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Glomerulotubular Injury and Phenotypic Switch of Kidney Cells in Diabetic Nephropathy

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Abstract: **The increased prevalence of diabetes mellitus (T2DM), and one of the complications, diabetic nephropathy (DN), constitutes a significant health care burden worldwide. Nephron, the kidney's structural and functional unit, includes two principal regions: glomerulus and tubule. Glomerular dysfunction and podocyte injury are significant factors in the development and progression of DN. Nevertheless, emerging evidence suggests that kidney tubular damage also contributes significantly to the pathogenesis of DN. To assess the contribution of both glomerular and tubular damage in DN's pathogenesis, we evaluated the functional and histochemical parameters and immunohistochemistry of the kidney biopsy specimens for EMT and fibrosis markers from 11 patients with confirmed diabetic nephropathy. Histopathological analysis revealed that besides significant glomerulosclerosis, kidneys are presented with tubular injury. These patients' glomeruli showed decreased epithelial markers' expression while expressing a more considerable number of mesenchymal markers. On the other hand, the tubular region displays an enhanced expression of the fibroblastic marker. Pathogenesis and proteinuria in DN patients could be contributed by the morphometric transformation of podocytes into mesenchymal form, tubular epithelial cells into fibroblasts. Our observations are significantly relevant for identifying novel therapeutics for both diagnosing and treating DN.**

Index Terms: **Diabetes, EMT, Fibroblast, Glomerulus, Nephropathy, Podocytes.**

I. INTRODUCTION

Vertebrate kidneys contribute to the body's homeostasis by regulating electrolyte, acid-base, and water balance with millions of nephrons' collective effort. The glomerulus and tubule are two regions of the nephron, while the former responsible for permselectivity, latter ensures selective reabsorption. Thus, the collective effort of the glomerulus and tubule determines the final composition of urine. The kidney's potential to excrete ultrafiltrated urine devoid of large molecules, including protein, is primarily determined by a three-layered glomerular filtration barrier (GFB). The three layers of GFB are the fenestrated endothelium of glomerular capillaries, glomerular basement membrane (GBM), and glomerular podocytes.

Podocytes are considered highly imperative for the normal function of GFB over the other components. Podocytes are visceral epithelial cells of the glomerulus, presented with a giant cell body and characteristic primary and secondary foot processes. With the help of foot processes, podocytes provide epithelial coverage to the capillaries. Podocytes injury is expected to impair the efficiency of glomerular permselectivity and is manifested by sclerosis and varying degree of albuminuria. Simultaneously, the kidney tubule is a layer of elongated tubular epithelial cells connected with the kidney capsule wall. The kidney tubular epithelial cells constitute the tubule's outer layer that plays a crucial role in absorbing glucose, amino acids, and primary urine proteins. At the same time, tubular cells elicit several essential functions such as regulating water, electrolyte, acid, and alkali balance. Injury to the kidney cells, including podocytes and tubular epithelial cells, during clinical conditions such as diabetic nephropathy (DN), compromise both kidney architecture and function.

DN is one of the most common complications in diabetic patients and the primary cause of end-stage kidney failure (ESKD) and is associated with increased mortality. 30-40% of diabetic patients (both type 1 and type2) develop DN, whereas a significant proportion of DN cases progresses to end-stage kidney disease (ESKD).

Although early stages of DN are asymptomatic, end-stage is being treated by either dialysis or kidney transplantation. DN'spathophysiological features include glomerulosclerosis, interstitial and tubular fibrosis, inflammation, thickening of GBM, decreased glomerular filtration rate (GFR), and overt proteinuria. Progressive proteinuria is a hallmark of DN, whereas patients initially show microalbuminuria (30-300 mg/24 hr) for an extended period (5-10 years), followed by

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macroalbuminuria (≥300 mg/ 24 hr). A proteinuric condition during DN suggests both impaired glomerular permselectivity and tubular reabsorption. Despite microalbuminuria or proteinuria, diabetic patients might have a standard glomerular structure with or without tubulointerstitial and arteriolar abnormalities (Rockwell,1994) .The clinical manifestations of DN are strongly related to the degree of mesangial expansion. Microscopic morphometric analysis has described both structural changes and the structural-functional relationships of DN. These structural changes may include injury to both podocytes and tubular epithelial cells. Podocytes (visceral epithelial cells) may undergo phenotypic conversion to mesenchymal cells and become motile and detach from GBM. Alternatively, tubular epithelial cells may transform into fibroblasts due to various noxious stimuli in the T2DM. Nevertheless, the simultaneous occurrence of both events was not demonstrated earlier in the glomeruli from DN subjects.

