duplex associates with RISC and becomes the functionally active miRNA [49]. The repression of gene expression is carried out by this miRNA-programmed RISC (miRISC) which has additional processing elements like Argonaute RISC catalytic component 2 (AGO2). AGO2 functions as a Dicer co-factor in pre-miRNA processing and is also loaded with the mature miRNA strand, making an active RISC [50, 51].

4.3.2. Function of miRNAs (Tuning)

The most important requirements for successful mRNA-miRNA interaction are a contiguous and perfect base pairing of the miRNA nt 2–8, potential mismatches to be present preferentially in the central region of the duplex precluding the AGO-mediated endonucleolytic cleavage of mRNA and at last reasonable complementarity to the miRNA 3′ half [52, 53]. MiRNAs bind to complementary sequences in the 3’UTR of target mRNAs and inhibit protein synthesis by either translation inhibition or deadenylation and subsequent degradation of the target mRNA [24].

In the first case, which account for the majority of the repression of protein production, miRNA is only partially complementary to its mRNA and mRNA levels are not affected. Translation inhibition mechanism remains in dispute. Translation may be hindered at initiation involving

cap-dependent processes or at a subsequent stage directed by various internal ribosome entry site *(*IRES) elements, including some that do not require any of the translation initiation factors [47, 54, 55]. MiRNAs might also cause retarded elongation by translating ribosomes, possibly coupled to premature termination or induce cotranslational degradation of nascent polypeptides [56, 57]. In the second case there is a near-perfect complementarity between miRNA and mRNA and the second undergoes either endonucleolytic cleavage by Ago2 or poly (A) removal by deadenylase **(Figure 4.2)** [54, 58].

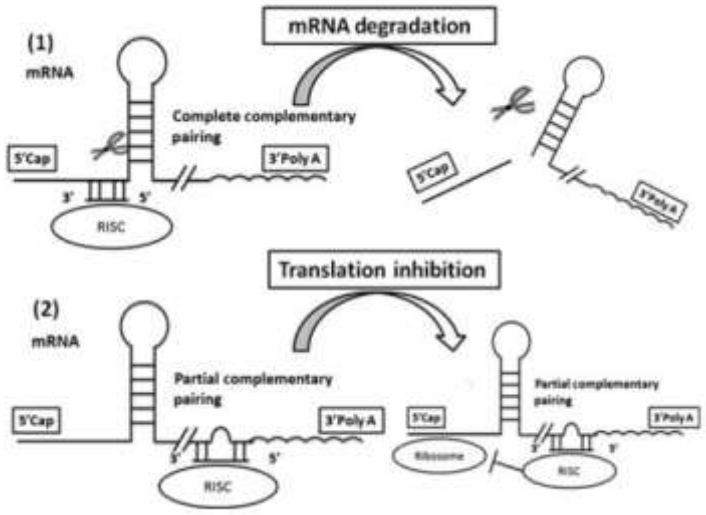


Figure 4.2. Micro-RNA post-transcriptional gene repression

4.4. MiRNAs and the kidney

Multiple studies have shown that a cluster of key miRs are highly expressed in the kidney and have a crucial role in kidney development and normal physiologic function. Altered miRNA expression can result in kidney disease such as diabetic nephritis, glomerulonephritis or cancer **(Table 4.1).** According to Sun et al, 6 miRNAs are expressed preferentially in the renal cortex and 11 in the renal medulla with functional implications [59, 60].

miR	Validated Targets	Expression and Relation to Kidney Disease
miR-15a	Cdc25A	Rat model of PKD
miR-17-92 cluster	PTEN and Bim	Enlarged kidney glomeruli, hypercellularity, mesangial expansion, proteinuria
miR-30 family	CTGF	High expression in kidney glomeruli (and loss in podocyte-specific Dicer KO)
miR-192	ZEB1 and ZEB2	Enhanced expression in diabetic mouse kidney and by TGF-β in mouse MC; enhanced expression by high glucose in human MCs
miR-200 family	ZEB1 and ZEB2	EMT in cultured kidney and cancer cells
miR-216a, miR-217 cluster	PTEN	Enhanced expression in diabetic mouse kidney and by TGF-β in mouse MCs
miR-377	PAK1 and MnSOD	Enhanced expression by high glucose in human MC

Table 4.1. MiRNAs with known function in kidney disease

As mentioned before, miRNAs play a critical role in renal development and function. In the growing mouse kidney, the expression of several miRNAs changes significantly during the phases of development, such as miR-30 and miR-200 [61-63]. These specific miRNAs have key roles in regulating the epithelial-mesenchymal transition through targeting transcriptional repressors of E-cadherin [64]. Removal of Dicer or Drosha from renal cell lines or animals promoted apoptosis, caused termination of nephrogenesis or dysplasia in early developing glomeruli [65, 66]. The pathways involved are TGF-β, Wnt and Notch signaling [22, 67, 68]. Dicer knock out in murine podocytes led to progressive glomerular and tubular injury ending with animal death by 4 weeks [69].

In diabetic nephropathy, miR-192 was found to control TGF-β induced collagen expression and to regulate fibrosis in the diabetic kidney [70-72]. MiR-29a as well as miR-377 have been associated to regulate collagen and fibronectin genes, respectively, under diabetic conditions [73-75]. Studies involved miR-15a to affect hepatic cystogenesis in a murine model of polycystic kidney disease, while cysts in kidney samples from patients with ADPKD had increased levels of miR-21 [76, 77]. In IgA nephropathy, miR148b and miR-155 may modulate IgA1 aberrant O-glycosylation providing a therapeutic target for the disease [78, 79]. In renal cell carcinoma, up-regulated miRNAs include miR-210, -155, -21, -142-3p, -185, -34, and -224, which down-regulate tumor suppressor genes and some are known to be related to HIF molecules and hypoxia. On the other hand, down-regulation of miR-149, -200c and -141 leads to activation of oncogenes [80, 81].

MiRs have also been studied in kidney transplantation. The combined downregulation of miRNA-15B, miRNA-16, miRNA-103A, miRNA-106A and miRNA-107 may help to identify severe T cell-mediated vascular rejection after renal transplantation [82]. Urinary miRs could be used as non-invasive biomarkers that provide information about the status of the transplanted kidney before irreversible damage has occurred through rejection, drug toxicity or other pathological events [83]. MiRs are also implicated in the pathogenesis of various types of acute kidney injury (AKI) [84]. A recent study showed changes both of plasma and kidney miR-192 levels in AKI and plasma miR-192 could be a predictor for ischemic AKI [85]. Expression of other miRs, like miR-146a and miR-324 has been associated with CKD progression and renal fibrosis [86, 87].

4.5. MiRNA in SLE and lupus nephritis

The role of miRNAs as transcriptional modulators in the pathogenesis of SLE has recently emerged and their regulatory effects on DNA methylation pathway, type I IFN pathway, estrogen and regulatory T-cells are becoming clear. MiRNA expression has been studied in peripheral blood mononuclear cells (PBMC), CD4 T cells, kidney biopsy tissue and Epstein-Barr virus transformed B-cell lines from patients with SLE [88-92].

Various studies show involvement of miRNAs in lupus nephritis **(Table 4.2)**. One study identified specific miRNAs in the peripheral blood of LN patients [92]. Dai et al reported the differential expression of miRNAs in kidney biopsy specimens from patients with lupus nephritis. MiRNA microarray data identified 30 downregulated and 36 upregulated miRNAs: miR-296, miR-150, and miR-365 were the most downregulated miRNAs, whereas miR-15, miR-124a, and miR-195 the most upregulated [89]. The same group had previously identified 16 differentially expressed miRNAs in PBMCs of SLE patients that are not included in the list of the 66 miRNAs in LN kidney biopsies [88]. MiR-let-7a has been found to be increased in the mesangial cells of NZB/W mice and to increase the expression of IL-6 in vitro [93, 94]. Another study by Lu et al confirmed that miR-146a is upregulated in glomerular tissue from LN patients and found that is not overexpressed in LN tubulointerstitial tissue [95]. MiR-638 expression was under expressed in glomerular tissue but higher in tubulointerstitial tissue and was positively correlated with proteinuria and SLE Disease Activity Index (SLEDAI) score [95]. MiR-150 was found significantly increased in renal biopsies with high chronicity index

and led to increased production of profibrotic molecules through downregulation of suppressor of cytokine signaling 1 (SOCS1), a negative regulator of fibrosis [96]. MiR-23b, which suppresses autoimmune inflammation by targeting proinflammatory cytokine-mediated signaling, was reported to be downregulated in the autoimmune prone MRL/lpr mouse strain. IL-17 was shown to cause this under expression of miR-23b in mouse primary kidney cells [97]. In podocytes, miR-26a was found to be down-regulated in LN, which in turn decreased the expression of genes associated with podocyte differentiation and cytoskeleton integrity [98].

Tissue	microRNA	Importance	Reference
Renal /PBMCs(H)	• 36up-regulated • 30down-regulated • hsa-miR-223 down regulated • 16 different miRNAs identified in PBMCs of SLE patients	miRNA organ/cell specific	[88, 89]
Renal (H+M)	Down-regulated miR-23b	IL17, TNFa,IL1β signaling inhibition	[97]
Renal (M)	Up-regulated let-7a	Promotion of IL 6 production Cell hyperplasia, proinflammatory response	[93, 94]
Renal (M)	Down-regulated miR-26a	Podocyte differentiation, cytoskeletal integrity	[98]
Renal (H)	Upregulated miR-150 in high chronicity index biopsies	Promotes renal fibrosis through downregulation of SOCS1	[96]
PBMCs	hsa-miR-371-5P, hsa-miR-423-5P, hsa-miR-638, hsa-miR-1224-3P and hsa-miR-663: differentially expressed in LN	First miRNAs associated with LN	[92]
Renal (H)	Deregulated expression of miR-638, miR-198, miR-146a	Different glomerular and tubulointerstitial expression Correlation with clinical disease activity	[95]

Table 4.2 MicroRNAs in lupus nephritis

5. Aim of the study

Emerging evidence has shown that aberrant miRNA expression influence a wide range of biological processes including immune cell differentiation, maturation and normal function and can be detected in tissues, serum or other body fluids. The role of miRNAs is evident in malignant as well as nonmalignant diseases including cancer, inflammatory and autoimmune diseases and there is accumulating evidence for an important role in SLE; to monitor disease activity and effects of treatment and to help understand the pathogenesis of the disease. MiRNA expression profiles of human or murine renal tissue have provided differentially expressed miRNAs in LN, suggesting that these patterns are cell or organ specific [89, 95, 96]. The studies that have examined miRNAs expression in kidney inflammatory lesions of SLE and their results are limited by the inclusion of only relatively mild forms of nephritis or the lack of comparison with disease-free control group [89, 96].

We aimed to identify micro-RNAs that are associated with acute inflammation in kidney biopsies from LN patients and to evaluate their potential implication in end-organ injury. More specifically, the objectives of this study are to characterize the miRNA signature in human LN and to explore their potential role as disease biomarkers. Additionally, we aim to identify important microRNA-gene pairs and construct gene networks and molecular pathways that are potentially involved in LN pathogenesis. We focused on miR-422a, which is the most upregulated miRNA that correlates with active histological lesions and its target KLK-4, a serine esterase with putative renoprotective properties.

6. Materials and methods

Human kidney tissue samples

Formalin-fixed paraffin-embedded (FFPE) kidney specimens from 20 patients with active LN were obtained at the 'Hippokration' Hospital of Thessaloniki. Twelve patients had proliferative nephritis (class III or IV [99]), five patients had membranous (class V) and three patients had mixed (V+III/ IV) nephritis **(Supplementary data, Table S1)**. The National Institutes of Health indexes were used to calculate activity and chronicity [100]. Twelve LN specimens (eight proliferative and four membranous) were used for miRNA profiling and RT-PCR. For the subsequent validation immunohistochemistry studies eight additional specimens were used. The controls included uninvolved parts of kidneys surgically resected due to renal tumor and with no histological evidence of renal disease or tumor (n = 3). The study was in accordance with the Declaration of Helsinki and was approved by the local ethics committees of the participating centers.

Animal models

New Zealand White (NZW), New Zealand Black (NZB) and NZB/NZW F1 (NZB/W) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and were housed in a pathogen free facility with 12-h light/dark cycles and unlimited access to food and water. The NZB/W strain included groups of females 8 weeks old (pre-disease control group without proteinuria) and 24 weeks old (proteinuria ≥300 mg/dl). As controls, NZW mice of the same age were used. Kidneys were removed from perfused mice and immediately snap frozen. All experiments were approved by the Veterinary Department of the Region of Crete and the University of Crete Medical School.

Micro-RNA profiling

FFPE kidney specimens (three to four 7–10 μm thick slides from each sample) were deparaffinized and total RNA was extracted with TRIzol® (Invitrogen) [101]. RNA concentration was determined with NanoDrop ND-1000 spectrophotometer and RNA quality was assessed by the OD 260/280 and 260/230 ratios. RNA (100 ng) was used for multiplex

reverse transcription reactions with micro-RNA-specific reverse transcriptase (RT) primers (Human Multiplex RT Set, Applied Biosystems). The expression of 365 micro-RNAs was analyzed by PCR using the TaqMan Low Density Array (TLDA) Human Micro-RNA Panel 1.0 (Applied Biosystems) in the Dana-Farber Molecular Diagnostics Facility. RNU48 expression was used for data normalization.

Prediction of potential mi-RNA targets and gene network analysis

Potential mi-RNA gene targets were identified using the following engines; miRDB (http://mirdb.org/miRDB/index.html), MicroCosm Targets (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/ targets/v5/) and TargetScan (http://www.targetscan.org/index.html). A potential gene target should be predicted by at least two out of three abovementioned engines and the targeted sequence be conserved among species. Experimentally validated targets were retrieved from the miRTarBase and miRecords. We analyzed gene ontology for each set of mi-RNA targets and their participation in biological pathways, gene networks and disease signatures, using the GeneCoDis and Ingenuity Pathway Analysis tools.

Transfection experiments

Human embryonic kidney 293 (HEK-293) cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with L-glutamine and 10% heat-inactivated fetal bovine serum (Life Technologies). Cells were transfected using the siPORT NeoFX agent (Invitrogen) with 50–75 nM of mirVana hsa-miR-422a micro-RNA mimic (miR-422a), hsa-miR-422a micro-RNA inhibitor (as-miR-422a) or their respective negative controls (miR-NC, as-miR-NC) (Ambion). Transfections were performed three to four times either in duplicates or triplicates. Cells were collected 48 hours post-transfection for total RNA or protein isolation.

3'-untranslated region luciferase assay

HEK-293 cells were plated for 24 hours and were cotransfected using Fugene6 reagent (Roche) with renilla reporter plasmid constructs (pLightSwitch) carrying the 3'-untranslated region (3'-UTR) of KLK4 (SwitchGear Genomics) plus miR-422a, as-miR-422a or their negative controls (NCs) [102]. After 36 h cell lysates were prepared and the luciferase assays were performed using the Dual-Luciferase Reporter Assay (Promega).

RT-PCR

TLDA results were confirmed with the mirVana qRT-PCR miRNA Detection Kit and qRT-PCR Primer Sets (Ambion). Total RNA was also extracted from mouse kidneys and HEK-293 cell lysates using TRIzol® and the RNAeasy Mini Kit (QIAGEN), respectively. Hsa-miR-422a was quantified with TaqMan Micro-RNA Assay (Applied Biosystems) using RNU48 as reference gene. Mmu-miR-378 (mmu-miR-378a-3p), which is the mouse paralog of hsa-miR-422a, was quantified with TaqMan Micro-RNA Assay (Applied Biosystems) using U6 snRNA for normalization. For mRNA quantification, reverse transcription was performed with the PrimeScript 1st strand cDNA Synthesis Kit (TAKARA). Human and mouse KLK4 was measured with TaqMan Assay (Applied Biosystems) using GAPDH as reference gene. CT values were normalized to endogenous control and the fold-change relative to a control sample ($2^{\Delta\Delta CT}$) was used for relative expression analysis.

Western blot

Mouse kidneys or transfected HEK-293 cells were lysed in RIPA buffer containing complete protease inhibitor cocktail (Roche). Proteins were separated by 10% sodium dodecyl sulphatepolyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. After blocking, membrane was incubated overnight at 4°C with rabbit polyclonal antibody to KLK4 (orb13527, Biorbyt Ltd) and mouse monoclonal antibody to β-actin (MAB1501, Millipore Corporation). Blots were probed with secondary horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratories) and membranes were visualized with the ECL Western Blotting Detection System (GE Healthcare Life Sciences). Protein density was quantified by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunohistochemistry

Paraffin-embedded renal tissue sections (4 μm thick) were deparaffinized in xylene and rehydrated in ethanol [103]. Rabbit polyclonal anti-KLK4 antibody (1:200) was applied overnight at 4°C. Heat-induced epitope retrieval was performed using a 10 mMcitrate buffer (pH 6.0). Detection of the immunoreaction was performed using an HRP-labeled polymer secondary antibody (Thermo Fisher Scientific), 3,3-diaminobenzidine/H2O2

as chromogen and hematoxylin as counterstain. Histopathology pictures were captured using Nikon Eclipse E-400 light microscope and the Nikon Digital sight, DS-SM, photographic system.

Statistical analysis

Data are presented as mean ± standard deviation or dot plots of individual values. Differences between groups were tested with the Mann–Whitney or Kruskal–Wallis tests, and correlations with the Spearman's test. Stepwise linear regression was performed to identify clinical or histological parameters independently associated with micro-RNAs levels. Differences in KLK4 protein expression (nominal parameter) between control and LN groups were analyzed using Fisher's exact test. P-value <0.05 (two-tailed) was considered statistically significant. All calculations were performed using the Statistical Package for Social Sciences.

7. Results

7.1. Microarray analysis reveals that human LN is characterized by distinct kidney mi-RNA expression profile

We first profiled the expression of 365 mi-RNAs in kidney biopsy samples of active LN patients. The mi-RNA analysis identified a total of 24 miRNAs with dysregulated (>2-fold) expression; 9 mi-RNAs were up-regulated and 15 were down-regulated compared to controls **(Figure 7.1 A and B)**. MiR-422a and miR-21 were the two most up-regulated and miR-26a and miR-133 the two most down-regulated mi-RNAs. These results were confirmed by RT-PCR in the original kidney specimens **(Figure 7.1 C)**. Histological analysis revealed dysregulated miRNA expression both in proliferative (class III–IV) and in membranous (class V) LN, with a trend for more disturbed expression in the latter group **(Figure 7.1 D)**. Although this is a small number of patients tested, multivariable linear regression showed that apart from the histological class of LN, presence of fibrinoid necrosis was an independent predictor of increased miR-422a/miR-21 and decreased miR-26a **(Table 7.1)**. The composite biopsy activity index was strong predictor of miR-133 intra-renal levels ($\beta = 0.78$).

