Identifying Functional Disease Drivers in Lupus Nephritis Associated with Glomerular Remodeling

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Abstract

Lupus Nephritis (LN) is an autoimmune disease that affects the healthy tissue of the kidney, leading to chronic inflammation and excessive leakage of protein in the urine (proteinuria). There is no cure; instead, current methods of treatment involve management of symptoms and slowing the progression toward renal failure. Although the genes linked to LN have long been been identified, limited research has highlighted which specific genes may be driving the physical changes caused by this disease.

This research used provided tissue samples from NZB/W female mice, a confirmed LN model, which had been previously sacrificed at 36 weeks at the first indication of excess proteinuria. The purpose of this study was to identify the specific morphological changes within the glomerulus that led to protein leakage, and then to use qPCR to identify the specific genes that are driving this conformational change.

Histochemistry with Trichrome staining revealed, for the first time, a significant expansion of the Bowman's capsule within the LN group which may correlate with excessive protein leakage. In addition, by correlating qPCR expression results with the increase in Bowman's space, this novel research revealed that *ROCK2, ICAM, cMET, TGFβ, acox,* and *Col1* may be disease drivers for LN.

Recent research has focused on understanding how disease progression differs from person to person. By having a better understanding of the underlying mechanisms of LN, precision medication can be tailored to each patient's genotype, thereby increasing the effectiveness of treatment.

Introduction

Systemic Lupus Erythematosus (SLE) is an autoimmune inflammatory disease in which the immune system targets healthy cells and tissues throughout the body. It is estimated that at least 5 million individuals worldwide develop some form of SLE,[1] and more than half of these patients then develop its renal manifestation, Lupus Nephritis (LN).[2] LN is characterized by the deposition of immune complexes at different locations within the glomerulus, leading to chronic inflammation of the kidneys and a buildup of excess waste. Additionally, LN is associated with frank proteinuria which is tubulo-toxic and only hastens end-stage renal disease. Thus, LN invariably leads to renal failure and the subsequent need for renal replacement therapy, including dialysis or transplantation.

Current methods of LN treatment involve immunosuppressants to weaken the activity of the immune system; specifically, these medications interfere with mitotic DNA synthesis, leading to apoptosis. Although these medications have been associated with clinical improvement of LN[3], a weakened immune system increases vulnerability to other infectious diseases. Additionally, many of these treatments may prove toxic to the kidneys. For instance, anti-inflammatory drugs can cause fluid retention and even interstitial fibrosis, leading to the degradation of the kidneys.[4]

As an alternative, researchers are looking into medications originally meant for other diseases to serve as potential treatments for LN. For instance, antimalarial agents have been found to decrease autoantibody production within SLE, increasing patient life expectancy.[5] However, these drugs are only effective when SLE is immediately diagnosed, and therefore, cannot treat more complex forms of the disease, such as LN.[6] Therefore, all of these treatments only delay renal failure; none cure LN.

Patients with LN also experience higher medical costs because of continuous hospitalization — even more than general SLE patients. Several studies have found that treatment itself can accumulate to over $80,000 per year, with annual pharmacy costs accounting up to 23% of direct costs.[7] Furthermore, the extreme effects that come with this disease force working patients into a greater rate of absenteeism, causing an additional economic burden of

$6,700 per year.[8] By creating more specialized treatments for LN, diseases could be treated more effectively, which, in turn, would lift the economic burden that comes with it.

Rationale

Research and medicine has recently been moving away from categorizing diseases in a "one size fits all" kind of way, and instead, has focused on understanding how disease progression differs from person to person. Since the genomic network of disease drivers of LN in one patient may differ from those in another, limiting treatment to only one type reduces its effectiveness. While the various genes linked to LN have long been identified,[20] no research has pinpointed one or more genes specifically driving the disease in terms of functional endpoints; in other words, there is incomplete understanding of functional genetic nodes in LN. And as the underlying mechanisms driving LN remain poorly understood, this leads to a lack of effective treatments. Having a better understanding of the genetic and morphological changes that drive LN could aid in the creation of precision medication that is specific to each individual's genotype.