Since podocyte contributes to the glomerular permselectivity and integrity of GBM, any insult to podocytes could contribute to altered glomerular morphology and proteinuria. Simultaneously, tubular injury and impaired reabsorption could also be anticipated during damage to tubular cells. This study demonstrated the phenotypic switch of podocytes (glomerular regions) and tubular epithelial cells (kidney tubule) in biopsy sections from T2DM patients.

II. METHODOLOGY

A. *Enrollment of Patients:*

We enrolled 11 patients with T2DM undergoing biopsy as recommended by the nephrologist. Inclusion criteria are diabetes with more than 15 years, persistently inadequate glycemic control, and proteinuria above 300 mg/24 hr. Exclusion criteria were hematuria, clinical and laboratory findings suggestive of non-diabetic glomerulopathy, and secondary renal damage due to hypertension. For the enrolled patients, 24 hours of urine and early morning spot urine were collected, and proteinuria was determined. Kidney biopsies of these 11 DN patients were collected and performed histopathological analysis. Biopsy specimens from kidney cancer patients who underwent nephrectomy for a localized kidney tumor were considered non-diabetic. The non-affected part of the kidney tissue was utilized for histopathological examinations. The kidney biopsy serial sections (4 μm) from paraffin-embedded tissues were prepared to perform morphometric analysis. The Institutional Review Board approved the study of GunturMedical College, Guntur, Andhra Pradesh, India (#GMC/IEC/120/2018). Our studies abide by the Declaration of Helsinki principle.

B. *Clinical Examination:*

Anthropometric measurements were recorded for the patients; Body mass index (BMI) was calculated using the formula: weight in kg and height in m^2. Blood pressure (BP) was checked thrice by a digital oscillometer (Omron Healthcare Co. Ltd.). Fasting blood glucose (FBG) was assessed in the whole blood using a glucometer (Accu-Chek Aviva, Roche *Diagnostics GmbH, Germany). HbA1c was estimated in whole blood using a bio-rad D-10 analyzer. Urinary albumin (number COD11573) and creatinine (number COD11502) levels were estimated using existing assay kits (Biosystems).*

C. *Morphometric analysis:*

For histological analysis, kidney cortical samples were fixed with 4% neutral buffered paraformaldehyde before embedding in paraffin. Paraffin-embedded tissues were sliced longitudinally into four μm thick sections, subjected to staining with haematoxylin and eosin for general evaluation of the cellular structure, periodic acid–Schiff (PAS) staining applied to provides excellent definitions for glomerular basement membrane, tubular basement membrane, and mesangium. Masson's trichrome staining is used to observe the extracellular mesangial volume, interstitial fibrosis percentage, and tubular atrophy (IFTA). At least 15 glomeruli per stained biopsy had to be counted for the measurement to be considered valid. The diagnosis and histological scoring for kidney damage were evaluated based on Tervaert et al.(2010). At least six glomeruli were captured for each biopsy sample and quantified for histopathological changes. We took images with a BX51 light microscope (Olympus, Tokyo) with appropriate filters. Histological positive staining intensity was quantified using software (Image J 1.44, NIH, USA) (Nishad et al., 2019; Kudose et al., 2018; Meyer, 2003).

D. *Nephroseq analysis:*

To validate the observations found in biopsy specimens holds with the existing database generated with data from human, we analysed the expression of the same set of genes Nephroseq database (https://nephroseq.org). The source for Nephroseq database is the University of Michigan O'Brien Renal Centre, Michigan, Ann Arbor, MI.

E. *Statistical analysis*:

Data are presented as mean±SD. For the statistical difference between the two groups of the data, Student's t-test was used to generate the P-values ($p \leq 0.05$). Statistical analyses were performed using GraphPad Prism V.6.0.