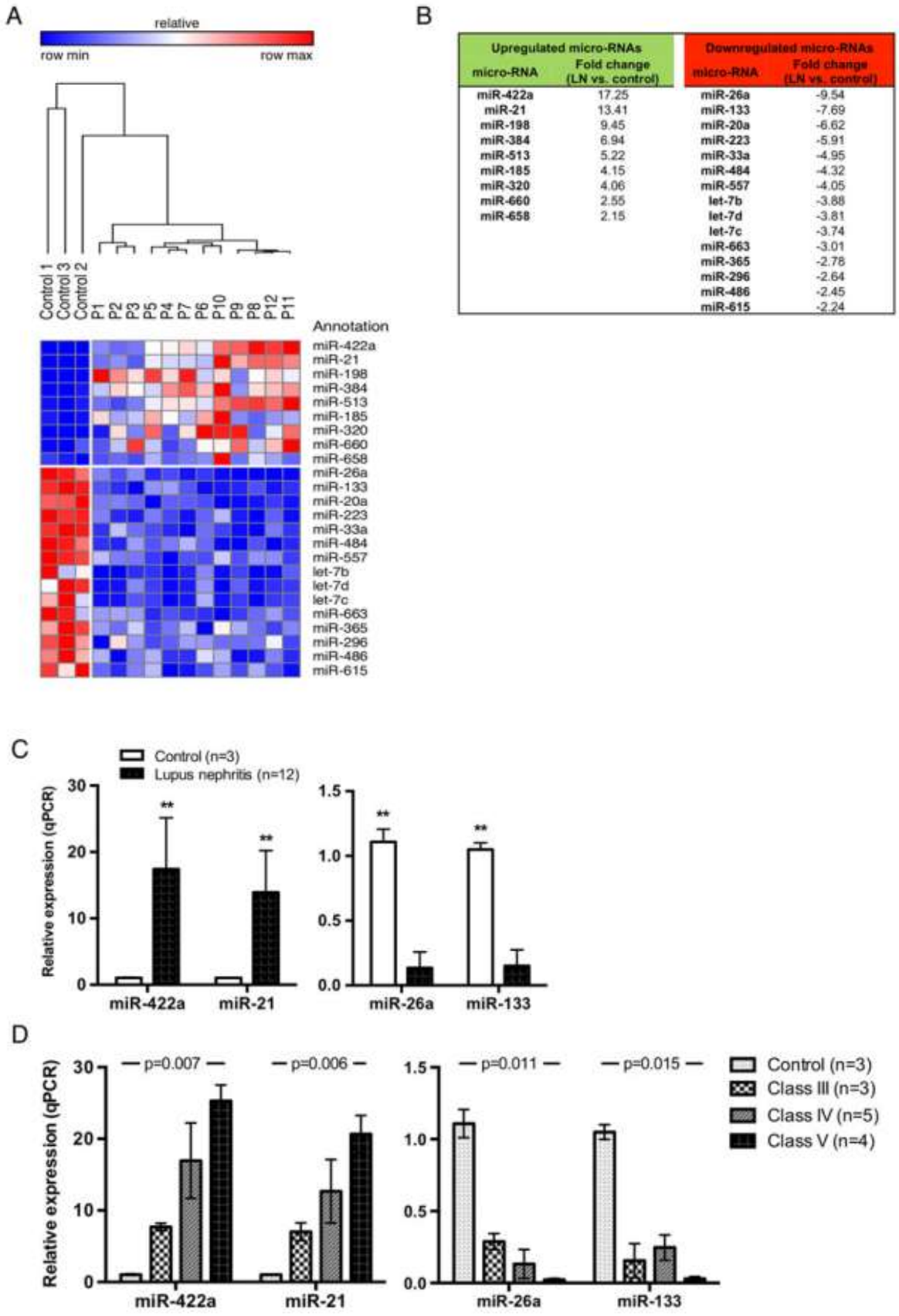


Figure 7.1. Human LN is characterized by distinct kidney micro-RNA expression profile

(A) Unsupervised hierarchical clustering analysis using the differentially expressed miRNAs can separate normal and active LN kidney samples. Heat map representation of the TLDA miRNA expression data in kidney specimens from 12 patients with active LN (P1 to P12) and three control samples (Control 1 to Control 3). Red represents higher mi-RNA expression and blue represents lower mi-RNA expression in LN compared with controls (or vice versa). The metric scale (top) represents the log10-fold change in mi-RNA expression using the group (LN or controls) with the lower expression as denominator. **(B)** Nine mi-RNAs were up-regulated by 2.2–17.3 times (green column) and 15 mi-RNAs were down-regulated by 2.2–9.5 times (red column) in LN versus unaffected renal samples. **(C)** Validation of the TLDA results for expression levels of miR-422a, miR-21, miR-26a and miR-133 by quantitative RT-PCR in the original dataset of LN and control kidney samples. Bars represent mean ± standard deviation (SD) of relative expression levels. **(D)** Expression levels (quantitative RT-PCR) of miR-422a, miR-21, miR-26a and miR-133 in controls and in LN kidney samples stratified according to the histological type of LN (class III, IV, V). Bars represent mean ± SD of relative expression levels. The Kruskal–Wallis test was used to test for statistically significant variation in miRNA expression across the four groups.

Micro-RNA	Independent variable (s)	Standardized β-coefficient	t-test	P-value
miR-422a	Proliferative (versus membranous) LN	−0.960	−6.690	<0.001
	Fibrinoid necrosis	0.626	4.361	0.002
miR-21	Proliferative (versus membranous) LN	−0.957	−6.060	<0.001
	Fibrinoid necrosis	0.571	3.615	0.007
miR-26a	Proliferative (versus membranous) LN	0.904	5.557	0.001
	Fibrinoid necrosis	−0.687	−4.221	0.003
miR-133	Activity index (0–24)	0.781	3.757	0.005

Multivariate stepwise linear regression analysis using each of the four micro-RNAs (miR-422a, miR-21, miR-26a, miR-133) as dependent variable. Demographic (gender, age), clinical parameters (urine sediment, serum creatinine, proteinuria), use of SLE treatments, histological class of LN (proliferative/membranous), the National Institutes of Health activity and chronicity indices (both the composite scores and the scores of individual features) were entered as independent variables. Only statistically significant ($P < 0.05$) associations are shown.

Table 7.1. Linear regression analysis for the identification of clinical and histological parameters associated with the expression levels of the two most up- and down-regulated miRNAs in LN.

7.2. Micro-RNAs that are dysregulated in LN are predicted to regulate gene networks involved in disease pathogenesis

MiRNAs with altered expression in LN are predicted to target genes involved in inflammatory signaling pathways, membrane transporters and cell homeostasis **(Figure 7.2 A)**. Pathway analysis based on these predictions showed enrichment for the TGF-β, protein kinase A, ERK/MAPK, NF-κB, HNF4A, Wnt/β-catenin signaling and also for the STAT3 and IL-4 pathways in active LN **(Figure 7.2 B)**. HNF4A was identified as a transcription factor key node, controlling several genes that are simultaneously regulated by the miRNAs with differential expression between LN and control kidneys **(Supplementary data, Figure S1)**. Combined gene network analysis identified individual kinases, miRNAs, proinflammatory cytokines and transcription factors as significant nodes involved in LN pathogenesis **(Figure 7.2 C)**. Integration analysis revealed that these pathways corresponded to pertinent disease toxicity lists, such as renal necrosis, glomerular injury, increased serum creatinine, cell death and survival, hematological cellular development as well as molecular mechanisms of cancer **(Supplementary data, Table S2)**. Thus, miRNAs with dysregulated expression in LN kidney lesions are predicted to regulate gene networks and molecular pathways with a plausible pathophysiological role.

A

Upregulated miRNAs	Selected gene targets
miR-422a	CYP8B1*, CYP7A1*, MLF1*, KLK4, TOB2, BMP2
miR-21	PDCD4*, BCL2*, PTEN*, TGFBI*, TIAM1, IL12A
miR-198	CCNT1*, NTRK3*, MET*, MYB*, HMGA1, STAT6, CDK4
miR-384	NTRK3*, CFTR, ESRRG, HDGF, MAPK9
miR-513	CD274*, GSTP1*, ITGA4, SMARCA1, CPD, GNRHR
miR-185	CDK6*, HMGA2*, DNMT1*, AKT1*, CASP14, WNT1
miR-320	TAC1*, MAPK1*, AQP1*, ATG7*, PBX3, IRF6, CCR7
miR-660	HUWE1*, NRCAM, MED8, IRS1, DNAJC3
miR-658	UPK1A, NES, TRAF6, CABP1
Downregulated miRNAs	**Selected gene targets**
miR-26a	HMGA1*, SMAD1*, EZH2*, MAP3K2*, TET2, LARP1
miR-133	KRT7*, CASP9*, BCL2L2*, IGF1R*, CLTA, PKHD1
miR-20a	HIF1A*, RUNX1*, CDKN1A*, E2F1*, PKD2, MAP3K2, EZH1, IRF9
miR-223	MEF2C*, ARID4B*, IL6*, RHOB*, SP3, PAX6
miR-33a	ABCA1*, SRC*, NPC1*, IRS2*, ABHD2, IL2RB
miR-484	FIS1*, HOXA5*, IL2*, PKD1*, IFNAR1, FGF1, TNFSF9
miR-557	CAMK4, FLT1, PRKCE, SP3
let-7b	CDC34*, NEDD4*, KRAS*, HMGA2, LIN28B, NPHP3
let-7d	HMGA2*, IL13*, BCL2*, BDNF*, CDC34, HIF1AN, SMC1A
let-7c	DICER1*, BCL2L1*, MYC*, HMGA2*, MAP4K3, IGDCC3, PTAFR
miR-663	JUNB*, KLF4*, JUND*, FOSB*, PAX2, SBF1, TGFB1
miR-365	CCND1*, IL6*, BAX*, NCOA4*, TBK1, HOXA9, LAMP2
miR-296	WNK4*, HOXA6*, KCNH1*, ARF1*, CX3CR1, BCAM, SERPINB2
miR-486	CD40*, TMED1*, NAT15, STIM1, AATK, MAPKBP1
miR-615	IGF2*, LCOR*, PBX3*, KRT19*, SEMA4B, RARA, KLF16

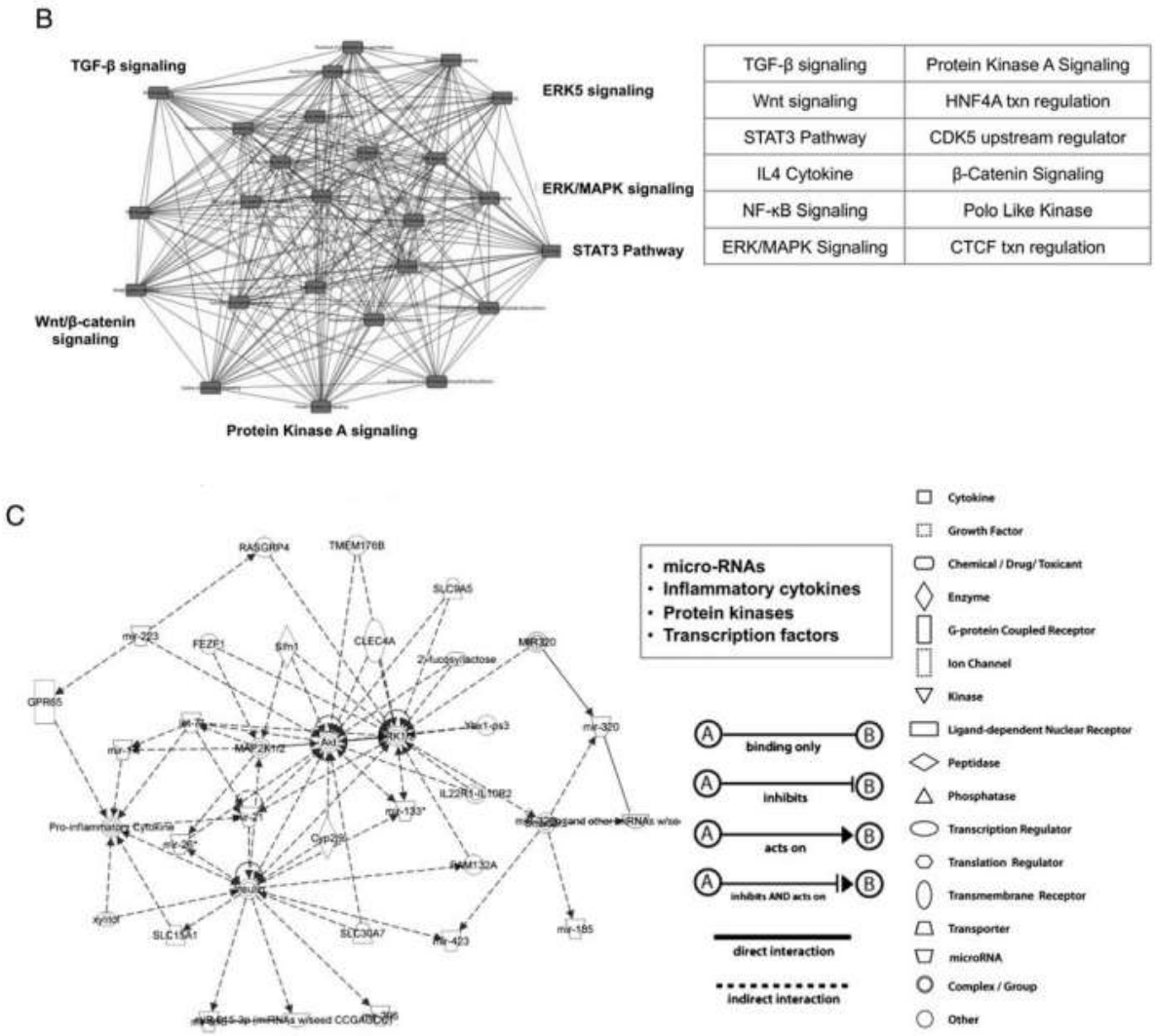
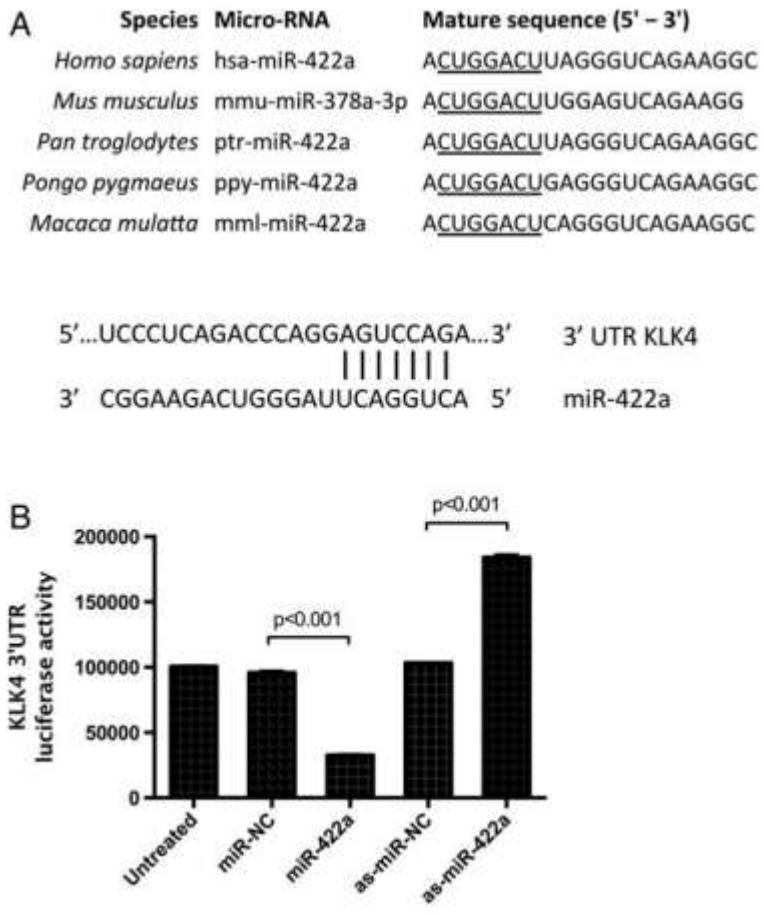


Figure 7.2. Micro-RNAs with altered expression in active LN are predicted to regulate gene networks and molecular pathways with a plausible pathophysiological role. (A) Predicted gene targets of the micro-RNAs with dysregulated (up- or down-regulated) expression in active LN compared with control kidney samples. Asterisks indicate experimentally validated gene targets according to the miRTarBase and miRecords databases. See more details in Materials and methods. **(B)** Significant canonical pathways (P-value <0.05) implicating predicted gene targets of LN-associated micro-RNAs, generated by Ingenuity Pathway Analysis. Pathway analysis shows enrichment for the TGF-β, protein kinase A, ERK/ MAPK, NF-κB, HNF4A, Wnt/β-catenin signaling and also for the STAT3 and IL-4 pathways in active LN. **(C)** Network analysis for top regulators of the predicted micro-RNA gene targets in LN identifies kinases, micro-RNAs, pro-inflammatory cytokines and transcription factors as significant nodes.

7.3. Mir-422a is upregulated in LN and regulates the expression of KLK4

MiR-422a is the miRNA with the highest expression (17.3-fold) in active LN. Bioinformatics analysis revealed that KLK4, which is a member of the KLK family of serine proteases, is the top-predicted gene target of miR-422a **(Figure 7.3 A)**. KLK4 is of particular interest since previous studies implicate KLK in various nephropathies including LN [104-106]. In order to validate that KLK4 is a direct target of miR-422a, HEK-293 cells were co-transfected with a plasmid containing firefly luciferase gene under the control of KLK4 3'-UTR and either miR-422a mimic (miR-422a) or miR-422a inhibitor (as-miR-422a) or the respective NCs. Overexpression of miR-422a suppressed luciferase activity by 65%, whereas inhibition of miR-422a induced luciferase activity by 80% **(Figure 7.3 B)**. We also examined the effect of miR-422a on KLK4 mRNA expression in HEK-293 cells. MiR-422a suppressed KLK4 mRNA by an average of 82%, whereas as-miR-422a caused a significant increase in KLK4 mRNA **(Figure 7.3 C)**. We conclude that KLK4 may be a direct target of miR-422a.



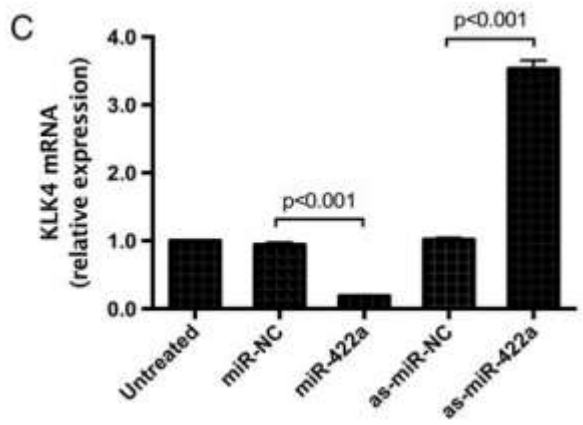


Figure 7.3. MiR-422a directly targets KLK4 mRNA. **(A)** 7mer seed sequence (underlined) of miR-422a is conserved across vertebrate species. TargetScan's predicted binding site of miR-422a within the UTR of human KLK4 mRNA. Lines represent interacting base pairs at the seed region of miR-422a. **(B)** HEK-293 cells were co-transfected with a plasmid containing Renilla luminescent reporter gene (RenSP) under the control of KLK4 30-UTR and either miR-422a mimic (miR-422a), or miR-422a inhibitor (as-miR-422a), or the respective NCs as described in Materials and methods. Luciferase activity was determined 36 h after transfection. Normalized luciferase activity is the Renilla/Firefly ratio of miR-422a-transfected reporter vector compared with the same NC vector. Data show the mean ± standard deviation (SD) from n = 3 independent experiments. **(C)** HEK-293 cells were transfected with 50–75 nM of miR-422a, as-miR-422a or their respective NCs (miR-NC, as-miR-NC), as described in Materials and methods. After 48 h, cells were collected for RNA extraction. KLK4 mRNA levels are significantly decreased in miR-422a-transfected cells, whereas they are significantly increased in as-miR-422a-transfected cells, as compared with their respective NC-transfected littermates. Bars represent the mean ± SD from n = 3 independent experiments.