There is a copious amount of previous research regarding the increased activity of certain genes in autoimmune diseases. For instance, the inhibition of *Col1* has been found to decrease the severity of LN, suggesting a role for this gene in the development of the disease.[9] An upregulation of *ICAM* has been strongly correlated with renal disease,[10] while the expression of *acox* may have a relationship with kidney fibrosis.[11] *ROCK* activity was discovered to be associated with the progression of chronic kidney disease[12]; recent studies have also found enhanced *ROCK* activity in patients with SLE[13], suggesting a potential link between autoimmunity and renal injury. *cMET* has been found to be related to acute kidney injury[14], whereas *TGFβ* has profibrotic effects.[15] Therefore, if these genes are upregulated in LN -- which also results in fibrosis and requires repair --, they could serve as potential drivers of this disease.

There is a need to specifically correlate the upregulation of these proteins with the morphological changes within the kidney, specifically the glomerulus, during LN. Excessive proteinuria is a major symptom of LN; however, the specific genetic drivers are unknown. As other proteinuric diseases have been discovered to be affiliated with glomerular remodeling, for

example both in minimal change disease (MCD)[16] and focal segmental glomerulosclerosis (FSGS)[17], it was hypothesized that this pattern is also present in LN. Thus, the purpose of our research was two-fold: to reveal the morphological cause of proteinuria during LN, and to identify at least a subset of disease driver genes associated with glomerular remodeling in a model of LN.

Methodology

Only banked Masson's Trichrome-stained kidney slides from a murine model of LN were used. Separately, I used RNA extracted from banked frozen renal homogenates (2016-005). Animals had been previously sacrificed and kidney samples had been previously collected for histopathological (10% formalin) or transcriptomic (liquid N_2) analysis.

Animals

Female C57BL/6 wild type mouse strains (control group) and lupus nephritis-generating NZB/W hybrid mice (experimental group) were purchased from Jackson Laboratory (Jackson Laboratory; Maine, U.S). NZB/W mice were used for the experimental group because they are a strain that has been found to spontaneously develop LN. In fact, seminal work by Berthier and colleagues indicates that there is remarkable transcriptomic and histopathological overlap between the kidneys of adult female NZB/W mice and LN patients.[21] Mice were fed a normal chow diet and were sacrificed at 36 weeks of age. 40 kidney slides were provided for each group and were stained with trichrome. Banked frozen renal homogenates from female age-matched wild-type sham mice and 36-week old female NZB/W mice were provided, as well.

Proteinuria Calculation

As described by Liu et al,[25] proteinuria can be determined using the following equation.

Proteinuria ($\mu g/day$) = (Bowman space (μm^2) -353.3)/36 (1).

Sacrifice

Animals were sacrificed by lab technicians on June 27, 2019. Upon sacrifice, kidneys were flash frozen in liquid nitrogen and stored at -80°C until cDNA synthesis. The other kidney halves were sent to Jackson Laboratories for histological sectioning and staining.

Histological staining

Tissues were sent to Jackson Laboratories (location) for histological sectioning and were stained with Trichrome.

Glomerular Analysis

Upon receiving sectioned kidney tissue, images were taken from each slide using an IX50 Olympus Microscope (Olympus; Tokoyo, Japan) focusing on glomerular structure. Area and diameter of the glomeruli and Bowman's capsule were quantified using ImageJ.

RNA Isolation

Sample RNA was isolated with a Qiagen RNeasy kit (Qiagen; Venlo, Netherlands) according to manufacturer's instructions. Once isolated, RNA concentration in each sample was determined using a Thermofisher Nanodrop LITE (Thermofisher; Massachusetts, U.S), and then stored at -80°C for coding DNA (cDNA) synthesis.

cDNA

RNA concentrations were used to generate equivalent levels of cDNA across the samples using the Thermofisher High-Capacity cDNA Reverse Transcription Kit and a BioRad S1000 Thermocycler (BioRad; California, U.S) following the cDNA thermal-cycling protocol. Samples were diluted at 1:5 with nuclease free H20 and stored at -20°C for analysis through SYBR-green based quantitative polymerase chain reaction (qPCR).

Primer Validation

Forward and reverse primers for all of the disease driver genes were designed using sequencing data from NCBI and using primer-design tools provided by Primer3Plus[27] set for qPCR settings (Table 1). Forward and reverse primers were ordered using Oligo-Sigma services (Millipore-Sigma; Massachusetts, U.S). Upon arrival the primers were reconstituted with nuclease free water for a 100µM concentration and validated using qPCR of a 1:2 serial dilution standard curve to ensure for optimal primer efficacy (90-110%), invalid primers were excluded.

qPCR

SYBR Green qPCR was performed with Thermofisher Power-Up SYBR Green Master-Mix, validated primers, and cDNA samples following SYBR Green protocol except for a 10µl reaction. 9µl of a mastermix of primer, cDNA, and water was added to the wells of a MicroAmp Endura Optical 96-well Clear Reaction Plate and 1 µl of sample cDNA was added in triplicate for each sample These plates were ran in a Thermofisher QuartStudio 3 qPCR machine following Power-SYBR Fast protocol.

