Paediatrica Indonesiana

p-ISSN 0030-9311; e-ISSN 2338-476X; Vol.62, No.6(2022). p.382-9; DOI: https://doi.org/10.14238/pi62.6.2022.382-9

Original Article

Effect of vitamin D analogues calcitriol and paricalcitol in a rat model of puromycin aminonucleoside-induced nephrotic syndrome

Hamdi Metin, Pelin Ertan, Ahmet Keskinoğlu, Elgin Türköz Uluer, Muhammet Burak Batır, Pembe Keskinoğlu, Damla Akoğulları, Fethi Sırrı Çam

Abstract

Background Renoprotective effects of vitamin D analogues have been shown in several experimental and clinical studies. The exact mechanism of the therapeutic effectiveness of these analogues in nephrotic syndrome remains unclear, and these are relatively few studies on potential treatment roles for vitamin D analogues in nephrotic-range proteinuria. İndicate similar efficacy of the vitamin D analogues calcitriol and paricalcitol in time-limited amelioration of proteinuria in nephrotic syndrome, yet suggest the likelihood of mechanisms other than direct upregulation of nephrin and podocin in podocytes underlie the renoprotective effects of vitamin D analogues.

Objective To investigate the effect of vitamin D analogues calcitriol and paricalcitol on urinary protein/creatinine ratio (UPCR) and renal podocin and nephrin expression in a rat model of puromycin aminonucleoside (PAN)-induced nephrotic syndrome (NS).

Methods A total of 28 male Wistar Albino rats were separated into 4 groups (n=7 for each) including CON [control; intraperitoneal (IP) saline injection], PAN (NS + IP saline injection), PAN-C (NS + IP 0.4 μg/kg/day calcitriol injection), and PAN-P (NS + IP 240 ng/kg/day paricalcitol injection). Nephrotic syndrome was induced via intravenous (IV) administration of 10mg/100gr PAN. The UPCR as well as histopathological, immuno-histochemical, and real time PCR analyses of kidney tissue specimens were recorded and analyzed among the 4 groups.

Results Median UPCR (Day 4) was significantly lower in both the PAN-C [1.45 (range 1.20-1.80)] and PAN-P [1.40 (range 1.10-1.80)] groups than in the PAN group [2.15 (range 2.00-2.40)] ($P<0.01$ for each). The PAN group had significantly higher mean UPCR than the CON group [1.75 (range 1.40-2.00); $P<0.05$]. No significant difference in UPCR was noted between groups on Day 7. Median podocin mRNA expression was significantly higher in the PAN-P group compared to the PAN group [22.55 (range 22.42-23.02) *vs.* 22.06 (range 21.81-22.06), respectively; ($P<0.01$)].

Conclusion Seven-day calcitriol and paricalcitol supplementation in a rat model of PAN-induced nephrotic syndrome have similar efficacy, in terms of temporary amelioration of proteinuria. **[Paediatr Indones. 2022;62:382-9; DOI: https://doi.org/10.14238/pi62.6.2022.382-9].**

Keywords: *nephrotic syndrome; Vit D analogues; paricalcitol; proteinuria; nephrin*

Nephrotic syndrome (NS) is one of the most prevalent glomerular diseases in children, characterized by relapsing episodes of edema, heavy proteinuria, and hypoalbuminemia.[1] Control of proteinuria is considered critical to managing glomerular diseases given its association with renal tubular damage and increased severity of chronic kidney disease.[2]

Proteinuria has also been associated with risk of deficiency in active vitamin D (Vit D3) and its metabolites, largely attributed to urinary loss of carrier vitamin D-binding protein (VDBP).[3-5] Hence, dysfunctional Vit D3 metabolism has consistently been reported in clinical and experimental studies in nephrotic syndrome. Paricalcitol [19-nor-1,25-dihydroxyvitamin D(2)] is a new vitamin D analogue that retains similar vitamin D biologic activity, but has a lower propensity for hypercalcemia than calcitriol

From the Department of Child Health and Diseases, Karabük Training and Research Hospital, Karabük, Turkey.