7.4. KLK 4 mRNA levels were reduced in human LN showing an inverse correlation with miR-422a

In accordance with our finding that KLK4 may be targeted by miR-422a, we interestingly found that KLK4 mRNA was reduced in human LN versus control kidneys **(Figure 7.4 A)**. Moreover, miR-422a showed significant inverse correlation with KLK4 expression levels within the LN samples **(Figure 7.4 B)**.

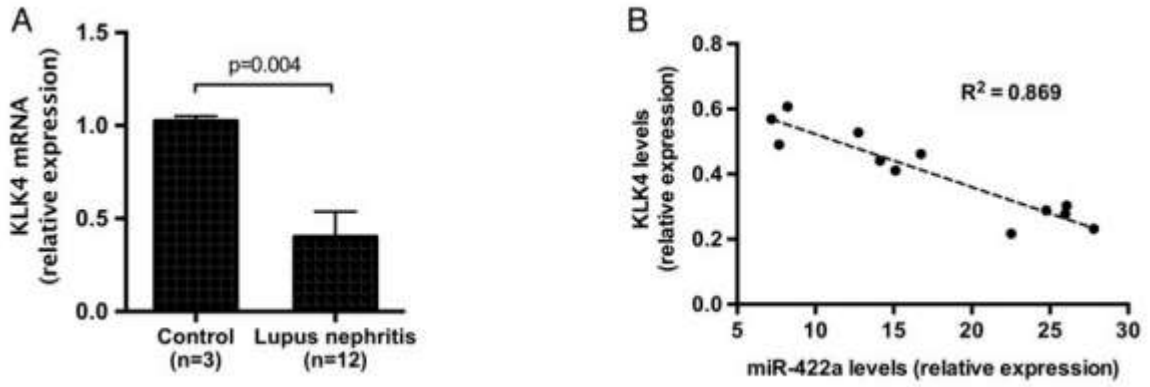


Figure 7.4. Deregulation of the miR-422a/KLK4 circuit in human LN. (A) Quantitative RT-PCR for KLK4 mRNA expression was performed in the original set of kidney specimens used for TLDA. KLK4 mRNA was significantly decreased by an average of 61% in active LN versus control kidney samples. Bars represent the mean ± standard deviation (SD) from n = 3 independent experiments. **(B)** Inverse correlation between intra-renal miR-422a and KLK4 mRNA levels in active LN. Analysis was performed within the subgroup of human LN kidney specimens (n = 12). The R-squared statistic is shown as a measure of how close the data are to the fitted regression line.

7.5. MiR-422a was upregulated and KLK4 downregulated in NZB/W F1 mice with proteinuria

The next aim was to test our results in NZB/W F1 mice, which spontaneously develop lupus and immune complex-mediated lupus glomerulonephritis closely resembling the human disease after the age of 5–6 months. We used 8-week-old pre-diseased and 24-week-old diseased NZB/W mice with overt proteinuria, which were compared against age-matched NZW control mice. RT-PCR in kidney tissue showed that miR-422a (mmu miR- 378) was significantly up-regulated in NZB/W F1 compared with NZW mice, both in the 8-week-old (by 4.1 times) and in the 24-week-old (by 9.4 times) **(Figure 7.5 C)**. An inverse correlation was observed with regards to KLK4 mRNA levels, which were reduced by an average of 3.4 and 7.6 times in young and old NZB/W1 mice, respectively, compared with NZW littermates. Immunoblotting in kidney lysates suggested diminished 28–30 kDa KLK4 protein expression in 24-week-old diseased NZB/W1 mice compared with 8-week-old pre-diseased and NZW control mice **(Figure 7.5 D)**. These findings implicate the miR-422a/KLK4 circuit in both human and murine LN.

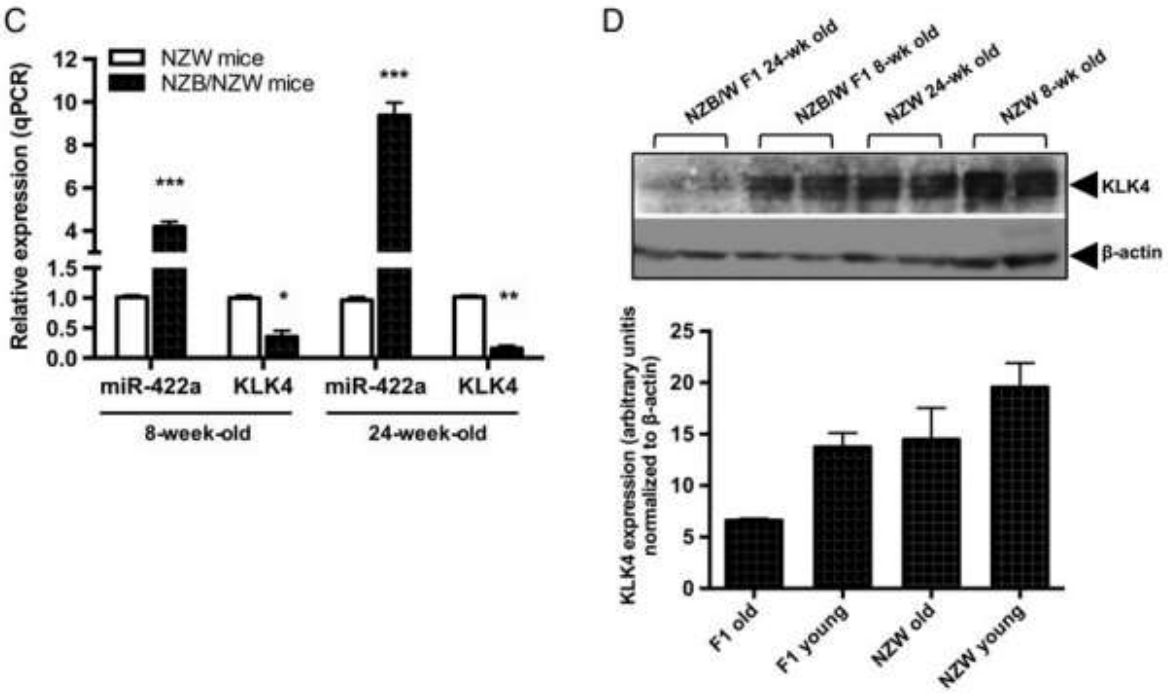


Figure 7.5. Deregulation of the miR-422a and KLK4 levels in murine LN (C) Quantitative RT-PCR for miR-422a (mmu-miR-378) and KLK4 mRNA was performed in kidney tissue from 8-week-old pre-diseased NZB/W F1 mice (n = 3), 24-week-old proteinuric NZB/W F1 mice (n = 4) and their age-matched NZW littermates (n = 3 in each group). Bars represent mean ± SD expression normalized to expression levels in control NZW mice across each age group and each gene. ***P < 0.001; **P < 0.01; *P < 0.05 compared with NZW. **(D)** Immunoblotting for KLK4 protein expression (28–30 kDa) was performed in kidney lysates from the same groups of mice as in (C). Protein densitometry was performed with ImageJ Software and KLK4 expression levels were normalized to β-actin protein levels. See Materials and methods for more details.

7.6. KLK4 expression is reduced in renal mesangial and tubular epithelial cells in murine and human LN.

Previous studies have shown that kallikreins are abundantly expressed and regulate the physiology of epithelial cells, including renal tubular epithelial cells. To gain insight into the renal expression and localization pattern of KLK4, we carried out an immunohistochemistry study in additional kidney specimens from NZB/W F1 mice and patients with active LN. KLK4 protein was strongly expressed (staining intensity 3+) mainly in the cytoplasm of renal tubular as well as mesangial cells in all specimens from unaffected NZW kidney and human control specimens **(Figure 7.6 A, top panel, and B)**. In contrast, KLK4 showed absent/faint (1+) or moderate (2+) expression in renal parenchymal cells of proteinuric NZB/W F1 and

most patients with active LN **(Figure 7.6 A, bottom panel, and B)**. Due to the relatively small sample size of human specimens, we could not detect any significant associations with LN histological or clinical variables.

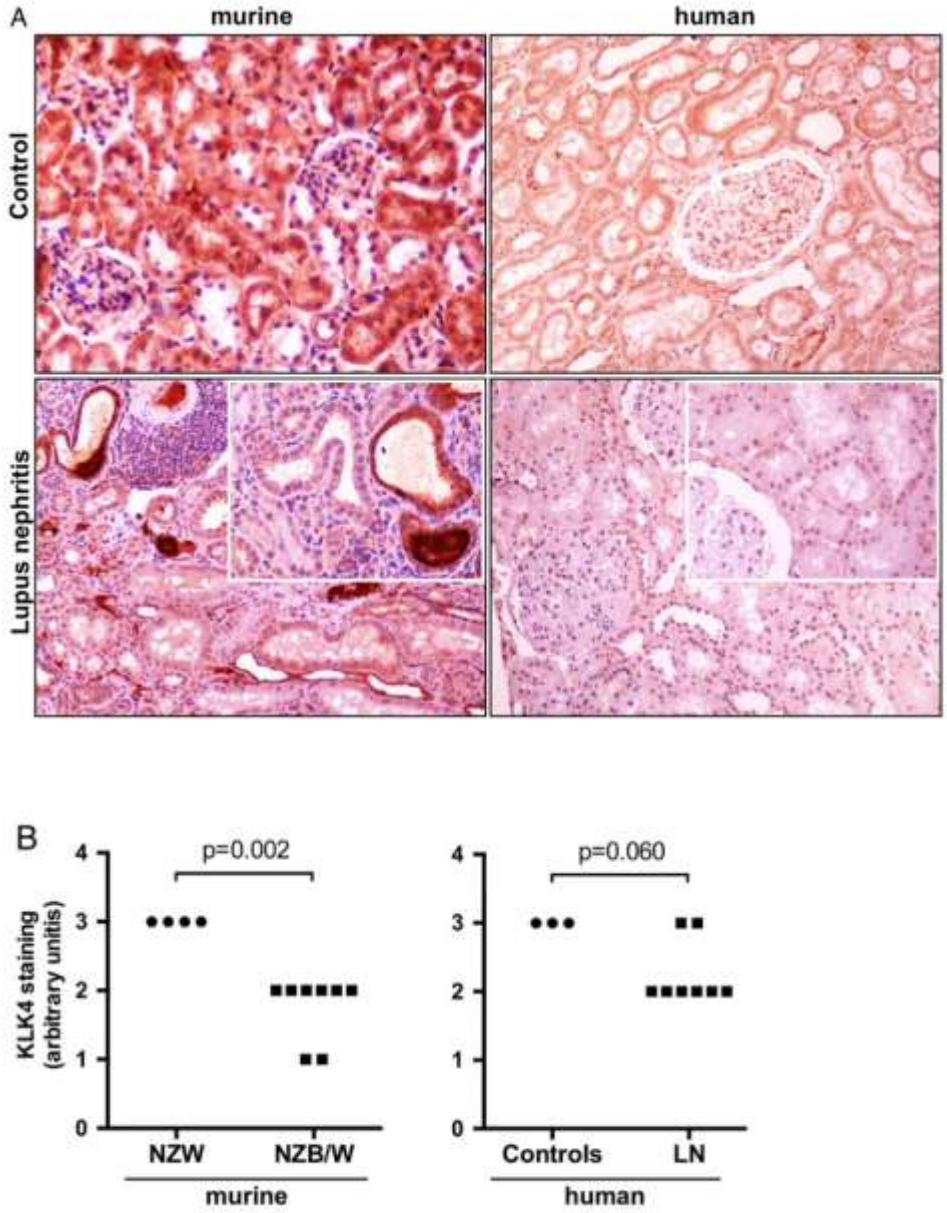


Figure 7.6. Reduced KLK4 protein expression by renal mesangial and tubular epithelial cells in murine and human LN. (A) Immunohistochemistry for KLK4 protein expression in human and murine control (unaffected) and inflamed LN kidney specimens. KLK4 was highly expressed in epithelial cells of all renal tubules and mesangial cells of glomeruli in control renal tissue without inflammation (upper panel). In contrast, absence, or low levels of KLK4 protein was observed in most renal tissues of NZB/W F1 mice or patients with active LN (lower panel). Notice, that a small minority of renal tubules retains normal KLK4 protein expression. See Materials and methods for more details. [DAB was used as chromogen, hematoxylin as counterstain; original magnification ×100 (insets magnification ×400)]. **(B)** Semi-quantification of the

immunohistochemistry results (1+ = faint/absent, 2+ = moderate staining, 3+ = strong staining) of KLK4 expression in murine (left panel) and human (right panel) kidney specimens. See Materials and methods for more details. In mice, all four control NZW kidneys had high KLK4 expression (staining 3+), whereas all eight NZW/BW F1 mice with proteinuria had low/moderate KLK4 expression (1+/2+) (Fisher's exact test, P = 0.002). In humans, high KLK4 expression was found in all three control kidney specimens as compared with only two out of eight LN specimens (Fisher's exact test, P = 0.060).

8. Discussion

In this study we sought to investigate the role of miRNAs in acute kidney inflammation associated with LN and to explore their potential implication in end-organ injury. We identified a set of differentially expressed miRNAs in active LN kidneys, compared to healthy controls. Interestingly, several of these miRNAs were predicted to interfere with major intracellular pathways and relevant processes such as renal necrosis and inflammation, glomerular injury and increased serum creatinine. Downstream molecular pathways include the TGF-β, protein kinase A, ERK/MAPK, NF-κB, HNF4A, Wnt/β-catenin, STAT3 and IL-4. This is consistent with the observation of Bethunaickan et al., who performed comparative mRNA profiling in the kidneys of three murine LN models and found TGF-β, STAT3, NF-κB and HNF4A transcription factor to be shared by all three strains [18].

The miRNA profiling was performed in 12 FFPE human kidney biopsy specimen and 3 control kidney tissue. Twenty-four miRNAs were differentially expressed in LN versus controls. The results were validated in vitro by using human cell culture and in vivo by means of a murine model of LN and were confirmed by carrying out Western blot and immunohistochemistry in both human and murine samples as well. Notably, active kidney lesions, particularly fibrinoid necrosis, were independent predictors of the two most up and down-regulated mi-RNAs. From a clinical standpoint high activity index and the presence of fibrinoid necrosis in kidney biopsy are adverse prognostic factors in LN, associated with increased risk for development of end-stage renal disease [100]. Validation in larger number of specimens will be required to explore the possible role of miR-422a as a biomarker of severe LN.

Several miRNAs have been identified in lupus kidneys. Dai et al. identified 36 up-regulated and 30 down-regulated miRNAs in fresh frozen kidney biopsy samples of patients with class II LN [89]. Lu et al examined the glomerular and tubulointerstitial expression of miRNA in different classes of LN patients. MiR-198 and miR-146a were highly expressed in both compartments, while miR-638 had lower expression at the glomerular level and their expression was correlated with the histological activity index, proteinuria as well as the SLEDAI score [95]. Zhou et al. compared 14 FFPE LN kidney biopsies with high (≥4) versus low (<4) chronicity index and identified 16 mi-RNAs with >2-fold increases. There was a strong correlation of miR-150 with changes in chronicity index [96]. They also showed that let-7i was up-regulated in LN with high compared to low chronicity index. We found that let-

7b/c/d were down-regulated in active LN and the discrepant findings could be explained by the different platforms used (Affymetrix microarrays versus TLDA) and the fact that our kidney biopsies comprised mainly of active LN (median activity index: 9) with lower chronicity scores compared to those used by Zhou et al. (median chronicity index: 3 versus 4 in our samples) [96].

In one study of B6.MRLc1 autoimmune glomerulonephritis, miR-146a was found increased and correlated with interstitial cell infiltration, tubular atrophy and fibrosis [86]. Reduced glomerular miR-26a in LN correlated with markers of podocyte injury [98]. In agreement, we found increased miR-198 and reduced miR-26a levels in active human LN and several miRNAs with differential expression in our dataset have been involved in other nephropathies as well. These include miR-21 in TGF-β mediated renal fibrosis, miR-133 in polycystic kidney disease, miR-223 in IgA nephropathy, miR-663 in hypertensive kidney disease and miR-296 in ischemic acute injury[107-112]. Let-7b and let-7c, both down-regulated in our active LN cohort, have been shown to exert renal antifibrotic effects [112].

The highest up-regulated miR-422a targeted KLK4, which expression was reduced in mesangial as well as tubular epithelial cells in active human and murine LN. This is consistent with previous studies that describe KLK expression in those cell types, which in turn are known to affect local immune response in glomerulonephritides such as LN [113-117]. It is not clear why KLK4/KLKs are expressed in specific resident renal cells. Moreover, two out of eight samples in human LN had normal KLK4 expression, which could be interpreted by the heterogeneity of the disease.

The renal KLK-kinin system is a multigene family of serine peptidases that plays an important role in inflammation, coagulation, angiogenesis and regulation of vessel tone and permeability [118-120]. The system originates from prekallikrein, which is being activated by factor XIIa and forms kallikrein. Tissue or serum kallikrein converts kininogen into kinins, including bradykinin and kallidin in humans. Kinins activate their receptors (B2R and B1R) and in turn several intracellular pathways, like PLA-2 or PI-PLC-dependent in diabetic nephropathy [121]. Kallikreins can also directly stimulate protease-activated receptors to initiate trans-plasma membrane signal transduction [120]. Kininases are a group of peptidases that rapidly hydrolyze kinins to inactive products. Kininase II is the same enzyme as angiotensin I-converting enzyme and there is much on the literature about the beneficial effects of ACEIs on renal diseases independently of their effect on blood pressure [122, 123].

The kallikrein-kinin system has been shown to have a protective role against ischemia – reperfusion renal injury, diabetic nephropathy, autosomal dominant polycystic kidney disease, chronic renal failure and hypertensive glomerulosclerosis [104, 106, 122, 124-128]. In diabetic nephropathy, the protective effect of KLK is believed to be carried out through inhibition of oxidative stress and inflammation, decrease of fibrosis and protection of the endothelial function [125]. In SLE, there are data to suggest the role of kallikreins as proinflammatory mediators implicated in the acute manifestations of LN, but also as disease genes playing a role in local tissue damage caused by the autoimmune response [105, 129-131]. In line with their role in the inflammatory cascade, higher levels of kininogen and tissue and urinary kallikrein were found in 30 patients with active lupus nephritis compared with age and sex matched controls [129].