Gene	Left Primer	Right Primer
ROCK1	AGCTGAATGACATGCAAGCG	CCAAAAGTTTTGCCCGCAAC
ROCK2	TGGCCCAGTTTGCATCTTTC	AGCAAGTTGTGTTCCCAACC
Col1	TCAGCTGCATACACAATGGC	ATTGCATTGCACGTCATCGC
acox	TACAGGTTTGCGCTGACATG	TGTTCTCACGATGCCAATGC
ICAM	ATCAGCGGCCAAAAATGTGG	TGGCTTCCCATCAACTTCAC
cMET	TTGCTGATTTCGGTCTTGCC	TGGTGAACTTCTGCGTTTGC
TGFB	ATTGCTGCAATCAGGACCAC	AAGTGCAATGCAGACGAAGC

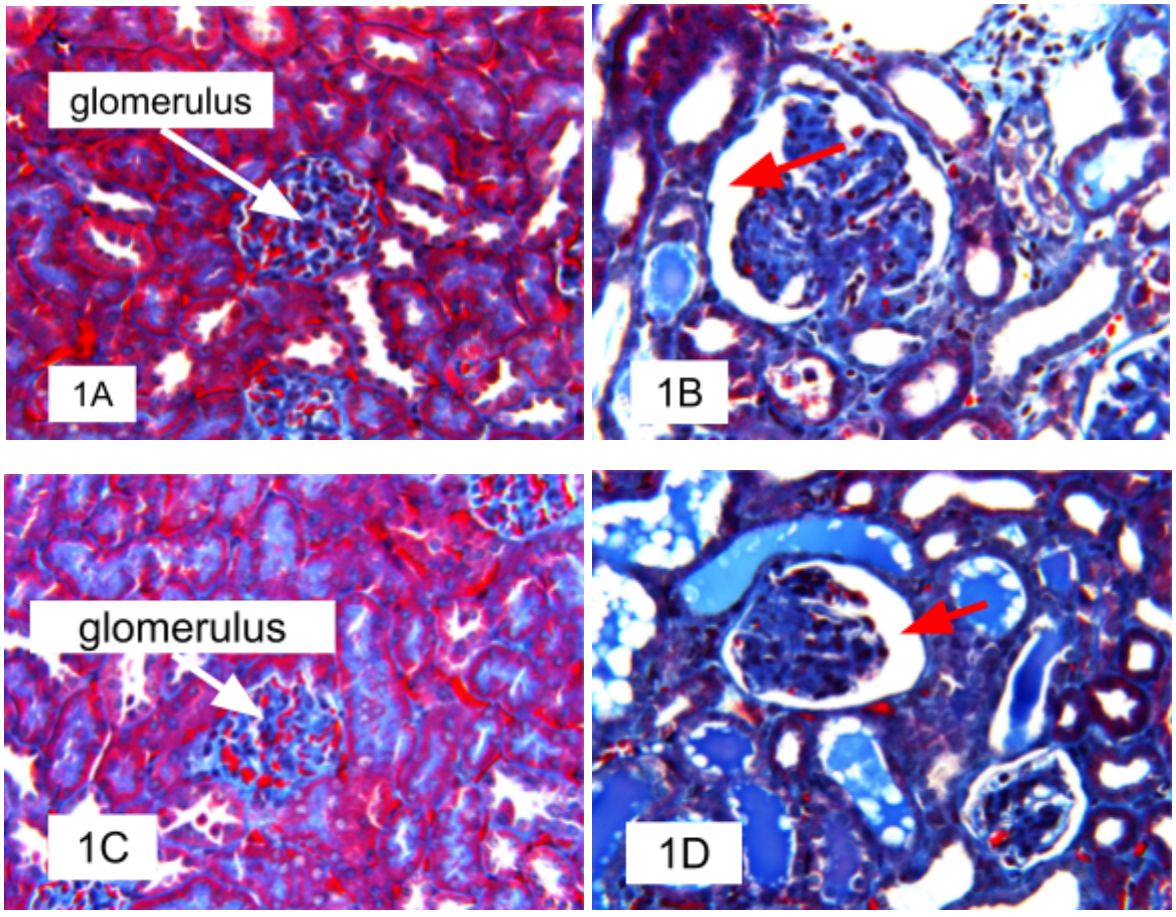
Table 1: Forward and reverse primers for all of the disease driver genes.