III. RESULTS

A. *Impaired renal function in diabetic subjects:*

Mean age of 11 diabetic patients was 61±5 years, BMI 29.7 ± 5.6, and HbA1c 9.1 ± 2%. Fasting glucose of diabetic patients significantly high (Fig.1A). Similarly, urinary albumin (302±71.87mg/24hrs.) and serum creatinine (3.87± 2.52 mg/dL)), and eGFR values (34.9±11.9ml/min/1.73m^2) suggests kidney functional parameters were in the progressive DN pathological range (Fig.1B-D). High fasting and postprandial

glucose levels and HbA1c (%) indicate poor glycemic control in these patients. These patients have ~15 yrs of diabetic history, and they are high overt proteinuria, suggesting that kidney function has been compromised and suffering from progressive DN.

Figure 1

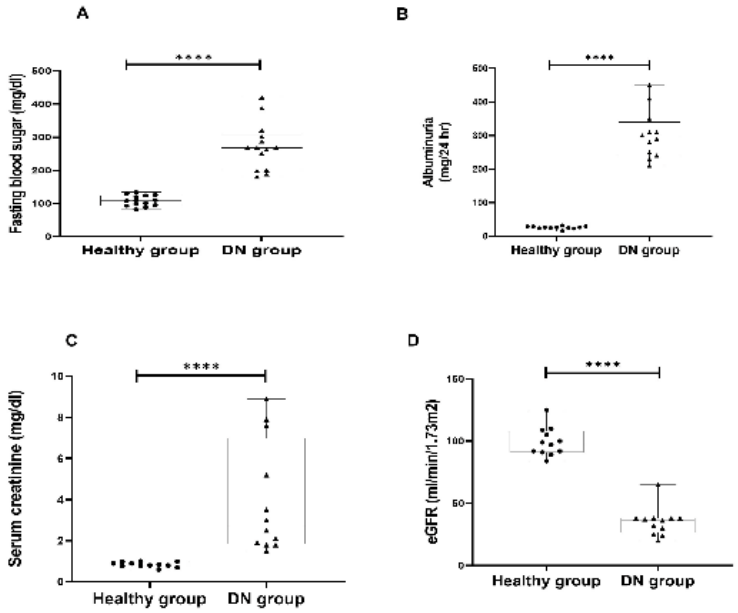

Figure 1: The kidney functional parameters in patients enrolled in the study:(A) Fasting blood glucose levels of healthy volunteers and patients with DN. (B) Albumin levels were measured in healthy volunteers and DN patients. (B) Creatinine levels were estimated in serum. (C) Glomerular filtration rate (GFR) was calculated in healthy volunteers and DN patients. Data from the DN group is significantly different from the Healthy group. ($p < 0.001$).

Figure 2

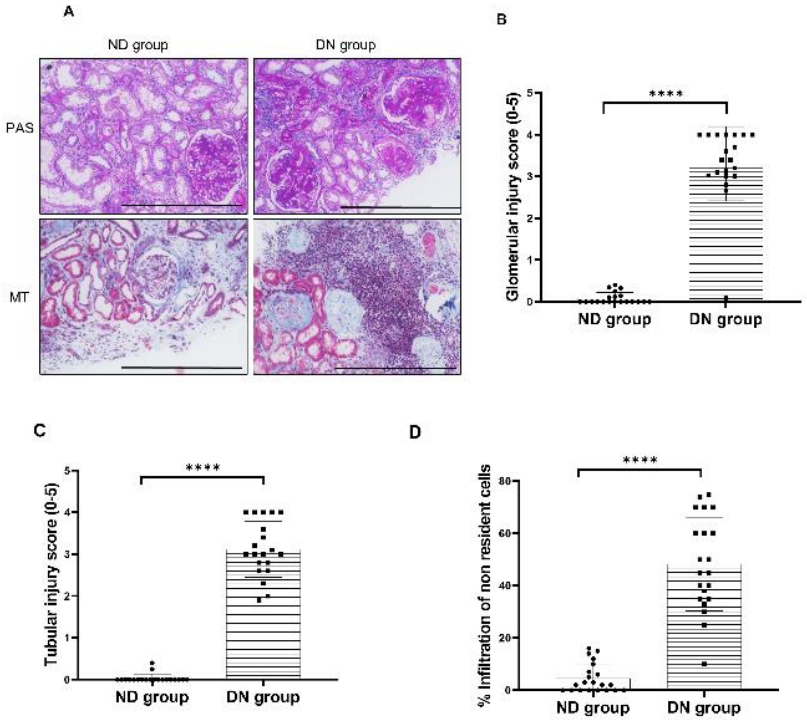

Figure 2: Quantification of kidney injury in DN patients. (A) Representative images of periodic acid Schiff's stain and Mason's trichrome staining of kidney sections from non-diabetic (ND) and diabetic nephropathy (DN) subjects. Glomerular injury score (B) and tubular injury score (C) were quantified in biopsy sections (n= 20). (D) % of infiltrated cells was quantified in ND and DN patients' specimens. Data from DN is significantly different from ND subjects ($p < 0.001$).