Liu et al compared the transcriptome in the renal cortex of mouse strains sensitive versus resistant to anti-GBM antibody-induced nephritis and identified tissue kallikreins as lupus-susceptibility genes. Fine-mapping studies of the mouse Sle3 lupus-susceptibility locus showed that it harbors the kallikrein cluster of genes and contributes to their reduced expression [105]. Kinin B2 receptor agonists and klk1 gene delivery ameliorated anti-GBM antibody-induced nephritis [105, 132]. Finally, klk1 overexpression in renal tubular epithelial cells or delivery of klk1 in inflamed kidneys by mesenchymal stem cells protected against nephritis in B6.Sle1.Sle3 LN mice [115, 133]. Moreover, polymorphisms in human KLK1 and KLK3 promoter were found to be associated with SLE and single-nucleotide polymorphisms in the KLK4 promoter, KLK5 and the KLK8-KLK11 block were more frequent in LN [105].

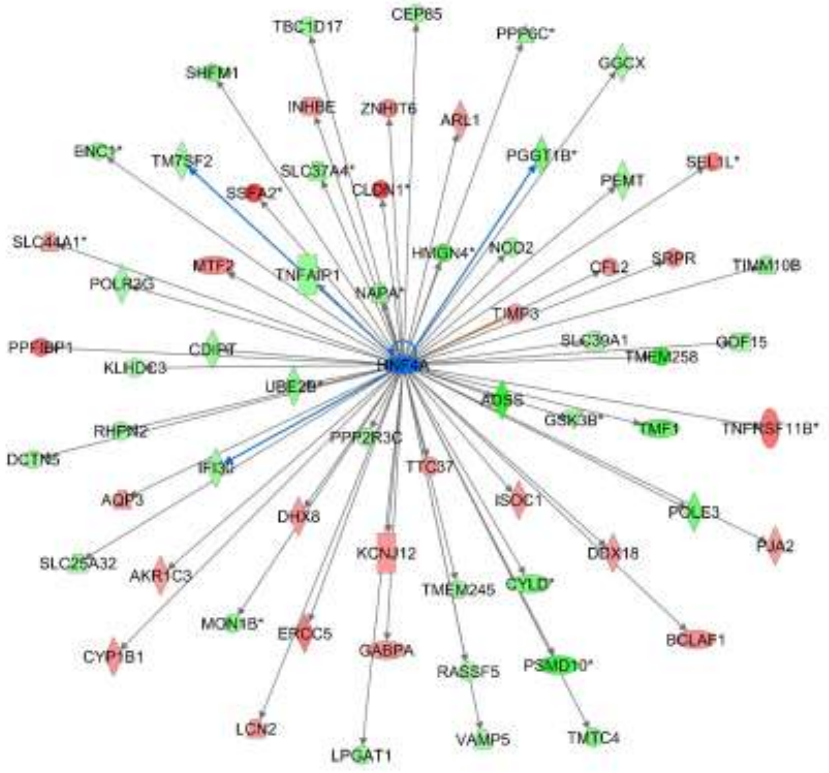
We used FFPE tissue samples in this study. While FFPE preserves tissue for histological analysis, nucleic acids may become irreversibly damaged and fragmented through formalin fixation and prolonged storage. However, mi-RNAs tend not to suffer from the fragmentation effect, probably due to their intrinsically shorter lengths and close association with large protein aggregates [134, 135]. Previous work in kidney, breast and colon cancer has shown that it is possible to perform genome-wide micro-RNAs screening using FFPE specimens [136, 137]. Studies comparing micro-RNA profiling between frozen and FFPE tissues have demonstrated high correlation with remarkable stability [138-140]. Finally, evidence suggests that PCR-based methods (including TLDA) are technically and biologically robust for analyzing micro-RNAs in FFPE samples [136, 141].

Conclusively, several micro-RNAs show altered expression in LN and are predicted to regulate molecular pathways with a plausible pathophysiological role. MiR-422a is overexpressed, correlates with active fibrinoid necrosis in inflamed kidneys and downregulates KLK4, a member of the kallikrein family with presumed renoprotective properties. These results coupled with evidence from in vivo animal studies suggest that the miR-422a/KLK4 axis could be exploited as therapeutic target in LN [115].

9. Supplementary material

Gender (female/male)	13 (65%) / 7 (35%)
Age (years)	36.0 ± 15.1
SLE duration (years)	4.4 ± 5.5
Proteinuria (g/24hr)	3.5 ± 3.0
Serum creatinine (mg/dl)	1.7 ± 2.0
Renal biopsy	
Class III	5 (25%)
Class IV	7 (35%)
Class V	5 (25%)
Class V + III/IV	3 (15%)
NIH activity index (0-24)	8.8 (1.0)
NIH chronicity index (0-12)	3.4 (0.7)
Treatment [2]	
None	9 (45%)
Glucocorticoids	10 (50%)
Methotrexate	1 (5%)
Azathioprine	3 (15%)
Mycophenolic acid	1 (5%)
Calcineurin inhibitor	2 (10%)

Supplementary Table S1. Clinical characteristics of the LN patients (n = 20) included in the study. It includes both patients who participated in the TLDA analysis and in the validation immunohistochemistry studies. The treatment is referred to the medication taken at the time of kidney biopsy. Data are presented as n (%) or mean ± SD.



Supplementary Figure S1. HNF4A controls genes that are regulated by differentially expressed miRs in LN

Renal Necrosis/Inflammation	**Hematological Cellular Development**
Glomerular Injury	**Increased Levels of Creatinine**
Cell Death and Survival	**Molecular Mechanisms of Cancer**

Supplementary Table S2. Disease toxicity lists generated through integration analysis of gene-targets of micro-RNAs with differential expression in LN versus control samples.

10. References

1. Tsokos, G.C., et al., *New insights into the immunopathogenesis of systemic lupus erythematosus.* Nat Rev Rheumatol, 2016. **12**(12): p. 716-730.
2. Lisnevskaia, L., G. Murphy, and D. Isenberg, *Systemic lupus erythematosus.* Lancet, 2014. **384**(9957): p. 1878-1888.
3. Hochberg, M.C., *Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus.* Arthritis Rheum, 1997. **40**(9): p. 1725.
4. Wahren-Herlenius, M. and T. Dorner, *Immunopathogenic mechanisms of systemic autoimmune disease.* Lancet, 2013. **382**(9894): p. 819-31.
5. Dorner, T., C. Giesecke, and P.E. Lipsky, *Mechanisms of B cell autoimmunity in SLE.* Arthritis Res Ther, 2011. **13**(5): p. 243.
6. Shao, W.H. and P.L. Cohen, *Disturbances of apoptotic cell clearance in systemic lupus erythematosus.* Arthritis Res Ther, 2011. **13**(1): p. 202.
7. Teruel, M. and M.E. Alarcon-Riquelme, *The genetic basis of systemic lupus erythematosus: What are the risk factors and what have we learned.* J Autoimmun, 2016. **74**: p. 161-175.
8. Alarcon, G.S., *Multiethnic lupus cohorts: what have they taught us?* Reumatol Clin, 2011. **7**(1): p. 3-6.
9. Hiraki, L.T., et al., *Clinical and laboratory characteristics and long-term outcome of pediatric systemic lupus erythematosus: a longitudinal study.* J Pediatr, 2008. **152**(4): p. 550-6.
10. Seligman, V.A., et al., *Demographic differences in the development of lupus nephritis: a retrospective analysis.* Am J Med, 2002. **112**(9): p. 726-9.
11. Tektonidou, M.G., A. Dasgupta, and M.M. Ward, *Risk of End-Stage Renal Disease in Patients With Lupus Nephritis, 1971-2015: A Systematic Review and Bayesian Meta-Analysis.* Arthritis Rheumatol, 2016. **68**(6): p. 1432-41.
12. de Holanda, M.I., et al., *The prevalence of HLA alleles in a lupus nephritis population.* Transpl Immunol, 2018.
13. Kaplan, M.J., *Neutrophils in the pathogenesis and manifestations of SLE.* Nat Rev Rheumatol, 2011. **7**(12): p. 691-9.
14. Nakou, M., et al., *Gene expression in systemic lupus erythematosus: bone marrow analysis differentiates active from inactive disease and reveals apoptosis and granulopoiesis signatures.* Arthritis Rheum, 2008. **58**(11): p. 3541-9.
15. Banchereau, R., et al., *Personalized Immunomonitoring Uncovers Molecular Networks that Stratify Lupus Patients.* Cell, 2016. **165**(3): p. 551-65.
16. Hakkim, A., et al., *Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis.* Proc Natl Acad Sci U S A, 2010. **107**(21): p. 9813-8.
17. Birmingham, D.J., et al., *Relationship of Circulating Anti-C3b and Anti-C1q IgG to Lupus Nephritis and Its Flare.* Clin J Am Soc Nephrol, 2016. **11**(1): p. 47-53.
18. Bethunaickan, R., et al., *Comparative transcriptional profiling of 3 murine models of SLE nephritis reveals both unique and shared regulatory networks.* PLoS One, 2013. **8**(10): p. e77489.
19. Ge, Y., et al., *Cgnz1 allele confers kidney resistance to damage preventing progression of immune complex-mediated acute lupus glomerulonephritis.* J Exp Med, 2013. **210**(11): p. 2387-401.
20. Berthier, C.C., et al., *Cross-species transcriptional network analysis defines shared inflammatory responses in murine and human lupus nephritis.* J Immunol, 2012. **189**(2): p. 988-1001.
21. Bethunaickan, R., et al., *A unique hybrid renal mononuclear phagocyte activation phenotype in murine systemic lupus erythematosus nephritis.* J Immunol, 2011. **186**(8): p. 4994-5003.

22. Bartel, D.P., *MicroRNAs: target recognition and regulatory functions.* Cell, 2009. **136**(2): p. 215-33.
23. Dugas, D.V. and B. Bartel, *MicroRNA regulation of gene expression in plants.* Curr Opin Plant Biol, 2004. **7**(5): p. 512-20.
24. Yates, L.A., C.J. Norbury, and R.J. Gilbert, *The long and short of microRNA.* Cell, 2013. **153**(3): p. 516-9.
25. Lee, R.C., R.L. Feinbaum, and V. Ambros, *The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14.* Cell, 1993. **75**(5): p. 843-54.
26. Reinhart, B.J., et al., *The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans.* Nature, 2000. **403**(6772): p. 901-6.
27. Wightman, B., I. Ha, and G. Ruvkun, *Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans.* Cell, 1993. **75**(5): p. 855-62.
28. Calin, G.A., et al., *Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers.* Proc Natl Acad Sci U S A, 2004. **101**(9): p. 2999-3004.
29. Emde, A. and E. Hornstein, *miRNAs at the interface of cellular stress and disease.* EMBO J, 2014. **33**(13): p. 1428-37.
30. Yeh, T.C., et al., *miR-151-3p Targets TWIST1 to Repress Migration of Human Breast Cancer Cells.* PLoS One, 2016. **11**(12): p. e0168171.
31. Rajewsky, N., *microRNA target predictions in animals.* Nat Genet, 2006. **38 Suppl**: p. S8-13.
32. Selbach, M., et al., *Widespread changes in protein synthesis induced by microRNAs.* Nature, 2008. **455**(7209): p. 58-63.
33. Lewis, B.P., et al., *Prediction of mammalian microRNA targets.* Cell, 2003. **115**(7): p. 787-98.
34. Xia, W., et al., *Integrative multi-omics analysis revealed SNP-lncRNA-mRNA (SLM) networks in human peripheral blood mononuclear cells.* Hum Genet, 2017. **136**(4): p. 451-462.
35. Luo, Q., et al., *Integrative analysis of long non-coding RNAs and messenger RNA expression profiles in systemic lupus erythematosus.* Mol Med Rep, 2018. **17**(3): p. 3489-3496.
36. Yang, H., et al., *Long noncoding RNA MALAT-1 is a novel inflammatory regulator in human systemic lupus erythematosus.* Oncotarget, 2017. **8**(44): p. 77400-77406.
37. Zhang, F., et al., *Identification of the long noncoding RNA NEAT1 as a novel inflammatory regulator acting through MAPK pathway in human lupus.* J Autoimmun, 2016. **75**: p. 96-104.
38. Kim, V.N., *MicroRNA biogenesis: coordinated cropping and dicing.* Nat Rev Mol Cell Biol, 2005. **6**(5): p. 376-85.
39. Saini, H.K., S. Griffiths-Jones, and A.J. Enright, *Genomic analysis of human microRNA transcripts.* Proc Natl Acad Sci U S A, 2007. **104**(45): p. 17719-24.
40. Lee, Y., et al., *MicroRNA maturation: stepwise processing and subcellular localization.* EMBO J, 2002. **21**(17): p. 4663-70.
41. Gregory, R.I., et al., *The Microprocessor complex mediates the genesis of microRNAs.* Nature, 2004. **432**(7014): p. 235-40.
42. Lee, Y., et al., *The nuclear RNase III Drosha initiates microRNA processing.* Nature, 2003. **425**(6956): p. 415-9.
43. Fletcher, C.E., et al., *A novel role for GSK3beta as a modulator of Drosha microprocessor activity and MicroRNA biogenesis.* Nucleic Acids Res, 2016.
44. Denli, A.M., et al., *Processing of primary microRNAs by the Microprocessor complex.* Nature, 2004. **432**(7014): p. 231-5.
45. Kim, Y.K., B. Kim, and V.N. Kim, *Re-evaluation of the roles of DROSHA, Export in 5, and DICER in microRNA biogenesis.* Proc Natl Acad Sci U S A, 2016. **113**(13): p. E1881-9.
46. Yi, R., et al., *Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs.* Genes Dev, 2003. **17**(24): p. 3011-6.
47. Pillai, R.S., C.G. Artus, and W. Filipowicz, *Tethering of human Ago proteins to mRNA mimics the miRNA-mediated repression of protein synthesis.* RNA, 2004. **10**(10): p. 1518-25.

48. Hutvagner, G., et al., *A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA.* Science, 2001. **293**(5531): p. 834-8.
49. Schwarz, D.S., et al., *Asymmetry in the assembly of the RNAi enzyme complex.* Cell, 2003. **115**(2): p. 199-208.
50. Diederichs, S. and D.A. Haber, *Dual role for argonautes in microRNA processing and posttranscriptional regulation of microRNA expression.* Cell, 2007. **131**(6): p. 1097-108.
51. Gan, H.H. and K.C. Gunsalus, *Assembly and analysis of eukaryotic Argonaute-RNA complexes in microRNA-target recognition.* Nucleic Acids Res, 2015. **43**(20): p. 9613-25.
52. Doench, J.G. and P.A. Sharp, *Specificity of microRNA target selection in translational repression.* Genes Dev, 2004. **18**(5): p. 504-11.
53. Grimson, A., et al., *MicroRNA targeting specificity in mammals: determinants beyond seed pairing.* Mol Cell, 2007. **27**(1): p. 91-105.
54. Humphreys, D.T., et al., *MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function.* Proc Natl Acad Sci U S A, 2005. **102**(47): p. 16961-6.
55. Kiriakidou, M., et al., *An mRNA m7G cap binding-like motif within human Ago2 represses translation.* Cell, 2007. **129**(6): p. 1141-51.
56. Nottrott, S., M.J. Simard, and J.D. Richter, *Human let-7a miRNA blocks protein production on actively translating polyribosomes.* Nat Struct Mol Biol, 2006. **13**(12): p. 1108-14.
57. Petersen, C.P., et al., *Short RNAs repress translation after initiation in mammalian cells.* Mol Cell, 2006. **21**(4): p. 533-42.
58. Filipowicz, W., S.N. Bhattacharyya, and N. Sonenberg, *Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight?* Nat Rev Genet, 2008. **9**(2): p. 102-14.
59. Sun, Y., et al., *Development of a micro-array to detect human and mouse microRNAs and characterization of expression in human organs.* Nucleic Acids Res, 2004. **32**(22): p. e188.
60. Tian, Z., et al., *MicroRNA-target pairs in the rat kidney identified by microRNA microarray, proteomic, and bioinformatic analysis.* Genome Res, 2008. **18**(3): p. 404-11.
61. Agrawal, R., U. Tran, and O. Wessely, *The miR-30 miRNA family regulates Xenopus pronephros development and targets the transcription factor Xlim1/Lhx1.* Development, 2009. **136**(23): p. 3927-36.
62. Li, Z., et al., *miR-200 family promotes podocyte differentiation through repression of RSAD2.* Sci Rep, 2016. **6**: p. 27105.
63. Wessely, O., R. Agrawal, and U. Tran, *MicroRNAs in kidney development: lessons from the frog.* RNA Biol, 2010. **7**(3): p. 296-9.
64. Park, S.M., et al., *The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2.* Genes Dev, 2008. **22**(7): p. 894-907.
65. Nagalakshmi, V.K., et al., *microRNA-dependent temporal gene expression in the ureteric bud epithelium during mammalian kidney development.* Dev Dyn, 2015. **244**(3): p. 444-56.
66. Zhdanova, O., et al., *The inducible deletion of Drosha and microRNAs in mature podocytes results in a collapsing glomerulopathy.* Kidney Int, 2011. **80**(7): p. 719-30.
67. Fu, Y., et al., *Interaction between Tbx1 and Hoxd10 and connection with TGFbeta-BMP signal pathway during kidney development.* Gene, 2014. **536**(1): p. 197-202.
68. Karner, C.M., et al., *Canonical Wnt9b signaling balances progenitor cell expansion and differentiation during kidney development.* Development, 2011. **138**(7): p. 1247-57.
69. Ho, J., et al., *Podocyte-specific loss of functional microRNAs leads to rapid glomerular and tubular injury.* J Am Soc Nephrol, 2008. **19**(11): p. 2069-75.
70. Kato, M., et al., *MicroRNA-192 in diabetic kidney glomeruli and its function in TGF-beta-induced collagen expression via inhibition of E-box repressors.* Proc Natl Acad Sci U S A, 2007. **104**(9): p. 3432-7.
71. Krupa, A., et al., *Loss of MicroRNA-192 promotes fibrogenesis in diabetic nephropathy.* J Am Soc Nephrol, 2010. **21**(3): p. 438-47.