Statistical Analysis

A *t*-test was used to determine significance between measured values of the Sham and NZB/W groups. A Pearson Product Moment Correlation was used to determine the strength of the association between the genes queried and the increase in Bowman's Space. A p-value ≤ 0.05 was considered to be statistically significant.

Results

As seen in Figure 1, NZB/W kidneys exhibited significant fibrosis evidenced by deep blue Masson's trichrome stain indicating collagen deposition across the entire renal parenchyma. Even more striking, however, was the change in glomerular morphology in juxtaposition to the sham. Both the Bowman's capsule and glomerulus had an exaggerated appearance, but perhaps most striking was the expansion of the Bowman's space (Figure 1).



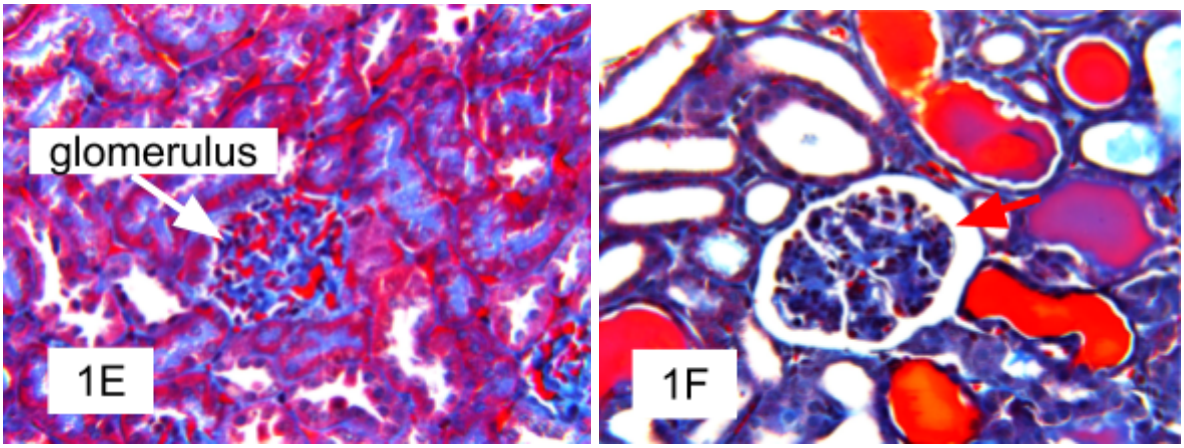


Figure 1: Representative cross sections of Masson's Trichrome-stain kidneys at 40x magnification. Intense blue color represents collagen deposition. 1A: Sham tissue. Glomerular diameter and area was measured for all tissue. 1B: NZB/W LN tissue. The arrow points to the increased area of the Bowman's Space. 1C: Sham tissue. The arrow points to the glomerulus. The Bowman's space is minimal. 1D: NZB/W LN tissue. The arrow points to the increased area of the Bowman's space. 1E: Sham tissue. The arrow points to the glomerulus. Again, the Bowman's space is minimal 1F: NZB/W LN tissue. The arrow points to the increased area of the Bowman's space.

Indeed, glomerular diameter, glomerular area, area of the Bowman's capsule and area of the Bowman's space was found to be increased in NZB/W kidneys, $p < .001$ (Figure 2).