Corresponding author: Hamdi Metin. Department of Child Health and Diseases, Karabük Training and Research Hospital, Karabük. Şirinevler Mahallesi, Alparslan Caddesi No 1 Merkez/Karabük, Turkey. Email: hamdimetin919@hotmail.com.

Submitted September 14, 2021. Accepted December 5, 2022.

[1, 25-dihydroxyvitamin D(3)].[6-8]

In a rat remnant kidney model, calcitriol was associated with reduction of proteinuria, prevention of podocyte injury, and attenuation of glomerulosclerosis.[9,10] Calcitriol was also reported to ameliorate proteinuria in IgA nephropathy in human.[11] In humans with adriamycin-induced NS, paricalcitol was also associated with reduction in proteinuria and kidney injury,[12] as well as amelioration of proteinuria in diabetic nephropathy[13] and in patients with chronic kidney disease (CKD).[14]

However, while renoprotective effects of Vit D analogues have been shown in several experimental and clinical studies,[15,16] the exact mechanism of the therapeutic effectiveness of these analogues in NS remains unclear, and these are relatively few studies on potential treatment roles for vitamin D analogues in nephrotic-range proteinuria.[17]

Proteinuria develops as a result of glomerular injury and impaired permeability due to disrupted structural integrity of the glomerular filtration barrier (basement membrane and slit diaphragm) maintained by podocytes in physiological conditions.[2,18] Podocyte-specific proteins, such as nephrin and podocin, are considered to be the key regulators for maintaining structural integrity of the slit diaphragm in recent studies.[2,19,20] However, while down-regulation of nephrin and podocin expression has been shown in human and experimental proteinuric glomerular diseases,[2,21,22] pathophysiologic factors playing a role in the regulation of nephrin and podocin expression remain largely unknown.[2,22] Therefore, we aimed to investigate for possible associations of administering Vit D analogues calcitriol and paricalcitol on UPCR and renal podocin and nephrin gene expression in a rat model of puromycin aminonucleoside (PAN)-induced nephrotic syndrome.

Methods

A total of 28 male Wistar Albino rats (6-8 weeks of age and weighing 200-300 g) were kept in a light- and temperature-controlled room with a 12 hours light-dark cycle, temperature of 22°C, and relative humidity of 30-70%. The animals were fed standard rat pellets and provided with water ad libitum. Approval for the study was granted by the Ethics Committee of Karabük Training and Research Hospital.

The rats were separated into 4 groups of 7 each, comprising control group (CON; intraperitoneal saline injection for 7 days), NS group (PAN; NS-induction + IP saline injection for 7 days), NS plus calcitriol group (PAN-C; NS-induction + IP 0.4 μg/kg/day calcitriol injection for 7 days), and NS plus paricalcitol group (PAN-P; NS-induction + IP 240 ng/kg/day paricalcitol injection for 7 days). Nephrotic syndrome was induced via IV (through the tail vein) administration of 10mg/100gr PAN (*Sigma*, #1499403, St. Louis, MO, USA) in the PAN, PAN-C, and PAN-P groups. Urinary protein/creatinine ratio was determined on days 0, 4, and 7 of the experimental period using urine specimens collected from each rat via bladder massage. At the end of experimental period, 7 days after NS-induction, all rats were sacrificed via cervical dislocation under 75 mg/kg ketamine (*Pfizer Inc.*, Ketalar vial, Istanbul, Turkey) and 10 mg/kg xylazine (*Bayer Inc.*, Rompun vial , Germany) anesthesia. Kidney tissue specimens were collected for histopathological, immunohistochemical, and real-time PCR analyses.