72. Putta, S., et al., *Inhibiting microRNA-192 ameliorates renal fibrosis in diabetic nephropathy.* J Am Soc Nephrol, 2012. **23**(3): p. 458-69.
73. Du, B., et al., *High glucose down-regulates miR-29a to increase collagen IV production in HK-2 cells.* FEBS Lett, 2010. **584**(4): p. 811-6.
74. Hsu, Y.C., et al., *Protective effects of miR-29a on diabetic glomerular dysfunction by modulation of DKK1/Wnt/beta-catenin signaling.* Sci Rep, 2016. **6**: p. 30575.
75. Wang, Q., et al., *MicroRNA-377 is up-regulated and can lead to increased fibronectin production in diabetic nephropathy.* FASEB J, 2008. **22**(12): p. 4126-35.
76. Lakhia, R., et al., *MicroRNA-21 Aggravates Cyst Growth in a Model of Polycystic Kidney Disease.* J Am Soc Nephrol, 2016. **27**(8): p. 2319-30.
77. Lee, S.O., et al., *MicroRNA15a modulates expression of the cell-cycle regulator Cdc25A and affects hepatic cystogenesis in a rat model of polycystic kidney disease.* J Clin Invest, 2008. **118**(11): p. 3714-24.
78. Serino, G., et al., *Abnormal miR-148b expression promotes aberrant glycosylation of IgA1 in IgA nephropathy.* J Am Soc Nephrol, 2012. **23**(5): p. 814-24.
79. Yang, D., et al., *The efficacy of tonsillectomy on clinical remission and relapse in patients with IgA nephropathy: a randomized controlled trial.* Ren Fail, 2016. **38**(2): p. 242-8.
80. Liu, H., et al., *Identifying mRNA targets of microRNA dysregulated in cancer: with application to clear cell Renal Cell Carcinoma.* BMC Syst Biol, 2010. **4**: p. 51.
81. Petrozza, V., et al., *Oncogenic MicroRNAs Characterization in Clear Cell Renal Cell Carcinoma.* Int J Mol Sci, 2015. **16**(12): p. 29219-25.
82. Matz, M., et al., *Identification of T Cell-Mediated Vascular Rejection After Kidney Transplantation by the Combined Measurement of 5 Specific MicroRNAs in Blood.* Transplantation, 2016. **100**(4): p. 898-907.
83. van de Vrie, M., et al., *Urinary MicroRNA as Biomarker in Renal Transplantation.* Am J Transplant, 2016.
84. Liu, Z., et al., *MicroRNAs in Pathogenesis of Acute Kidney Injury.* Nephron, 2016. **134**(3): p. 149-153.
85. Zhang, L., et al., *Implications of dynamic changes in miR-192 expression in ischemic acute kidney injury.* Int Urol Nephrol, 2016.
86. Ichii, O., et al., *Altered expression of microRNA miR-146a correlates with the development of chronic renal inflammation.* Kidney Int, 2012. **81**(3): p. 280-92.
87. Macconi, D., et al., *MicroRNA-324-3p promotes renal fibrosis and is a target of ACE inhibition.* J Am Soc Nephrol, 2012. **23**(9): p. 1496-505.
88. Dai, Y., et al., *Microarray analysis of microRNA expression in peripheral blood cells of systemic lupus erythematosus patients.* Lupus, 2007. **16**(12): p. 939-46.
89. Dai, Y., et al., *Comprehensive analysis of microRNA expression patterns in renal biopsies of lupus nephritis patients.* Rheumatol Int, 2009. **29**(7): p. 749-54.
90. Stagakis, E., et al., *Identification of novel microRNA signatures linked to human lupus disease activity and pathogenesis: miR-21 regulates aberrant T cell responses through regulation of PDCD4 expression.* Ann Rheum Dis, 2011. **70**(8): p. 1496-506.
91. Tang, Y., et al., *MicroRNA-146A contributes to abnormal activation of the type I interferon pathway in human lupus by targeting the key signaling proteins.* Arthritis Rheum, 2009. **60**(4): p. 1065-75.
92. Te, J.L., et al., *Identification of unique microRNA signature associated with lupus nephritis.* PLoS One, 2010. **5**(5): p. e10344.
93. Chafin, C.B., et al., *MicroRNA-let-7a promotes E2F-mediated cell proliferation and NFkappaB activation in vitro.* Cell Mol Immunol, 2014. **11**(1): p. 79-83.
94. Chafin, C.B., et al., *MicroRNA-let-7a expression is increased in the mesangial cells of NZB/W mice and increases IL-6 production in vitro.* Autoimmunity, 2013. **46**(6): p. 351-62.

95. Lu, J., et al., *Glomerular and tubulointerstitial miR-638, miR-198 and miR-146a expression in lupus nephritis.* Nephrology (Carlton), 2012. **17**(4): p. 346-51.
96. Zhou, H., et al., *miR-150 promotes renal fibrosis in lupus nephritis by downregulating SOCS1.* J Am Soc Nephrol, 2013. **24**(7): p. 1073-87.
97. Zhu, S., et al., *The microRNA miR-23b suppresses IL-17-associated autoimmune inflammation by targeting TAB2, TAB3 and IKK-alpha.* Nat Med, 2012. **18**(7): p. 1077-86.
98. Ichii, O., et al., *Decreased miR-26a expression correlates with the progression of podocyte injury in autoimmune glomerulonephritis.* PLoS One, 2014. **9**(10): p. e110383.
99. Weening, J.J., et al., *The classification of glomerulonephritis in systemic lupus erythematosus revisited.* J Am Soc Nephrol, 2004. **15**(2): p. 241-50.
100. Austin, H.A., 3rd, et al., *Prognostic factors in lupus nephritis. Contribution of renal histologic data.* Am J Med, 1983. **75**(3): p. 382-91.
101. Ma, Z., et al., *Profiling and discovery of novel miRNAs from formalin-fixed, paraffin-embedded melanoma and nodal specimens.* J Mol Diagn, 2009. **11**(5): p. 420-9.
102. Koukos, G., et al., *MicroRNA-124 regulates STAT3 expression and is down-regulated in colon tissues of pediatric patients with ulcerative colitis.* Gastroenterology, 2013. **145**(4): p. 842-52 e2.
103. Drakos, E., et al., *c-Jun expression and activation are restricted to CD30+ lymphoproliferative disorders.* Am J Surg Pathol, 2007. **31**(3): p. 447-53.
104. Kakoki, M., et al., *Bradykinin B1 and B2 receptors both have protective roles in renal ischemia/reperfusion injury.* Proc Natl Acad Sci U S A, 2007. **104**(18): p. 7576-81.
105. Liu, K., et al., *Kallikrein genes are associated with lupus and glomerular basement membrane-specific antibody-induced nephritis in mice and humans.* J Clin Invest, 2009. **119**(4): p. 911-23.
106. Zhang, J.J., et al., *Tissue kallikrein attenuates salt-induced renal fibrosis by inhibition of oxidative stress.* Kidney Int, 2004. **66**(2): p. 722-32.
107. Bao, H., et al., *MiR-223 downregulation promotes glomerular endothelial cell activation by upregulating importin alpha4 and alpha5 in IgA nephropathy.* Kidney Int, 2014. **85**(3): p. 624-35.
108. Ben-Dov, I.Z., et al., *Urine microRNA as potential biomarkers of autosomal dominant polycystic kidney disease progression: description of miRNA profiles at baseline.* PLoS One, 2014. **9**(1): p. e86856.
109. Cantaluppi, V., et al., *Microvesicles derived from endothelial progenitor cells protect the kidney from ischemia-reperfusion injury by microRNA-dependent reprogramming of resident renal cells.* Kidney Int, 2012. **82**(4): p. 412-27.
110. Liu, G., et al., *miR-21 mediates fibrogenic activation of pulmonary fibroblasts and lung fibrosis.* J Exp Med, 2010. **207**(8): p. 1589-97.
111. Marques, F.Z., et al., *Gene expression profiling reveals renin mRNA overexpression in human hypertensive kidneys and a role for microRNAs.* Hypertension, 2011. **58**(6): p. 1093-8.
112. Wang, B., et al., *Transforming growth factor-beta1-mediated renal fibrosis is dependent on the regulation of transforming growth factor receptor 1 expression by let-7b.* Kidney Int, 2014. **85**(2): p. 352-61.
113. Benigni, A., et al., *Involvement of renal tubular Toll-like receptor 9 in the development of tubulointerstitial injury in systemic lupus.* Arthritis Rheum, 2007. **56**(5): p. 1569-78.
114. Pawluczyk, I.Z., et al., *Kallikrein gene 'knock-down' by small interfering RNA transfection induces a profibrotic phenotype in rat mesangial cells.* J Hypertens, 2008. **26**(1): p. 93-101.
115. Shao, X., et al., *Inducible expression of kallikrein in renal tubular cells protects mice against spontaneous lupus nephritis.* Arthritis Rheum, 2013. **65**(3): p. 780-91.
116. Timoshanko, J.R. and P.G. Tipping, *Resident kidney cells and their involvement in glomerulonephritis.* Curr Drug Targets Inflamm Allergy, 2005. **4**(3): p. 353-62.
117. Wilkinson, R., et al., *Activated human renal tubular cells inhibit autologous immune responses.* Nephrol Dial Transplant, 2011. **26**(5): p. 1483-92.

118. Borgono, C.A. and E.P. Diamandis, *The emerging roles of human tissue kallikreins in cancer.* Nat Rev Cancer, 2004. **4**(11): p. 876-90.
119. Caliendo, G., et al., *Kallikrein protease activated receptor (PAR) axis: an attractive target for drug development.* J Med Chem, 2012. **55**(15): p. 6669-86.
120. Ramsay, A.J., et al., *Kallikrein-related peptidase 4 (KLK4) initiates intracellular signaling via protease-activated receptors (PARs). KLK4 and PAR-2 are co-expressed during prostate cancer progression.* J Biol Chem, 2008. **283**(18): p. 12293-304.
121. Kakoki, M. and O. Smithies, *The kallikrein-kinin system in health and in diseases of the kidney.* Kidney Int, 2009. **75**(10): p. 1019-30.
122. Liu, W., R.C. Stanton, and Z. Zhang, *The kallikrein-kinin system in diabetic kidney disease.* Curr Opin Nephrol Hypertens, 2017. **26**(5): p. 351-357.
123. Shen, B. and S.S. El-Dahr, *Cross-talk of the renin-angiotensin and kallikrein-kinin systems.* Biol Chem, 2006. **387**(2): p. 145-50.
124. Braun, C., et al., *Activity and functional significance of the renal kallikrein-kinin-system in polycystic kidney disease of the rat.* Kidney Int, 2002. **61**(6): p. 2149-56.
125. Liu, W., et al., *Exogenous kallikrein protects against diabetic nephropathy.* Kidney Int, 2016. **90**(5): p. 1023-1036.
126. Liu, Y., et al., *Blockade of endogenous tissue kallikrein aggravates renal injury by enhancing oxidative stress and inhibiting matrix degradation.* Am J Physiol Renal Physiol, 2010. **298**(4): p. F1033-40.
127. Park, S.S., et al., *Bradykinin prevents reperfusion injury by targeting mitochondrial permeability transition pore through glycogen synthase kinase 3beta.* J Mol Cell Cardiol, 2006. **40**(5): p. 708-16.
128. Wolf, W.C., et al., *Human tissue kallikrein gene delivery attenuates hypertension, renal injury, and cardiac remodeling in chronic renal failure.* Kidney Int, 2000. **58**(2): p. 730-9.
129. Dellalibera-Joviliano, R., M.L. Reis, and E.A. Donadi, *Kinin system in lupus nephritis.* Int Immunopharmacol, 2001. **1**(9-10): p. 1889-96.
130. Lee, Y.H., et al., *Angiotensin-converting enzyme insertion/deletion polymorphism and systemic lupus erythematosus: a metaanalysis.* J Rheumatol, 2006. **33**(4): p. 698-702.
131. Parsa, A., et al., *Association of angiotensin-converting enzyme polymorphisms with systemic lupus erythematosus and nephritis: analysis of 644 SLE families.* Genes Immun, 2002. **3 Suppl 1**: p. S42-6.
132. Li, Q.Z., et al., *The lupus-susceptibility gene kallikrein downmodulates antibody-mediated glomerulonephritis.* Genes Immun, 2009. **10**(5): p. 503-8.
133. Li, Y., et al., *Kallikrein transduced mesenchymal stem cells protect against anti-GBM disease and lupus nephritis by ameliorating inflammation and oxidative stress.* PLoS One, 2013. **8**(7): p. e67790.
134. Dijkstra, J.R., et al., *MicroRNA expression in formalin-fixed paraffin embedded tissue using real time quantitative PCR: the strengths and pitfalls.* J Cell Mol Med, 2012. **16**(4): p. 683-90.
135. Pritchard, C.C., H.H. Cheng, and M. Tewari, *MicroRNA profiling: approaches and considerations.* Nat Rev Genet, 2012. **13**(5): p. 358-69.
136. Hui, A.B., et al., *Robust global micro-RNA profiling with formalin-fixed paraffin-embedded breast cancer tissues.* Lab Invest, 2009. **89**(5): p. 597-606.
137. Zaravinos, A., et al., *New miRNA profiles accurately distinguish renal cell carcinomas and upper tract urothelial carcinomas from the normal kidney.* PLoS One, 2014. **9**(3): p. e91646.
138. Romero-Cordoba, S., et al., *Identification and pathway analysis of microRNAs with no previous involvement in breast cancer.* PLoS One, 2012. **7**(3): p. e31904.
139. Xi, Y., et al., *Systematic analysis of microRNA expression of RNA extracted from fresh frozen and formalin-fixed paraffin-embedded samples.* RNA, 2007. **13**(10): p. 1668-74.

140. Zhang, X., et al., *An array-based analysis of microRNA expression comparing matched frozen and formalin-fixed paraffin-embedded human tissue samples.* J Mol Diagn, 2008. **10**(6): p. 513-9.
141. Kolbert, C.P., et al., *Multi-platform analysis of microRNA expression measurements in RNA from fresh frozen and FFPE tissues.* PLoS One, 2013. **8**(1): p. e52517.

11. Ευχαριστίες

Η εργασία αυτή εκπονήθηκε στο εργαστήριο Αυτοανοσίας και φλεγμονής με την υποστήριξη και συνδρομή πολλών ανθρώπων τους οποίους θα ήθελα να ευχαριστήσω.

Αρχικά θα ήθελα να ευχαριστήσω το καθηγητή κ. Δημήτρη Μπούμπα για την επίβλεψη, τη καθοδήγηση και τη στήριξη όλα τα χρόνια. Ιδιαίτερα επίσης ευχαριστώ τον Γ. Μπερτσιά για την πολύτιμη βοήθεια στο σχεδιασμό των πειραμάτων, την καθοδήγηση και την ουσιαστική συμβολή του στη διεκπεραίωση και ολοκλήρωση της εργασίας.

Η συνεργασία με το καθηγητή Δ. Ηλιόπουλο, αναπληρωτή καθηγητή Ιατρική του πανεπιστημίου UCLA, που μας παραχώρησε τεχνικές microarrays, ήταν καταλυτική για την διενέργεια και ολοκλήρωση της εργασίας. Ευχαριστώ επίσης το καθηγητή Γ. Γουλιέλμο για τη βοήθεια του και καθοδήγηση από τα αρχικά βήματα στο εργαστήριο καθώς και όλους τους καθηγητές του μεταπτυχιακού προγράμματος σπουδών 'Μοριακή βάση των ασθενειών του ανθρώπου'.

Πολλές ευχαριστίες οφείλω στα άτομα που έχουν στελεχώσει όλο αυτό το διάστημα το εργαστήριο Αυτοανοσίας και φλεγμονής και ιδιαίτερα τον Ηλία Σταγάκη για τη στήριξη, τη καθοδήγηση και τις χρήσιμες υποδείξεις και τη Χριστιάννα Χουλάκη για την βοήθεια της σε ότι χρειαζόμουν. Επίσης ευχαριστώ τους φίλους και συμφοιτητές μου από το μεταπτυχιακό πρόγραμμα σπουδών για τη συμπαράσταση.

Τέλος, ευχαριστώ τον Κρίτωνα και την οικογένεια μου για την κατανόηση και τη υπομονή τους όλα αυτά τα χρόνια.

Nephrol Dial Transplant (2015) 0: 1–11
doi: 10.1093/ndt/gfv374

ndt Nephrology Dialysis Transplantation

Original Article

Micro-RNA analysis of renal biopsies in human lupus nephritis demonstrates up-regulated miR-422a driving reduction of kallikrein-related peptidase 4

Eleni Krasoudaki[1], Aggelos Banos[1,2], Elias Stagakis[1], Konstantinos Loupasakis[3], Elias Drakos[4], Vaios Sinatkas[4], Amalia Zampoulaki[1], Aikaterini Papagianni[5], Dimitrios Iliopoulos[6], Dimitrios T. Boumpas[1,2,7,*] and George K. Bertsias[1,8,*]

[1]Laboratory of Autoimmunity and Inflammation, Institute of Molecular Biology-Biotechnology, FORTH, Iraklio, Greece, [2]Biomedical Research Foundation of the Academy of Athens, Iraklio, Greece, [3]Hospital for Special Surgery, Weill Cornell Medical College, New York, NY, USA, [4]Department of Pathology, University of Crete Medical School, Iraklio, Greece, [5]Department of Nephrology, Aristotle University of Thessaloniki Medical School, Thessaloniki, Greece, [6]Center for Systems Biomedicine, Division of Digestive Diseases, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, CA, USA, [7]4th Department of Medicine, Attikon University Hospital, University of Athens Medical School, Athens, Greece and [8]Rheumatology, Clinical Immunology and Allergy, University of Crete Medical School, Iraklio, Greece

Correspondence and offprint requests to: George K. Bertsias; E-mail: gbert@med.uoc.gr
*These authors contributed equally.

ABSTRACT

Background. Aberrancies in gene expression in immune effector cells and in end-organs are implicated in lupus pathogenesis. To gain insights into the mechanisms of tissue injury, we profiled the expression of micro-RNAs in inflammatory kidney lesions of human lupus nephritis (LN).

Methods. Kidney specimens were from patients with active proliferative, membranous or mixed LN and unaffected control tissue. Micro-RNAs were quantified by TaqMan Low Density Arrays. Bioinformatics was employed to predict gene targets, gene networks and perturbed signaling pathways. Results were validated by transfection studies (luciferase assay, real-time PCR) and in murine LN. Protein expression was determined by immunoblotting and immunohistochemistry.

Results. Twenty-four micro-RNAs were dysregulated (9 up-regulated, 15 down-regulated) in human LN compared with control renal tissue. Their predicted gene targets participated in pathways associated with TGF-β, kinases, NF-κB, HNF4A, Wnt/β-catenin, STAT3 and IL-4. miR-422a showed the highest upregulation (17-fold) in active LN and correlated with fibrinoid necrosis lesions ($\beta = 0.63$, $P = 0.002$). In transfection studies, miR-422a was found to directly target kallikrein-related peptidase 4 (*KLK4*) mRNA. Concordantly, *KLK4* mRNA was significantly reduced in the kidneys of human and murine LN and correlated inversely with miR-422a levels. Immunohistochemistry confirmed reduced KLK4 protein expression in renal mesangial and tubular epithelial cells in human and murine LN.

Conclusions. KLK4, a serine esterase with putative renoprotective properties, is down-regulated by miR-422a in LN kidney suggesting that, in addition to immune activation, local factors may be implicated in the disease.

Keywords: autoimmune, epigenetic, glomerulonephritis, inflammation, tissue injury

INTRODUCTION

Systemic lupus erythematosus (SLE) is characterized by dysfunction of the innate and adaptive immunity [1] and kidney involvement in SLE [lupus nephritis (LN)] predisposes to substantial morbidity and mortality [2]. The pathological hallmark of LN is deposition of mesangial, subendothelial and/or subepithelial immune complexes with complement activation,

activation of renal endothelial and parenchymal cells and recruitment of leukocytes that propagate local inflammatory responses [3].

Although immune system activation is *sine qua non* for development of LN [3], the renal tissue response to immunological insult is crucial in determining the disease outcome. Gene expression studies in LN have identified gene networks that involve not only immune cell infiltration and activation, but also extracellular matrix formation, response to endoplasmic reticulum stress and hypoxia, renal tissue repair and fibrosis [4–6]. After intergrossing the C57L/J *Cgnz1* locus to NZM2328 lupus-prone mice, hybrids exhibited immune complex kidney deposits with complement activation and acute glomerulonephritis; nevertheless, they were resistant to development of severe proteinuria and end-stage renal disease [7]. The *Cgnz1* locus contains mitochondrial, autophagy-related and cell survival genes that might regulate podocyte survival. Thus, both autoimmunity and end-organ damage may contribute to pathogenesis and progression of nephritis [7].