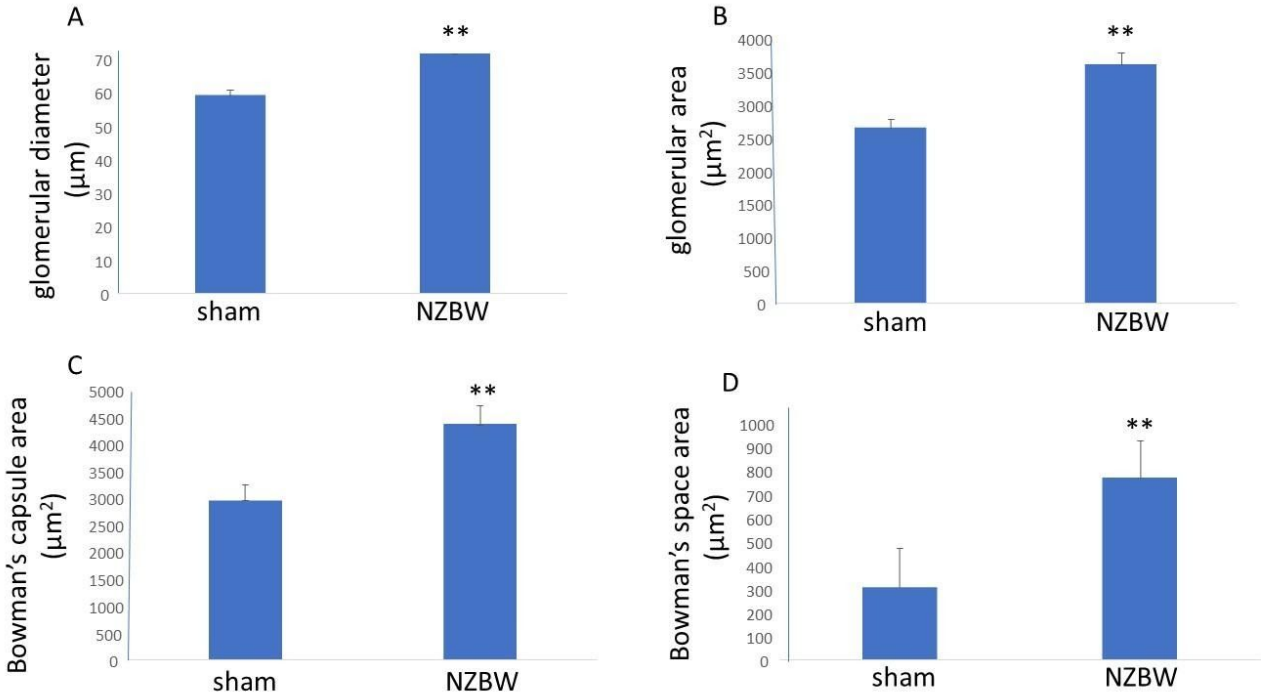


Figure 2: Remodeling of Glomerulus and Bowman's Capsule. NZB/W mice experienced an increase in (A) glomerular diameter, (B) glomerular area, (C) Bowman's capsule area and (D) area of the Bowman's space. **, p<0.01 vs. sham

Next, I confirmed that expression levels of genes previously reported to be elevated in this model of LN, were indeed increased. As seen in Figure 3, *ROCK2, ICAM, cMET, TGFβ, acox, Col1,* and *ROCK1* were all found to be upregulated in the LN model, confirming previous research.

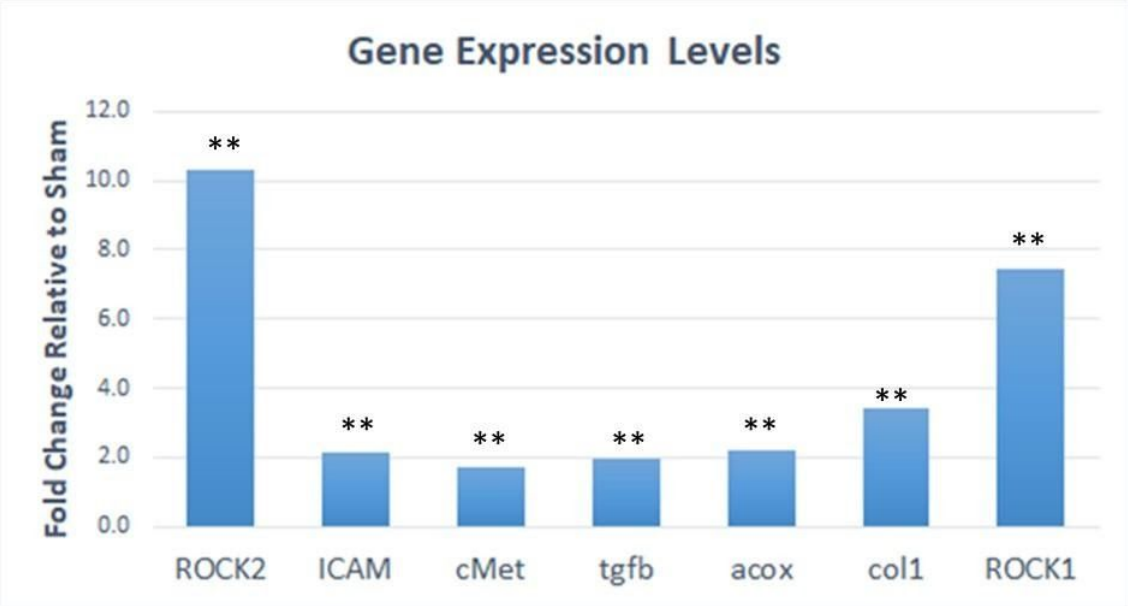


Figure 3: Fold change in gene expression analysis by RT-qPCR. Tissue expression profiles of LN disease driver genes in the NZB/W LN murine model. Data represent the fold change in expression of the analyzed transcripts relative to the Sham group. All increases were found to be significant, $p < 0.01$.