Figure 3

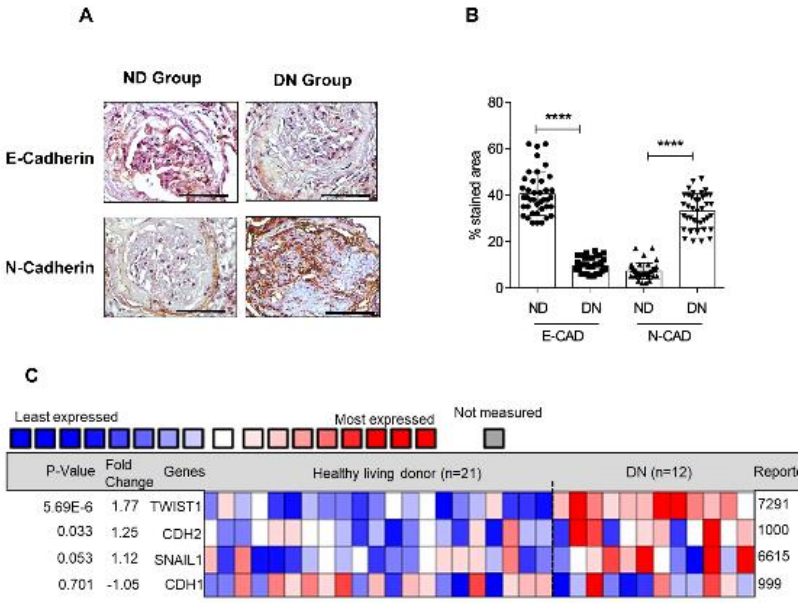

Figure 3: Quantification of epithelial-mesenchymal markers in glomerulus from DN patients, A) Staining for E-cadherin (epithelial marker) and N-cadherin (mesenchymal marker) in the glomerular region of non-diabetic (ND) and diabetic nephropathy (DN) subjects. (B) Staining intensity for E-cad and N-cad was quantified using Image-J (NIH, USA) and presented as a bar diagram (n= ~40 sections) (p <0.001). (C) Expression of EMT markers in glomeruli from healthy living donors vs. DN patents (Data mining in the Nephroseq database (University of Michigan O'Brien renal center, Ann Arbor, USA).