Micro-RNAs fine-tune the expression of genes involved in diverse physiological processes [8]. They show abundant expression in the kidney, play a critical role in its development and function [9] and have been implicated in kidney disorders such as polycystic kidney disease, hypertensive nephrosclerosis, diabetic nephropathy, obstructive nephropathy, immune-mediated renal diseases, kidney transplant rejection and renal cell carcinoma [10].

We [11] and others [12] have previously characterized the micro-RNA signature in SLE patients. These studies revealed altered expression of micro-RNAs that regulate DNA methylation, type I interferon signaling, T-cell activation, plasma cell differentiation and regulatory T-cell function [12]. Notwithstanding, very few studies [13–16] have examined micro-RNAs expression in kidney inflammatory lesions of SLE and their results are limited by the inclusion of only relatively mild forms of nephritis [13], or the lack of comparison with disease-free control group [15].

Herein, we aimed to identify micro-RNAs associated with acute inflammation in kidney biopsies from LN patients and to evaluate their potential implication in end-organ injury. Our results show several dysregulated micro-RNAs in LN that are predicted to regulate molecular pathways with a putative pathophysiological role. We show that miR-422a is most overexpressed in LN, correlates with active severe histological lesions and downregulates kallikrein-related peptidase 4 (KLK4), a serine esterase with putative renoprotective properties. These data corroborate previous findings on the possible role of kallirein family protein in LN and reiterate the involvement of local factors within the kidney in determining the disease phenotype.

MATERIALS AND METHODS

Human kidney tissue samples

Formalin-fixed paraffin-embedded (FFPE) kidney specimens were obtained from 20 patients with active LN at the 'Hippokration' Hospital of Thessaloniki. Twelve patients had proliferative (class III or IV [17]), five patients had pure membranous (class V) and three patients had mixed (V+III/IV) nephritis (Supplementary data, Table S1). The National Institutes of Health activity and chronicity indexes [18] were calculated. Twelve LN specimens (eight proliferative, four membranous) were used for micro-RNA profiling and RT-PCR. Eight additional specimens were used in validation immunohistochemistry studies. Uninvolved parts of kidneys surgically resected due to renal tumor and with no histological evidence of renal disease or tumor were used as controls ($n = 3$). The study was in accordance with the Declaration of Helsinki and was approved by the local ethics committees of the participating centers.

Animal models

New Zealand White (NZW), New Zealand Black (NZB) and NZB/NZW F1 (NZB/W) mice were from The Jackson Laboratory (Bar Harbor, ME, USA), and were housed in a pathogen-free facility with 12-h light/dark cycles and unlimited access to food and water. The NZB/W strain included groups of females 8 weeks old (pre-disease control group without proteinuria) and 24 weeks old (proteinuria ≥300 mg/dL). NZW mice of the same age were used as controls. Kidneys were removed from perfused mice and immediately snap frozen. All experiments were approved by the Veterinary Department of the Region of Crete and the University of Crete Medical School.

micro-RNA profiling

FFPE kidney specimens (three to four 7–10 μm thick slides from each sample) were deparaffinized and total RNA was extracted with TRIzol® (Invitrogen) [19]. RNA concentration was determined with NanoDrop ND-1000 spectrophotometer, and RNA quality was assessed by the OD 260/280 and 260/230 ratios. RNA (100 ng) was used for multiplex reverse transcription reactions with micro-RNA-specific reverse transcriptase (RT) primers (Human Multiplex RT Set, Applied Biosystems). The expression of 365 micro-RNAs was tested by PCR using the TaqMan Low Density Array (TLDA) Human Micro-RNA Panel 1.0 (Applied Biosystems) in the Dana-Farber Molecular Diagnostics Facility. RNU48 expression was used for data normalization.

Prediction of potential micro-RNA targets and gene network analysis

Potential micro-RNA gene targets were identified using the miRDB (http://mirdb.org/miRDB/index.html), MicroCosm Targets (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) and TargetScan (http://www.targetscan.org/index.html) engines. A potential gene target should be predicted by at least two out of three abovementioned engines and the targeted sequence be conserved among species. Experimentally validated targets were retrieved from the miRTarBase and miRecords. We analyzed gene ontology for each set of micro-RNA targets and their participation in biological pathways, gene-networks and disease signatures, using the GeneCoDis and Ingenuity Pathway Analysis tools.

Transfection experiments

Human embryonic kidney 293 (HEK-293) cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with L-glutamine and 10% heat-inactivated fetal bovine serum

(Life Technologies). Cells were transfected using the siPORT NeoFX agent (Invitrogen) with 50–75 nM of *mir*Vana hsa-miR-422a micro-RNA mimic (miR-422a), hsa-miR-422a micro-RNA inhibitor (as-miR-422a) or their respective negative controls (miR-NC, as-miR-NC) (Ambion). Transfections were performed three to four times, in duplicates or triplicates. Cells were collected 48 h post-transfection for total RNA or protein isolation.

3′-untranslated region luciferase assay

HEK-293 cells were plated for 24 h and then were co-transfected using Fugene6 reagent (Roche) with renilla reporter plasmid constructs (pLightSwitch) carrying the 3′-untranslated region (3′-UTR) of *KLK4* (SwitchGear Genomics) plus miR-422a, as-miR-422a or their negative controls (NCs) [20]. After 36 h, cell lysates were prepared and luciferase assays were performed using the Dual-Luciferase Reporter Assay (Promega).

RT-PCR

TLDA results were confirmed with the mirVana qRT-PCR miRNA Detection Kit and qRT-PCR Primer Sets (Ambion). Total RNA was also extracted from mouse kidneys and HEK-293 cell lysates using TRIzol® and the RNAeasy Mini Kit (QIAGEN), respectively. hsa-miR-422a was quantified with TaqMan Micro-RNA Assay (Applied Biosystems) using RNU48 as reference gene. mmu-miR-378 (mmu-miR-378a-3p), which is the mouse paralog of hsa-miR-422a, was quantified with TaqMan Micro-RNA Assay (Applied Biosystems) using U6 snRNA for normalization. For mRNA quantification, reverse transcription was performed with the PrimeScript 1st strand cDNA Synthesis Kit (TAKARA). Human and mouse KLK4 was measured with TaqMan Assay (Applied Biosystems) using GAPDH as reference gene. C_T values were normalized to endogenous control and the fold-change relative to a control sample ($2^{\Delta\Delta CT}$) was used for relative expression analysis.

Western blot

Mouse kidneys or transfected HEK-293 cells were lysed in RIPA buffer containing complete protease inhibitor cocktail (Roche). Proteins were separated by 10% sodium dodecyl sulphatepolyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. After blocking, membrane was incubated overnight at 4°C with rabbit polyclonal antibody to KLK4 (orb13527, Biorbyt Ltd) and mouse monoclonal antibody to β-actin (MAB1501, Millipore Corporation). Blots were probed with secondary horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratories), and membranes were visualized with the ECL Western Blotting Detection System (GE Healthcare Life Sciences). Protein density was quantified by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunohistochemistry

Paraffin-embedded renal tissue sections (4 μm thick) were deparaffinized in xylene and rehydrated in ethanol [21]. Rabbit polyclonal anti-KLK4 antibody (1:200) was applied overnight at 4°C. Heat-induced epitope retrieval was performed using a 10 mM citrate buffer (pH 6.0). Detection of the immunoreaction was performed using an HRP-labeled polymer secondary antibody (Thermo Fisher Scientific), 3,3-diaminobenzidine/H_2O_2 as chromogen and hematoxylin as counterstain. Histopathology pictures were captured using Nikon Eclipse E-400 light microscope and the Nikon Digital sight, DS-SM, photographic system.

Statistical analysis

Data are presented as mean ± standard deviation or dot plots of individual values. Differences between groups were tested with the Mann–Whitney or Kruskal–Wallis tests, and correlations with the Spearman's test. Stepwise linear regression was performed to identify clinical or histological parameters independently associated with micro-RNAs levels. Differences in KLK4 protein expression (nominal parameter) between control and LN groups were analyzed using Fisher's exact test. P-value <0.05 (two-tailed) was considered statistically significant. All calculations were performed using the Statistical Package for Social Sciences.

RESULTS

Human LN is characterized by distinct kidney micro-RNA expression profile

To gain insight into the molecular pathways implicated in lupus renal tissue injury, we profiled the expression of 365 micro-RNAs in kidney biopsy samples of active LN patients. We identified 24 micro-RNAs with dysregulated (>2-fold) expression in LN (9 up-regulated, 15 down-regulated) (Figure 1A and B). Results for the two most up-regulated (miR-422a, miR-21) and the two most down-regulated (miR-26a, miR-133) micro-RNAs were confirmed by RT-PCR (Figure 1C) in the original kidney specimens.

Analysis according to the histological type revealed dysregulated micro-RNA expression both in proliferative (class III–IV) and in membranous (class V) LN, with a trend for more disturbed expression in the latter group (Figure 1D). Although this is a small number of patients tested, multivariable linear regression showed that apart from the histological class of LN, presence of fibrinoid necrosis was an independent predictor of increased miR-422a/miR-21 and decreased miR-26a (Table 1). The composite biopsy activity index was strong predictor of miR-133 intra-renal levels ($\beta = 0.78$). We found no significant associations between levels of micro-RNAs and chronicity lesions in kidney biopsy, renal function, proteinuria, urine sediment analysis or administered treatments (data not shown).

Dysregulated micro-RNAs are predicted to regulate gene networks with a plausible pathophysiological role in LN

Several of the micro-RNAs with altered expression in LN are predicted or have been shown to target genes involved in inflammatory/cytokine signaling pathways, membrane transporters and cell homeostasis (Figure 2A). Canonical pathway analysis based on these predictions showed enrichment for the TGF-β, protein kinase A, ERK/MAPK, NF-κB, HNF4A,

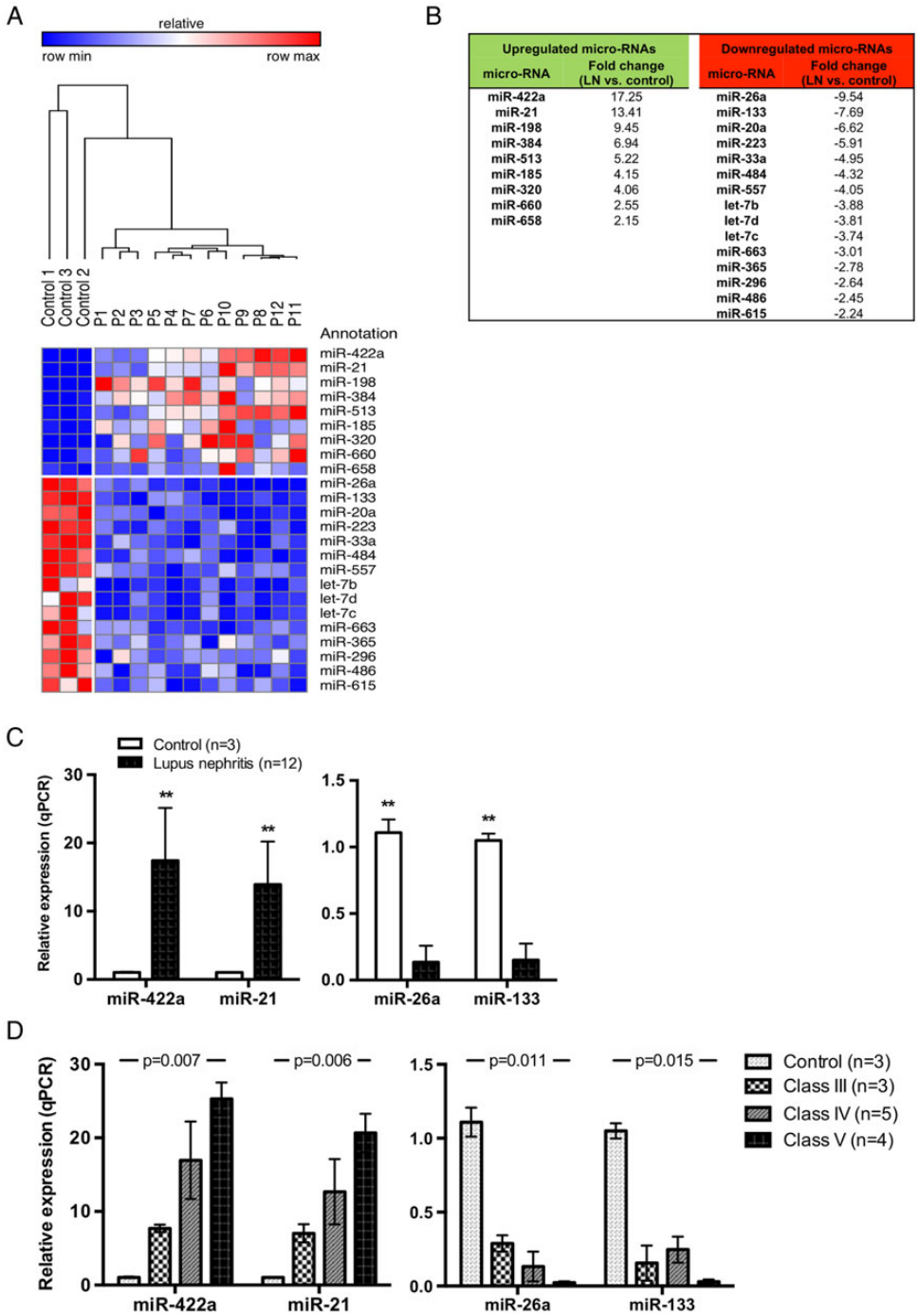


FIGURE 1: Human LN is characterized by distinct kidney micro-RNA expression profile. (**A**) Unsupervised hierarchical clustering analysis using the differentially expressed micro-RNAs can separate normal and active LN kidney samples. Heat map representation of the TLDA micro-RNA expression data in kidney specimens from 12 patients with active LN (P1 to P12) and three control samples (Control 1 to Control 3). Red represents higher micro-RNA expression and blue represents lower micro-RNA expression in LN compared with controls (or *vice versa*). The metric scale (top) represents the $\log_{10}$-fold change in micro-RNA expression using the group (LN or controls) with the lower expression as denominator. (**B**) Nine micro-RNAs were up-regulated by 2.2–17.3 times (green column) and 15 micro-RNAs were down-regulated by 2.2–9.5 times (red column) in LN versus unaffected renal samples. (**C**) Validation of the TLDA results for expression levels of miR-422a, miR-21, miR-26a and miR-133 by quantitative RT-PCR in the original dataset of LN and control kidney samples. Bars represent mean ± standard deviation (SD) of relative expression levels. (**D**) Expression levels (quantitative RT-PCR) of miR-422a, miR-21, miR-26a and miR-133 in controls and in LN kidney samples stratified according to the histological type of LN (class III, IV, V). Bars represent mean ± SD of relative expression levels. The Kruskal–Wallis test was used to test for statistically significant variation in micro-RNA expression across the four groups.

Wnt/β-catenin signaling and also for the STAT3 and IL-4 pathways in active LN (Figure 2B). HNF4A was identified as a transcription factor key node, controlling several genes (Supplementary data, Figure S1) that are simultaneously regulated by the micro-RNAs with differential expression between LN and control kidneys in our study. Combined gene network

Table 1. Linear regression analysis for the identification of clinical and histological parameters associated with the expression levels of the two most up- and down-regulated micro-RNAs in LN

Micro-RNA	Independent variable (s)	Standardized β-coefficient	t-test	P-value
miR-422a	Proliferative (versus membranous) LN	−0.960	−6.690	<0.001
	Fibrinoid necrosis	0.626	4.361	0.002
miR-21	Proliferative (versus membranous) LN	−0.957	−6.060	<0.001
	Fibrinoid necrosis	0.571	3.615	0.007
miR-26a	Proliferative (versus membranous) LN	0.904	5.557	0.001
	Fibrinoid necrosis	−0.687	−4.221	0.003
miR-133	Activity index (0–24)	0.781	3.757	0.005

Multivariate stepwise linear regression analysis using each of the four micro-RNAs (miR-422a, miR-21, miR-26a, miR-133) as dependent variable. Demographic (gender, age), clinical parameters (urine sediment, serum creatinine, proteinuria), use of SLE treatments, histological class of LN (proliferative/membranous), the National Institutes of Health activity and chronicity indices (both the composite scores and the scores of individual features) were entered as independent variables. Only statistically significant ($P < 0.05$) associations are shown.

analysis identified individual kinases, micro-RNAs, pro-inflammatory cytokines and transcription factors as significant nodes involved in LN pathogenesis (Figure 2C). Finally, integration analysis revealed that the abovementioned pathways corresponded to pertinent disease toxicity lists, namely renal necrosis/inflammation, glomerular injury, increased serum creatinine, cell death and survival, hematological cellular development and molecular mechanisms of cancer (Supplementary data, Table S2). Thus, micro-RNAs with altered expression in LN kidney lesions are predicted to regulate gene networks and molecular pathways with a plausible pathophysiological role.

miR-422a directly regulates KLK4 expression. We focused on miR-422a, the micro-RNA with the highest overexpression (17.3-fold) in active LN. Bioinformatics analysis revealed KLK4, a member of the kallikrein family of serine proteases having diverse physiological functions, as the top-predicted gene target of miR-422a (Figure 3A). KLK4 was also considered of particular interest in view of previous studies implicating kallikreins in nephropathies including LN [22–24]. To validate the prediction that KLK4 is a direct target of miR-422a, HEK-293 cells were co-transfected with a plasmid containing firefly luciferase gene under the control of KLK4 3′-UTR and either miR-422a mimic (miR-422a) or miR-422a inhibitor (as-miR-422a) or the respective NCs. Overexpression of miR-422a suppressed luciferase activity by 65%, whereas inhibition of miR-422a induced luciferase activity by 80% (Figure 3B). We also examined the effect of miR-422a on *KLK4* mRNA expression in HEK-293 cells. miR-422a suppressed *KLK4* mRNA by an average of 82%, whereas as-miR-422a caused a significant increase in *KLK4* mRNA (Figure 3C). Together, KLK4 may be a direct target of miR-422a.

The miR-422a/KLK4 circuit is dysregulated in human and murine LN. In line with our observation that KLK4 may be targeted by miR-422a, we found significantly reduced *KLK4* mRNA in human LN versus control kidneys (Figure 4A). Accordingly, miR-422a showed significant inverse correlation with KLK4 expression levels within the LN samples (Figure 4B).

We sought to validate our results in NZB/W F1 mice, which spontaneously develop lupus and immune complex-mediated lupus glomerulonephritis closely resembling the human disease after the age of 5–6 months. We used 8-week-old pre-diseased and 24-week-old diseased NZB/W mice with overt proteinuria, which were compared against age-matched NZW control mice. RT-PCR in kidney tissue showed that miR-422a (mmu-miR-378) was significantly up-regulated in NZB/W F1 compared with NZW mice, both in the 8-week-old (by 4.1 times) and in the 24-week-old (by 9.4 times) (Figure 4C). An inverse correlation was observed with regards to *KLK4* mRNA levels, which were reduced by an average of 3.4 and 7.6 times in young and old NZB/W1 mice, respectively, compared with NZW littermates. Immunoblotting in kidney lysates suggested diminished 28–30 kDa KLK4 protein expression in 24-week-old diseased NZB/W1 mice compared with 8-week-old pre-diseased and NZW control mice (Figure 4D). These findings implicate the miR-422a/KLK4 circuit in both human and murine LN.