The leakage of proteins is a hallmark feature of LN, and research led by Liu and colleagues[23] at the lab had found proteinuria to be increased in the LN sample at 36 weeks, p-value < 0.01 (Figure 4), indicating that glomerular remodeling had begun. Mice had then been sacrificed and subsequent tissue had been provided for my study so that histology and genetic drivers could be compared.

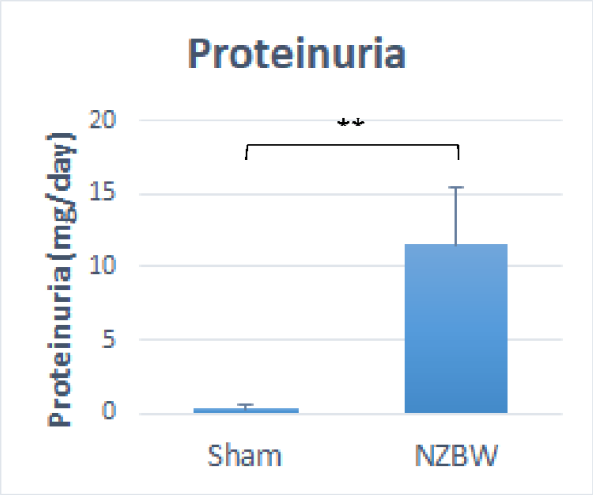
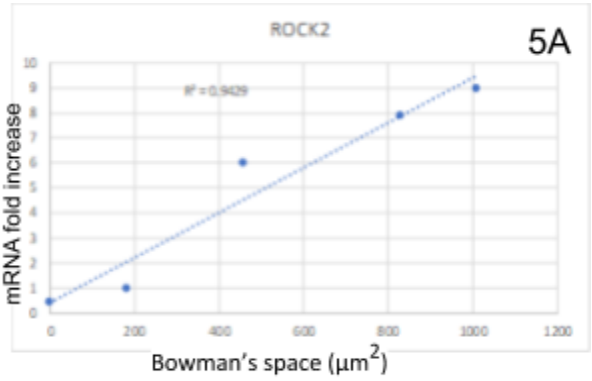
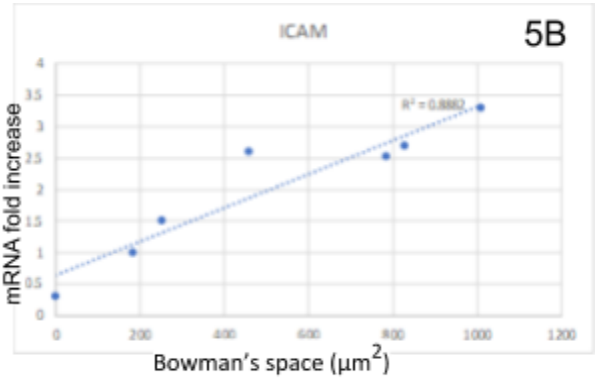


Figure 4: Proteinuria is significantly increased in the NZBW model of LN. **, $p<0.01$.

Increased Bowman's spacing has also been found to drive proteinuria.[24] To correlate which of these disease-driving genes was associated with glomerular remodeling during LN, the qPCR results were then compared against Bowman's Space values. *ROCK2, ICAM, cMET, TGFβ, acox,* and *Col1* increase in fold expression were found to have a significant and direct correlation with the increase in Bowman's Space (Figure 5), $p < 0.05$. By contrast, *ROCK1* increase in fold expression showed no correlation with the Bowman's space (Figure 6).