Figure 4

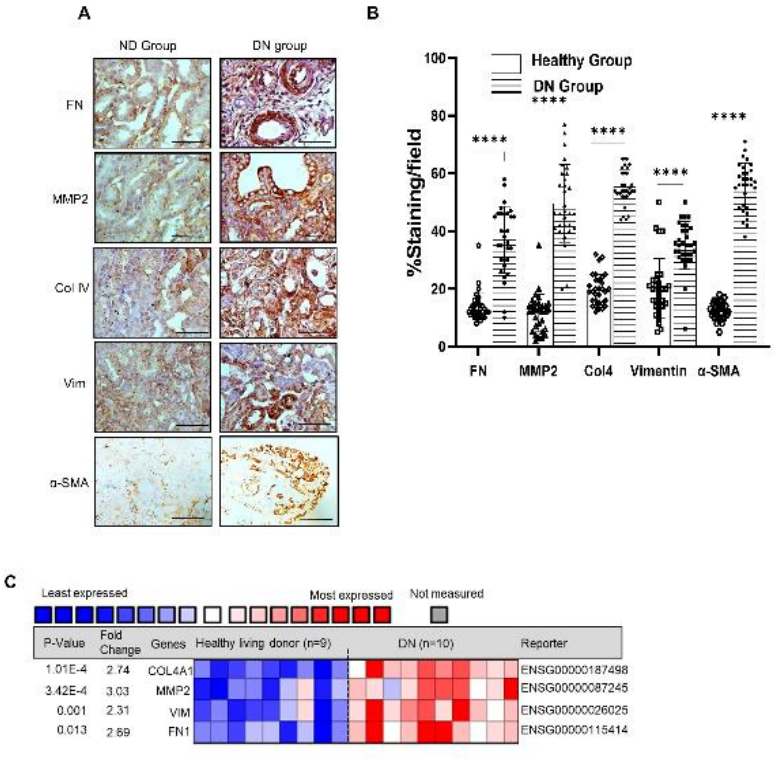

Figure 4*:* Quantification of fibroblast markers in the tubular region from DN patients**.** (A), (B), Representative images of staining for fibronectin (FN), MMP2, Collagen IV (Col IV), Vimentin (Vim), and α-Smooth Muscle Actin (SMA) in a tubular region of non-diabetic (ND) and diabetic nephropathy (DN) subjects. Staining intensity was quantified using Image-J (NIH, USA) and presented as a bar diagram (n= ~40 sections) (p <0.001). (C) Expression of fibroblastic markers from healthy living donors vs. DN patents (Data mining in the Nephroseq database, U of Michigan O'Brien renal center, Ann Arbor, USA).

B. Severe glomerular and tubular injury in DN patients:

Since diabetic patients showed macroalbuminuria, we wanted to perform a histological examination to assess nephropathy intensity. We performed PAS and Mason's trichrome stainings (Fig.2A) and evaluated both glomerular and tubular injury in these patients. One of the characteristic features of DN is the infiltration of non-resident cells into glomeruli. Therefore, we

counted % of in filtered cells into the glomeruli of these patients and found that ~50% of glomerular cells are non-resident cells compared with ~5% of non-resident cells in non-diabetic individuals (Fig.2D)

C. Attenuated expression of epithelial markers in glomerular regions in DN patients:

As discussed in the introduction, the appearance of protein in the urine indicates a structural and functional artifact, particularly in the glomerular area. Podocytes are the primary cell type and account for ~40% of all glomerular cells. In response to injury, Podocytes undergo a morphologic switch known as epithelial to mesenchymal transition (EMT), during which they attain an embryonic form by shedding their epithelial features. It is conceivable that podocytes, undergone EMT, abandon their complex architecture and relinquish their specialized functions, leading to proteinuria. Since we observed proteinuria in these patients and few resident cells in the glomerulus, we suspect that podocytes might have undergone EMT. When we stained for E-cadherin, a putative epithelial marker, we observed decreased staining (Fig.3A).

On the other hand, N-cadherin (a mesenchymal marker) expression enhanced, suggesting loss of epithelial features and gain of mesenchymal features (Fig.3A&B). We also performed a meta-analysis of the Nephroseq database for EMT markers. Nephroseq analysis (Fig.3C) reveals a marginally decreased expression of E-cadherin (CDH1; -1.05) but an enhanced expression of N-cadherin (CDH2; 1.25), SNAIL1 (1.12), and TWIST. (1.77). Together Nephroseq analysis and histochemical data suggest podocytes undergo EMT in DN patients.

D. Elevated fibrotic markers in tubular regions in diabetic patients:

Recent studies suggest that increased glomerular leakage and impaired tubular reabsorption are accountable for albuminuria in the early DN(Zeni et al., 2017). Further, tubulocentric pathology and treatment for DN were proposed(Zeni et al., 2017). Since we observed tubular injury in these patients (Fig2C), we investigated whether tubular cells have undergone any phenotypic transformation. Interestingly, we observed enhanced expression of fibrotic markers such as fibronectin (FN), MMP2, collagen IV, vimentin, and α-SMA in both proximal and distal tubules (Fig.4A&B). Thesignificantinduction of these markers suggests that the transformation of tubular cells to fibroblasts. We also performed a meta-analysis using the Nephroseq database for fibroblasts markers. Nephroseq analysis (Fig.4C) reveals the significant induction of the above-mentioned fibrotic markers suggesting our biopsy data correlates with data mined from the database (Fig.4B vs. Fig.4C). Together our data suggest tubular cells from DN patients could transform into fibroblasts.