Reduced KLK4 expression by renal mesangial and tubular epithelial cells in murine and human LN. Previous studies have shown that kallikreins are abundantly expressed and regulate the physiology of epithelial cells, including renal tubular epithelial cells. To gain insight into the renal expression and localization pattern of KLK4, we carried out an immunohistochemistry study in additional kidney specimens from NZB/W F1 mice and patients with active LN. KLK4 protein was strongly expressed (staining intensity 3+) mainly in the cytoplasm of renal tubular as well as mesangial cells in all specimens from unaffected NZW kidney and human control specimens (Figure 5A, top panel, and B). In contrast, KLK4 showed absent/faint (1+) or moderate (2+) expression in renal parenchymal cells of proteinuric NZB/W F1 and most patients with active LN (Figure 5A, bottom panel, and B). Due to the relatively small sample size of human specimens, we could not detect any significant associations with LN histological or clinical variables.

DISCUSSION

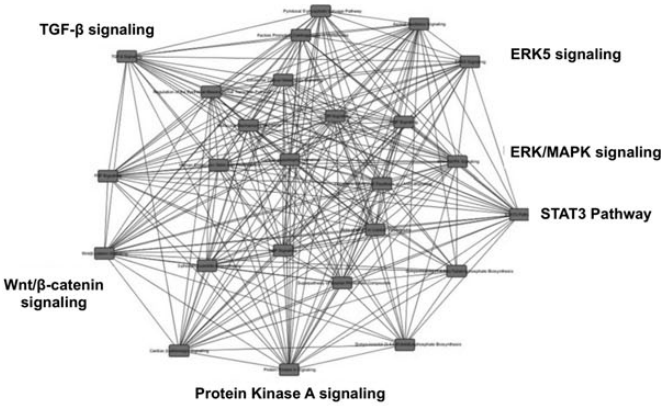
We have identified micro-RNAs with aberrant expression in active LN kidneys, which are predicted to interfere with major intracellular pathways and relevant processes such as renal necrosis and inflammation, glomerular injury and increased serum creatinine. miR-422a, the micro-RNA with the highest upregulation in LN, correlated with active severe histological lesions and targeted *KLK4* mRNA. Accordingly, KLK4 levels were reduced in kidneys of human and murine LN, suggesting possible involvement of the miR-422a/KLK4 axis in LN.

The development and outcome of LN is determined by both immune system activation and the renal tissue response to inflammation [7, 25, 26]. To understand the molecular mechanisms of lupus kidney injury, we studied the intra-renal expression of micro-RNAs in active LN. Twenty-four micro-RNAs were differentially expressed in LN versus control kidney

A

Upregulated miRNAs	Selected gene targets
miR-422a	CYP8B1*, CYP7A1*, MLF1*, KLK4, TOB2, BMP2
miR-21	PDCD4*, BCL2*, PTEN*, TGFBI*, TIAM1, IL12A
miR-198	CCNT1*, NTRK3*, MET*, MYB*, HMGA1, STAT6, CDK4
miR-384	NTRK3*, CFTR, ESRRG, HDGF, MAPK9
miR-513	CD274*, GSTP1*, ITGA4, SMARCA1, CPD, GNRHR
miR-185	CDK6*, HMGA2*, DNMT1*, AKT1*, CASP14, WNT1
miR-320	TAC1*, MAPK1*, AQP1*, ATG7*, PBX3, IRF6, CCR7
miR-660	HUWE1*, NRCAM, MED8, IRS1, DNAJC3
miR-658	UPK1A, NES, TRAF6, CABP1
Downregulated miRNAs	**Selected gene targets**
miR-26a	HMGA1*, SMAD1*, EZH2*, MAP3K2*, TET2, LARP1
miR-133	KRT7*, CASP9*, BCL2L2*, IGF1R*, CLTA, PKHD1
miR-20a	HIF1A*, RUNX1*, CDKN1A*, E2F1*, PKD2, MAP3K2, EZH1, IRF9
miR-223	MEF2C*, ARID4B*, IL6*, RHOB*, SP3, PAX6
miR-33a	ABCA1*, SRC*, NPC1*, IRS2*, ABHD2, IL2RB
miR-484	FIS1*, HOXA5*, IL2*, PKD1*, IFNAR1, FGF1, TNFSF9
miR-557	CAMK4, FLT1, PRKCE, SP3
let-7b	CDC34*, NEDD4*, KRAS*, HMGA2, LIN28B, NPHP3
let-7d	HMGA2*, IL13*, BCL2*, BDNF*, CDC34, HIF1AN, SMC1A
let-7c	DICER1*, BCL2L1*, MYC*, HMGA2*, MAP4K3, IGDCC3, PTAFR
miR-663	JUNB*, KLF4*, JUND*, FOSB*, PAX2, SBF1, TGFB1
miR-365	CCND1*, IL6*, BAX*, NCOA4*, TBK1, HOXA9, LAMP2
miR-296	WNK4*, HOXA6*, KCNH1*, ARF1*, CX3CR1, BCAM, SERPINB2
miR-486	CD40*, TMED1*, NAT15, STIM1, AATK, MAPKBP1
miR-615	IGF2*, LCOR*, PBX3*, KRT19*, SEMA4B, RARA, KLF16

B



TGF-β signaling	Protein Kinase A Signaling
Wnt signaling	HNF4A txn regulation
STAT3 Pathway	CDK5 upstream regulator
IL4 Cytokine	β-Catenin Signaling
NF-κB Signaling	Polo Like Kinase
ERK/MAPK Signaling	CTCF txn regulation

C

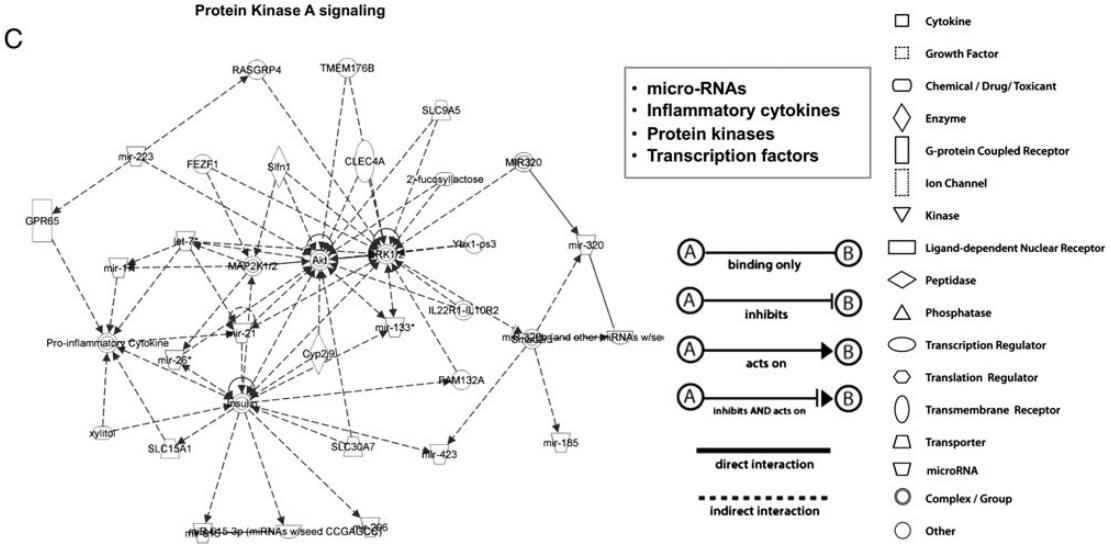


FIGURE 2: Micro-RNAs with altered expression in active LN are predicted to regulate gene networks and molecular pathways with a plausible pathophysiological role. (**A**) Predicted gene targets of the micro-RNAs with dysregulated (up- or down-regulated) expression in active LN compared with control kidney samples. Asterisks indicate experimentally validated gene targets according to the miRTarBase and miRecords databases. See more details in Materials and methods. (**B**) Significant canonical pathways (P-value <0.05) implicating predicted gene targets of LN-associated micro-RNAs, generated by Ingenuity Pathway Analysis. Pathway analysis shows enrichment for the TGF-β, protein kinase A, ERK/MAPK, NF-κB, HNF4A, Wnt/β-catenin signaling and also for the STAT3 and IL-4 pathways in active LN. (**C**) Network analysis for top regulators of the predicted micro-RNA gene targets in LN identifies kinases, micro-RNAs, pro-inflammatory cytokines and transcription factors as significant nodes.

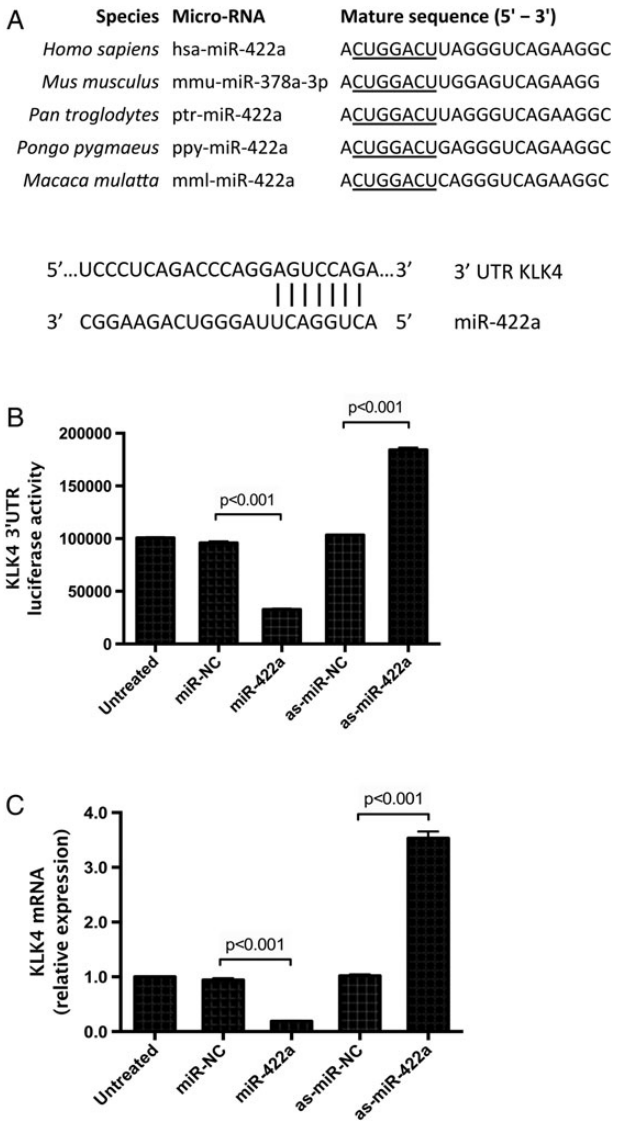


FIGURE 3: miR-422a directly targets *KLK4* mRNA. (**A**) 7mer seed sequence (underlined) of miR-422a is conserved across vertebrate species. TargetScan's predicted binding site of miR-422a within the UTR of human *KLK4* mRNA. Lines represent interacting base pairs at the seed region of miR-422a. (**B**) HEK-293 cells were co-transfected with a plasmid containing Renilla luminescent reporter gene (RenSP) under the control of *KLK4* 3′-UTR and either miR-422a mimic (miR-422a), or miR-422a inhibitor (as-miR-422a), or the respective NCs as described in Materials and methods. Luciferase activity was determined 36 h after transfection. Normalized luciferase activity is the Renilla/Firefly ratio of miR-422a-transfected reporter vector compared with the same NC vector. Data show the mean ± standard deviation (SD) from $n = 3$ independent experiments. (**C**) HEK-293 cells were transfected with 50–75 nM of miR-422a, as-miR-422a or their respective NCs (miR-NC, as-miR-NC), as described in Materials and methods. After 48 h, cells were collected for RNA extraction. *KLK4* mRNA levels are significantly decreased in miR-422a-transfected cells, whereas they are significantly increased in as-miR-422a-transfected cells, as compared with their respective NC-transfected littermates. Bars represent the mean ± SD from $n = 3$ independent experiments.

tissue. Notably, active kidney lesions, particularly fibrinoid necrosis, were independent predictors of the two most up- and down-regulated micro-RNAs. From a clinical standpoint, high activity index and presence of fibrinoid necrosis in kidney biopsy are adverse prognostic factors in LN [27], associated with increased risk for development of end-stage renal disease. Validation in larger number of specimens will be required to explore the possible role of miR-422a as a biomarker of severe LN.

Dysregulated micro-RNAs in LN were predicted to regulate downstream genes and molecular pathways including the TGF-β, protein kinase A, ERK/MAPK, NF-κB, HNF4A, Wnt/β-catenin, STAT3 and IL-4. Our results agree with those of Bethunaickan *et al.* [6], who performed comparative mRNA profiling in the kidneys of three murine LN models and found TGF-β, STAT3, NF-κB and HNF4A transcription factor to be shared by all three strains.

Few studies have so far examined the expression of micro-RNAs in lupus kidneys [13]. Lu *et al.* [14] found increased expression of miR-638, miR-198 and miR-146a. Zhou *et al.* [15] compared LN kidney biopsies with high (≥4) versus low (<4) chronicity index and identified 16 micro-RNAs with differential expression; miR-150 showed the strongest correlation with renal chronicity scores. Other researchers found increased miR-146a in B6.MRLc1 autoimmune glomerulonephritis, which correlated with interstitial cell infiltration, tubular atrophy and fibrosis [28]. Reduced glomerular miR-26a in LN correlated with markers of podocyte injury [29].

In agreement, we found increased miR-198 and reduced miR-26a levels in active human LN. Several other micro-RNAs with differential expression in our dataset have been involved in non-malignant nephropathies. These include miR-21 in TGF-β mediated renal fibrosis [30], miR-133 in polycystic kidney disease [31], miR-223 in IgA nephropathy [32], miR-663 in hypertensive kidney disease [33] and miR-296 in ischemic acute injury [34]. Let-7b and let-7c, both down-regulated in our active LN cohort, have been shown to exert renal anti-fibrotic effects [35].

The present work represents the first micro-RNA analysis in active LN, addressing not only fibrosis but also renal injury in general. Comparing our data with those of Zhou *et al.* [15], only let-7 was common. Specifically, Zhou *et al.* showed that let-7i was up-regulated in LN with high versus low chronicity index, whereas we found that let-7b/c/d were down-regulated in active LN. The discrepant findings could be explained by the different platforms used (Affymetrix microarrays versus TLDA) and the fact that our kidney biopsies comprised mainly of active LN (median activity index: 9) with lower chronicity scores than those of Zhou *et al.* [15] (median chronicity index: 3 versus 4 in our samples).

miR-422a had the highest upregulation and targeted KLK4 in active LN. These findings were confirmed in NZB/W LN mice and by the reduced KLK4 protein expression by tubular epithelial and mesangial cells in human and murine LN. These results are in concert with the previously described expression of KLKs in the aforementioned cell types [36, 37], whereas no expression has been reported in other glomerular cell types. The molecular basis for the discrepant expression of KLKs/KLK4 in different kidney resident cells remains elusive and further studies will be required. Nonetheless, both tubular epithelial and mesangial cells are implicated in the pathogenesis of glomerulonephritides such as LN, by orchestrating local

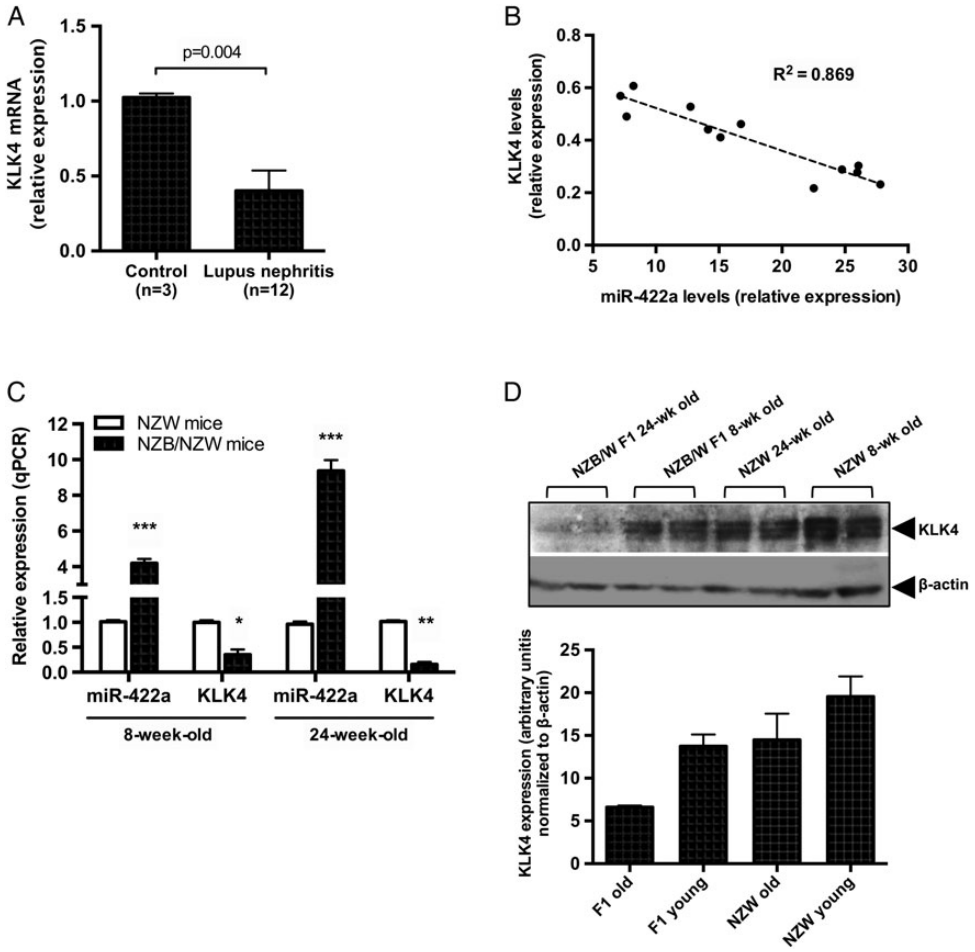


FIGURE 4: Deregulation of the miR-422a/KLK4 circuit in human and murine LN. (**A**) Quantitative RT-PCR for *KLK4* mRNA expression was performed in the original set of kidney specimens used for TLDA. *KLK4* mRNA was significantly decreased by an average of 61% in active LN versus control kidney samples. Bars represent the mean ± standard deviation (SD) from $n = 3$ independent experiments. (**B**) Inverse correlation between intra-renal miR-422a and *KLK4* mRNA levels in active LN. Analysis was performed within the subgroup of human LN kidney specimens ($n = 12$). The R-squared statistic is shown as a measure of how close the data are to the fitted regression line. (**C**) Quantitative RT-PCR for miR-422a (mmu-miR-378) and *KLK4* mRNA was performed in kidney tissue from 8-week-old pre-diseased NZB/W F1 mice ($n = 3$), 24-week-old proteinuric NZB/W F1 mice ($n = 4$) and their age-matched NZW littermates ($n = 3$ in each group). Bars represent mean ± SD expression normalized to expression levels in control NZW mice across each age group and each gene. ***P < 0.001; **P < 0.01; *P < 0.05 compared with NZW. (**D**) Immunoblotting for KLK4 protein expression (28–30 kDa) was performed in kidney lysates from the same groups of mice as in (C). Protein densitometry was performed with ImageJ Software and KLK4 expression levels were normalized to β-actin protein levels. See Materials and methods for more details.

immune responses and affecting the biology of intraglomerular cells [38–40]. Two out of eight human LN samples had normal KLK4 protein expression, which could be explained by the heterogeneity of the disease.