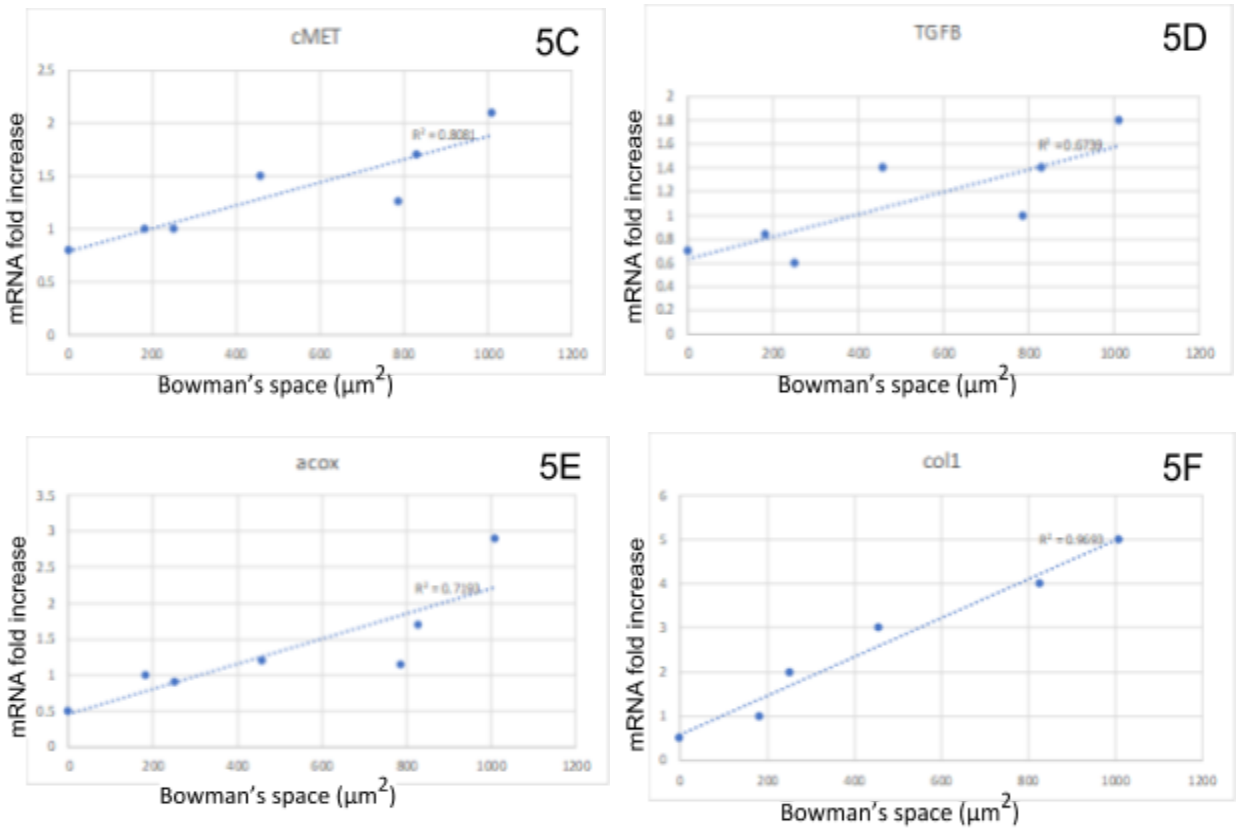


Figure 5: Correlation of Protein Fold Change with Bowman's Space. 5A: *ROCK2* vs Bowman's space, $R^2 = 0.9429$. 5B: *ICAM* vs Bowman's space, $R^2 = 0.8882$. 5C: *cMET* vs Bowman's space, $R^2 = 0.8081$. 5D: *TGFβ* vs Bowman's space, $R^2 = 0.6739$. 5E: *acox* vs Bowman's space, $R^2 = 0.7193$. 5F: *Col1* vs Bowman's space, $R^2 = 0.9693$. $p < 0.05$ for all graphs.