DISCUSSION

Diabetes is presented with high morbidity and often progresses to chronic kidney disease and accounts for significant ESKD and dialysis cases. With the increasing prevalence of T2DM, its complications DN impose a tremendous burden on individuals, families, and the health care sector. Although there is extensive information on diagnosis and biomarkers of diabetic kidney diseases, it is still increasingly clear whether the glomerulus or tubules are more critical in DN's development and progression. Glomerulosclerosis was considered a primary event of the pathogenesis in DN. Other glomerular changes such as thickening of the basement membrane and mesangial expansion, and podocytopathy are prominent manifestations during DN.Although glomerular centric pathological events undoubtedly contribute to DN's pathology, there is growing evidence to suggest a significant role for the proximal tubules as drivers of proteinuria and other clinical manifestations of DN. In the present, we noticed that DN patients showed both glomerular and tubular injury, as evidenced by PAS and Mason's trichrome staining. Indeed, we saw the loss of epithelial markers and mesenchymal markers in glomerular cells and fibrotic markers in tubular cells. This evidence of both glomerular cells and tubular cells' transition to mesenchymal and fibrotic phenotype, respectively, provides a new perspective on the pathophysiology of DN. This novel observation provides new diagnosis and therapeutic approaches by targeting both glomerular epithelial cells and tubular cells in DN.

Both glomerular filtration barrier (consist of podocytes, basement membrane, and endothelium) and tubular epithelium work in concert to ensure the ultrafiltration of plasma and reabsorption of primary urine, respectively, from becoming urine. Thus, both glomerulus and tubule regulate the final composition of urine. Besides being a part of the filtration apparatus, glomerular podocytes elicit several functions to maintain the glomerular function's integrity. Podocytes provide epithelial coverage to the glomerular capillaries and oppose hydrostatic pressure to facilitate glomerular filtration and are thus indispensable to renal filtration. However, podocytes are exposed to a myriad of hematological agents and noxious stimuli due to diabetes. A body of experimental data demonstrated high glucose, glucose-derived advanced glycation end-products (AGEs), activation of sorbitol, RAAS, Growth hormone (GH), and TGF-β, etc., were contributed to the altered signaling events thus pathology of DN. Accumulating evidence suggests that glomerular podocytes undergo EMT during various insults such as high glucose, AGEs, GH, and TGF-β (Anil Kumar et al., 2014). Podocytes

might majorly contribute to the EMT phenomenon; we observed glomeruli from DN patients as they are the glomerulus' predominant epithelial cells. EMT of podocytes might compromise their ability to offer epithelial coverage to the capillaries and oppose hydrostatic pressure, resulting in impaired glomerular filtration.EMT of podocytes could be a reason for podocytes' appearance in urine from patients with advanced DN (Reidy & Susztak, 2009). The two parts of the kidney tubule, proximal and distal, have specialized functions. The proximal part is concerned with the reabsorption of several primary urine components to maintain homeostasis; in contrast, the distal tubule is associated with complete water, electrolyte, and acid-base balance regulation. Recent studies suggest that in addition to increased glomerular leakage, impaired tubular reabsorption is also accountable for DN's albuminuria(Zeni et al., 2017). DN's tubular manifestations include tubular atrophy, interstitial fibrosis, and thickening of the tubular basement membrane, correlated with kidney dysfunction and proteinuria. It is noteworthy that the proximal tubule injury can trigger glomerular dysfunction and such retrograde trafficking has implications in the progression of DN(Hasegawa et al., 2013).Glomerular podocytes and proximal tubular cells work in association with complementing each other's biology. Hasegawa et al. demonstrated that proximal tubules communicate with podocytes by releasing nicotinamide mononucleotide(Hasegawa et al., 2013).Proximal tubule-specific Sirt1 helps maintain glomerular NMN concentrations, thus preserving the glomerular podocyte function and offering protection against diabetic kidney disease(Hasegawa et al., 2013). These observations suggest that the injury to tubular cells significantly affects the glomerular function and promotes DN progression.

Additionally, the injured proximal tubule epithelium triggers the inflammatory response, which leads to tubulointerstitial fibrosis, tubular atrophy that, in turn, promotes secondary glomerulosclerosis(Lee et al., 2012). Therefore, the observed tubular and glomerular injury in DN patients from our study reveals that DN's pathological evaluation needs to be comprehensive and evaluate the tubular injury and not only glomerulus. In summary, our study provides evidence for significant tubular damage in addition to glomerular injury. It suggests a need for diagnostic evaluation and therapeutic advances in DN towards both glomerulus and tubule.

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