The kallikrein-kinin system comprises a multigene family of serine proteases with pleiotropic effects in inflammation, oxidative stress, coagulation and vascular function [41]. KLKs act by producing active kinin peptides from their kininogen precursors that activate kinin receptors, and by directly stimulating protease-activated receptors [41]. Previous work has demonstrated a protective role of the kallikrein-kinin system against renal ischemic injury [22], diabetic [42] and hypertensive nephropathy [24]. By comparing the transcriptome in the renal cortex of mouse strains sensitive versus resistant to anti-GBM antibody-induced nephritis, Liu *et al.* [23] found that several of the under-expressed genes in the sensitive strains belonged to the kallikrein family. Fine-mapping studies of the mouse *Sle3* lupus-susceptibility locus revealed that it harbors the kallikrein cluster of genes and contributes to their reduced expression [23]. Kinin B2 receptor agonists [23] and *klk1* gene delivery [43] ameliorated anti-GBM antibody-induced nephritis. Finally, *klk1* overexpression in renal tubular epithelial cells [37] or delivery of *klk1* in inflamed kidneys by mesenchymal stem cells [44] protected against nephritis in B6.*Sle1*.*Sle3* LN mice.

In humans, polymorphisms in *KLK* genes, particularly in *KLK1* and in *KLK3* promoter, were associated with SLE; haplotypes harboring the *KLK4* promoter, *KLK5* and the *KLK8-KLK11* block were more frequent in LN [23]. Our study provides further support for the role of KLK4 in LN and proposes a mechanism according to which miR-422a overexpression leads to *KLK4* downregulation. Additional studies

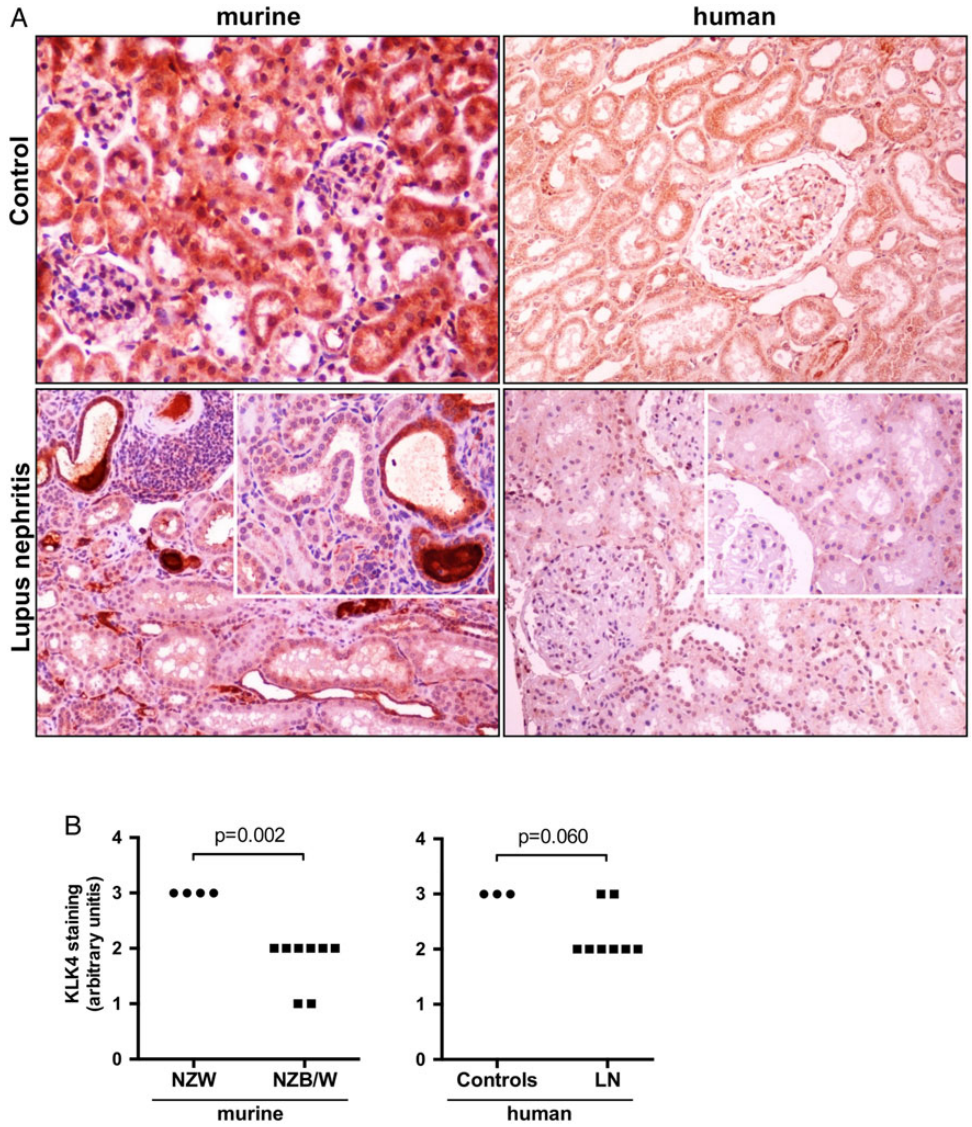


FIGURE 5: Reduced KLK4 protein expression by renal mesangial and tubular epithelial cells in murine and human LN. (**A**) Immunohistochemistry for KLK4 protein expression in human and murine control (unaffected) and inflamed LN kidney specimens. KLK4 was highly expressed in epithelial cells of all renal tubules and mesangial cells of glomeruli in control renal tissue without inflammation (upper panel). In contrast, absence, or low levels of KLK4 protein was observed in most renal tissues of NZB/W F1 mice or patients with active LN (lower panel). Notice, that a small minority of renal tubules retains normal KLK4 protein expression. See Materials and methods for more details. [DAB was used as chromogen, hematoxylin as counterstain; original magnification ×100 (insets magnification ×400)]. (**B**) Semi-quantification of the immunohistochemistry results (1+ = faint/absent, 2+ = moderate staining, 3+ = strong staining) of KLK4 expression in murine (left panel) and human (right panel) kidney specimens. See Materials and methods for more details. In mice, all four control NZW kidneys had high KLK4 expression (staining 3+), whereas all eight NZW/BW F1 mice with proteinuria had low/moderate KLK4 expression (1+/2+) (Fisher's exact test, P = 0.002). In humans, high KLK4 expression was found in all three control kidney specimens as compared with only two out of eight LN specimens (Fisher's exact test, P = 0.060).

will be required to unravel the physiologic role of KLK4 and how its reduced expression contributes to LN pathogenesis.

We used FFPE tissue samples in this study. While FFPE preserves tissue for histological analysis, nucleic acids may become irreversibly damaged and fragmented through formalin fixation and prolonged storage. However, micro-RNAs tend not to suffer from the fragmentation effect, probably due to their intrinsically shorter lengths and close association with large protein aggregates [45, 46]. Previous work in kidney, breast and colon cancer has shown that it is possible to perform genome-wide micro-RNAs screening using FFPE specimens [47, 48]. Studies comparing micro-RNA profiling between frozen and FFPE tissues have demonstrated high correlation with remarkable stability [49–51]. Finally, evidence suggests that PCR-based methods (including TLDA) are technically and biologically robust for analyzing micro-RNAs in FFPE samples [47, 52].

Conclusively, several micro-RNAs show altered expression in LN and are predicted to regulate molecular pathways with a plausible pathophysiological role. miR-422a is overexpressed, correlates with active fibrinoid necrosis in inflamed kidneys,

and downregulates KLK4, a member of the kallikrein family with presumed renoprotective properties. These results coupled with evidence from *in vivo* animal studies [37] suggest that the miR-422a/KLK4 axis could be exploited as therapeutic target in LN.

SUPPLEMENTARY DATA

Supplementary data are available online at http://ndt.oxfordjournals.org.

ACKNOWLEDGEMENTS

This work was implemented by the Greek General Secretariat of Research and Technology 'Aristeia' action of the Operational Program 'Education and Lifelong Learning' and is co-funded by the European Social Fund (ESF) and National Resources (Aristeia I 2344 to D.T.B.). A.B. (post-doctoral researcher) was supported by the Aristeia I 2344 action. G.K.B. was supported by the Greek State Scholarships Foundation-IKY (Post-doc Fellowships of Excellence—Siemens). We wish to thank Christianna Choulaki for technical support.

CONFLICT OF INTEREST STATEMENT

The results presented in this paper have not been published previously in whole or part, except in abstract format.

REFERENCES

1. Wahren-Herlenius M, Dorner T. Immunopathogenic mechanisms of systemic autoimmune disease. Lancet 2013; 382: 819–831
2. Borchers AT, Leibushor N, Naguwa SM *et al.* Lupus nephritis: a critical review. Autoimmun Rev 2012; 12: 174–194
3. Lech M, Anders HJ. The pathogenesis of lupus nephritis. J Am Soc Nephrol 2013; 24: 1357–1366
4. Berthier CC, Bethunaickan R, Gonzalez-Rivera T *et al.* Cross-species transcriptional network analysis defines shared inflammatory responses in murine and human lupus nephritis. J Immunol 2012; 189: 988–1001
5. Bethunaickan R, Berthier CC, Ramanujam M *et al.* A unique hybrid renal mononuclear phagocyte activation phenotype in murine systemic lupus erythematosus nephritis. J Immunol 2011; 186: 4994–5003
6. Bethunaickan R, Berthier CC, Zhang W *et al.* Comparative transcriptional profiling of 3 murine models of SLE nephritis reveals both unique and shared regulatory networks. PLoS One 2013; 8: e77489
7. Ge Y, Jiang C, Sung SS *et al.* Cgnz1 allele confers kidney resistance to damage preventing progression of immune complex-mediated acute lupus glomerulonephritis. J Exp Med 2013; 210: 2387–2401
8. Yates LA, Norbury CJ, Gilbert RJ. The long and short of microRNA. Cell 2013; 153: 516–519
9. Ma L, Qu L. The function of microRNAs in renal development and pathophysiology. J Genet Genomics 2013; 40: 143–152
10. Khella HW, Bakhet M, Lichner Z *et al.* MicroRNAs in kidney disease: an emerging understanding. Am J Kidney Dis 2013; 61: 798–808
11. Stagakis E, Bertsias G, Verginis P *et al.* Identification of novel microRNA signatures linked to human lupus disease activity and pathogenesis: miR-21 regulates aberrant T cell responses through regulation of PDCD4 expression. Ann Rheum Dis 2011; 70: 1496–1506
12. Frangou EA, Bertsias GK, Boumpas DT. Gene expression and regulation in systemic lupus erythematosus. Eur J Clin Invest 2013; 43: 1084–1096
13. Dai Y, Sui W, Lan H *et al.* Comprehensive analysis of microRNA expression patterns in renal biopsies of lupus nephritis patients. Rheumatol Int 2009; 29: 749–754
14. Lu J, Kwan BC, Lai FM *et al.* Glomerular and tubulointerstitial miR-638, miR-198 and miR-146a expression in lupus nephritis. Nephrology (Carlton) 2012; 17: 346–351
15. Zhou H, Hasni SA, Perez P *et al.* miR-150 promotes renal fibrosis in lupus nephritis by downregulating SOCS1. J Am Soc Nephrol 2013; 24: 1073–1087
16. Zhu S, Pan W, Song X *et al.* The microRNA miR-23b suppresses IL-17-associated autoimmune inflammation by targeting TAB2, TAB3 and IKK-alpha. Nat Med 2012; 18: 1077–1086
17. Weening JJ, D'Agati VD, Schwartz MM *et al.* The classification of glomerulonephritis in systemic lupus erythematosus revisited. J Am Soc Nephrol 2004; 15: 241–250
18. Austin HA, 3rd, Muenz LR, Joyce KM *et al.* Prognostic factors in lupus nephritis. Contribution of renal histologic data. Am J Med 1983; 75: 382–391
19. Ma Z, Lui WO, Fire A *et al.* Profiling and discovery of novel miRNAs from formalin-fixed, paraffin-embedded melanoma and nodal specimens. J Mol Diagn 2009; 11: 420–429
20. Koukos G, Polytarchou C, Kaplan JL *et al.* MicroRNA-124 regulates STAT3 expression and is down-regulated in colon tissues of pediatric patients with ulcerative colitis. Gastroenterology 2013; 145: 842–852
21. Drakos E, Leventaki V, Schlette EJ *et al.* c-Jun expression and activation are restricted to CD30+ lymphoproliferative disorders. Am J Surg Pathol 2007; 31: 447–453
22. Kakoki M, McGarrah RW, Kim HS *et al.* Bradykinin B1 and B2 receptors both have protective roles in renal ischemia/reperfusion injury. Proc Natl Acad Sci USA 2007; 104: 7576–7581
23. Liu K, Li QZ, Delgado-Vega AM *et al.* Kallikrein genes are associated with lupus and glomerular basement membrane-specific antibody-induced nephritis in mice and humans. J Clin Invest 2009; 119: 911–923
24. Zhang JJ, Bledsoe G, Kato K *et al.* Tissue kallikrein attenuates salt-induced renal fibrosis by inhibition of oxidative stress. Kidney Int 2004; 66: 722–732
25. Clynes R, Dumitru C, Ravetch JV. Uncoupling of immune complex formation and kidney damage in autoimmune glomerulonephritis. Science 1998; 279: 1052–1054
26. Xie C, Zhou XJ, Liu X *et al.* Enhanced susceptibility to end-organ disease in the lupus-facilitating NZW mouse strain. Arthritis Rheum 2003; 48: 1080–1092
27. Austin HA, 3rd, Muenz LR, Joyce KM *et al.* Diffuse proliferative lupus nephritis: identification of specific pathologic features affecting renal outcome. Kidney Int 1984; 25: 689–695
28. Ichii O, Otsuka S, Sasaki N *et al.* Altered expression of microRNA miR-146a correlates with the development of chronic renal inflammation. Kidney Int 2012; 81: 280–292
29. Ichii O, Otsuka-Kanazawa S, Horino T *et al.* Decreased miR-26a expression correlates with the progression of podocyte injury in autoimmune glomerulonephritis. PLoS One 2014; 9: e110383
30. Liu G, Friggeri A, Yang Y *et al.* miR-21 mediates fibrogenic activation of pulmonary fibroblasts and lung fibrosis. J Exp Med 2010; 207: 1589–1597
31. Ben-Dov IZ, Tan YC, Morozov P *et al.* Urine microRNA as potential biomarkers of autosomal dominant polycystic kidney disease progression: description of miRNA profiles at baseline. PLoS One 2014; 9: e86856
32. Bao H, Chen H, Zhu X *et al.* MiR-223 downregulation promotes glomerular endothelial cell activation by upregulating importin alpha4 and alpha5 in IgA nephropathy. Kidney Int 2014; 85: 624–635
33. Marques FZ, Campain AE, Tomaszewski M *et al.* Gene expression profiling reveals renin mRNA overexpression in human hypertensive kidneys and a role for microRNAs. Hypertension 2011; 58: 1093–1098
34. Cantaluppi V, Gatti S, Medica D *et al.* Microvesicles derived from endothelial progenitor cells protect the kidney from ischemia-reperfusion injury by microRNA-dependent reprogramming of resident renal cells. Kidney Int 2012; 82: 412–427
35. Wang B, Jha JC, Hagiwara S *et al.* Transforming growth factor-beta1-mediated renal fibrosis is dependent on the regulation of transforming growth factor receptor 1 expression by let-7b. Kidney Int 2014; 85: 352–361

36. Pawluczyk IZ, Tan EK, Lodwick D *et al.* Kallikrein gene 'knock-down' by small interfering RNA transfection induces a profibrotic phenotype in rat mesangial cells. J Hypertens 2008; 26: 93–101
37. Shao X, Yang R, Yan M *et al.* Inducible expression of kallikrein in renal tubular cells protects mice against spontaneous lupus nephritis. Arthritis Rheum 2013; 65: 780–791
38. Benigni A, Caroli C, Longaretti L *et al.* Involvement of renal tubular Toll-like receptor 9 in the development of tubulointerstitial injury in systemic lupus. Arthritis Rheum 2007; 56: 1569–1578
39. Timoshanko JR, Tipping PG. Resident kidney cells and their involvement in glomerulonephritis. Curr Drug Targets Inflamm Allergy 2005; 4: 353–362
40. Wilkinson R, Wang X, Roper KE *et al.* Activated human renal tubular cells inhibit autologous immune responses. Nephrol Dial Transplant 2011; 26: 1483–1492
41. Caliendo G, Santagada V, Perissutti E *et al.* Kallikrein protease activated receptor (PAR) axis: an attractive target for drug development. J Med Chem 2012; 55: 6669–6686
42. Kakoki M, Sullivan KA, Backus C *et al.* Lack of both bradykinin B1 and B2 receptors enhances nephropathy, neuropathy, and bone mineral loss in Akita diabetic mice. Proc Natl Acad Sci USA 2010; 107: 10190–10195
43. Li QZ, Zhou J, Yang R *et al.* The lupus-susceptibility gene kallikrein down-modulates antibody-mediated glomerulonephritis. Genes Immun 2009; 10: 503–508
44. Li Y, Raman I, Du Y *et al.* Kallikrein transduced mesenchymal stem cells protect against anti-GBM disease and lupus nephritis by ameliorating inflammation and oxidative stress. PLoS One 2013; 8: e67790
45. Dijkstra JR, Mekenkamp LJ, Teerenstra S *et al.* MicroRNA expression in formalin-fixed paraffin embedded tissue using real time quantitative PCR: the strengths and pitfalls. J Cell Mol Med 2012; 16: 683–690
46. Pritchard CC, Cheng HH, Tewari M. MicroRNA profiling: approaches and considerations. Nat Rev Genet 2012; 13: 358–369
47. Hui AB, Shi W, Boutros PC *et al.* Robust global micro-RNA profiling with formalin-fixed paraffin-embedded breast cancer tissues. Lab Invest 2009; 89: 597–606
48. Zaravinos A, Lambrou GI, Mourmouras N *et al.* New miRNA profiles accurately distinguish renal cell carcinomas and upper tract urothelial carcinomas from the normal kidney. PLoS One 2014; 9: e91646
49. Romero-Cordoba S, Rodriguez-Cuevas S, Rebollar-Vega R *et al.* Identification and pathway analysis of microRNAs with no previous involvement in breast cancer. PLoS One 2012; 7: e31904
50. Xi Y, Nakajima G, Gavin E *et al.* Systematic analysis of microRNA expression of RNA extracted from fresh frozen and formalin-fixed paraffin-embedded samples. RNA 2007; 13: 1668–1674
51. Zhang X, Chen J, Radcliffe T *et al.* An array-based analysis of microRNA expression comparing matched frozen and formalin-fixed paraffin-embedded human tissue samples. J Mol Diagn 2008; 10: 513–519
52. Kolbert CP, Feddersen RM, Rakhshan F *et al.* Multi-platform analysis of microRNA expression measurements in RNA from fresh frozen and FFPE tissues. PLoS One 2013; 8: e52517

Received for publication: 18.7.2015; Accepted in revised form: 5.10.2015