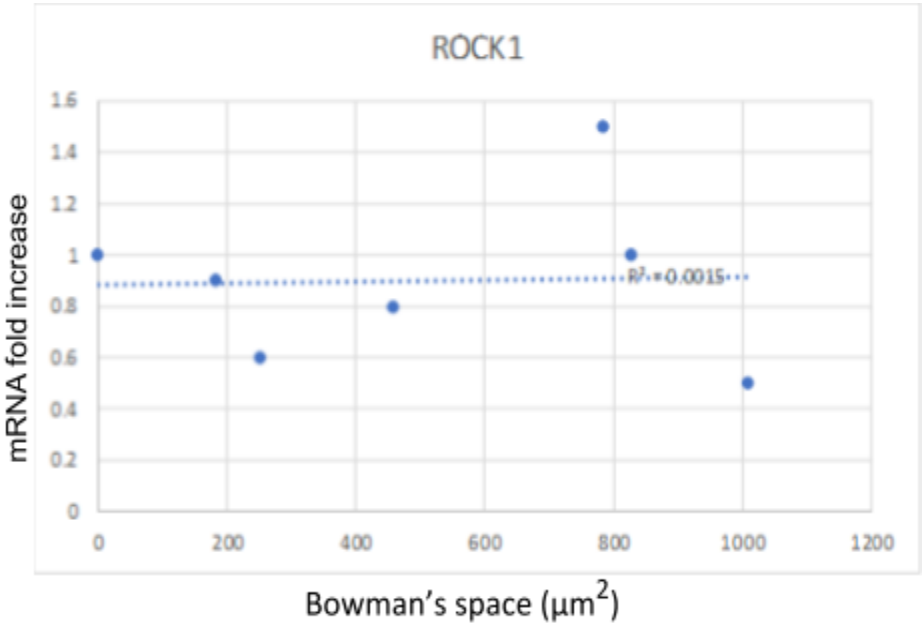


Figure 6: The correlation of *ROCK1* fold change vs Bowman's space, $R^2 = 0.0015$.

Discussion

In a clinically relevant model of LN, this study found glomerular remodeling evidenced by significant expansion of the Bowman's capsule, blossoming of the glomeruli and expansion of the Bowman's space. Transcriptomic analysis of the kidneys indicates that a set of key genes may serve as functional disease drivers. Expression levels of each of these genes directly correlates with the area of the Bowman's space which, in turn, informs proteinuria in this model of LN.

This novel finding suggested that, unlike other proteinuric diseases, the increased area of the Bowman's space may be correlated with the leakage of proteins into the urine in LN models. Minimal change disease lacks visible glomerular remodeling when observed through light microscopy; the effacement of the epithelial tissue leads to the loss of the normal charge barrier, resulting in protein leakage. FSGS is characterized by a collagenous scarring in only a portion of the glomerulus. However, LN proteinuria is due to the glomerular blossoming itself, not the scarring, that allows the protein to leak through the drastic increase in Bowman's Spacing.

In addition, this research project focused on identifying the specific disease drivers leading to glomerular remodeling. Although previous research had already linked the increased expressions of *ROCK2, ICAM, cMET, TGFβ, acox, ROCK1,* and *Col1* to LN, this novel research was able to further clarify the level of importance of each of these genes. By correlating the expression levels of each protein versus the area of the Bowman's space, *ROCK1* was ruled out as a driver of glomerular hypertrophy, as the correlation coefficient was close to zero. Meanwhile, *ROCK2, ICAM, cMET, TGFβ, acox,* and *Col1* had significant correlation values with the increased area of Bowman's Space, which suggests that these genes may be disease drivers for LN and are potentially strong targets for precision medicine.

Currently there is no cure for LN. The proteinuria due to glomerular hypertrophy and remodeling of LN is toxic to the proximal tubular epithelial cells of the renal tubules. Previous research has shown that proteinuria leads to nephropathy due to increased inflammatory cytokine

signaling, altered gene signaling, and apoptosis.[18] Thus medical treatments focus on relief from lupus symptoms and slowing the eventual degradation of the kidney.

The murine model of NZB/W has long been shown to be a clinically relevant model of SLE.[19] Therefore, these novel findings can be used as a springboard for other research that focuses on inhibiting the expression of these proteins within murine models of LN. If future research can show that specific protein inhibition prevents glomerular remodeling and thus attenuates proteinuria, this would confirm that one of these proteins is indeed a driver of LN. From here, the next step would be to create medications that target these proteins. This form of treatment could open the door for new ways of categorizing this disease; for instance, instead of simply being labeled as "LN," it would be labeled as "*ROCK2*-Driven Lupus Nephritis." This method of diagnosis allows for the integration of genomic variability among individuals, thus creating more effective precision treatments.

Conclusion

There were several limitations to this study that must be considered. The only primers used were those that had been previously validated to bind properly. Therefore, it is possible that there are other genes also drive renal dysfunction in LN; this study was limited to only those that were validated. Future studies can focus on improving the creation of primers for other genes previously correlated with LN. Furthermore, while this research quantified the overexpression of specific proteins within the NZB/W LN model, it did not specifically show the localization of the gene products within the glomerulus. Future research can focus either on isolating only the glomerular tissue before using qPCR to quantify the amount of mRNA present, or perhaps use immunofluorescence to show increased expression of the disease driving proteins within the glomerulus.

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