Kidney tissue sections (5µm thick) were incubated at 60°C overnight for deparaffinization, fixed in 10% buffered formalin, and embedded in paraffin for serial sectioning. Longitudinal sections were stained with hematoxylin and eosin (HE) for histopathological analysis. For immunohistochemical staining, sections were incubated at 60°C overnight, stored in xylene, rehydrated through a descending series of ethanols, then washed with distilled water for 10 min. For antigen retrieval, sections were treated with trypsin (*Invitrogen 00-3008*, Camarillo, CA) at 37°C for 10 min, then washed with phosphate-buffered saline (PBS). Sections were incubated in 3% H_2O_2 (*Merck*, K31355100, Darmstadt, Germany) for 5 minutes at room temperature to quench endogenous peroxidase activity, then incubated for 1 h with blocking serum (*Invitrogen,* 85-9043), according to the manufacturer's instructions.

Primary antibodies [anti-nephrin (*Novus Biologicals*, NBP1-19742, USA) and anti-podocin (*Abcam*, ab93650, USA)] were applied overnight at 4°C. The sections were incubated with biotinylated IgG for 30 minutes, followed by three washes in PBS, then incubated with streptavidin-peroxidase conjugate (*Invitrogen*, 85-9043) for 30 minutes and washed three

times with PBS. To visualize immunolabeling, sections were incubated with 3,3'-diaminobenzidine (*Novex®* DAB San Diego, CA, USA) for 5 minutes. Sections were counterstained with Mayer's hematoxylin (*J. T. Baker*, 02274390059, Deventer, The Netherlands) and mounted with mounting medium. Negative controls were treated as described above, but were incubated with rabbit IgG or mouse IgG instead of the primary antibodies. All sections were evaluated using a light microscope (*Olympus Corp.*, Olympus BX43, Tokyo, Japan). Immunoreactivity intensities were scored from 0 to 3 by observers blinded to the experimental information (0: no change; 1: mild; 2: moderate; 3: severe).

Kidney tissue specimens were placed in RNAlater solution (*Sigma*, #R0901) for storage until PCR analysis was performed based on reduction, replacement and refinement. The RNA isolation was followed by cDNA synthesis (equivalent RNA 30 ng/μL) and measurement of expression levels with use of nephrin- and podocin-specific primers in real-time PCR method. Total RNA extraction from whole kidney tissue specimens placed in RNAlater solution was performed using with the *PureLink®* RNA Mini Kit (*Thermo Fisher Scientific*, 12183555). Reverse transcription of the extracted RNA was performed using a high capacity cDNA reverse transcription kit (*Applied Biosystems*, Foster City CA, USA) according to manufacturer's recommendations in a SensoQuest (*Göttingen*, Germany) thermal cycler. RT-PCR analysis from the reverse transcribed RNA specimens was performed using forward PodF1 primer 5'-ACCTCCACACCCTTCAGTCT-3', reverse PodR1 primer 5'-CCTGAGTTCTGTTGCTGGGA-3', forward NephF1 5'- GCCCTGCCTGAAAACCTGA-3', reverse NephR1 5'-TACCTCGGGAAGCCTGGG-3' and *QuantiTect SYBR Green PCR Kit* (*Qiagen*, 204143). Separately prepared PodF1, PodR1, *QuantiTect SYBR Green* and NephF1, NephR1, *QuantiTect SYBR Gree*n mixtures were analyzed in the *Rotor-Gene Q* (*Qiagen*, Hilden, Germany) for the evaluation of podocin and nephrin RNA expression levels. β-microtubulin (B2M) (B2MF1 5'-TCTCTCTTTCTGGCCTGGA-3', B2MR1 5'-TGTCGGATGGATGAAACCC-3') and hypoxanthine phosphoribosyl transferase (HPRT1) (HPRT1F1 5'-CGTCTTGCTCGAGATGTGAT-3', HPRT1R1 5'-TTCAGTGCTTTGATGTAATCCAG-3') were used as housekeeping genes to normalize the expression changes. The related forward and reverse primers were synthesized by *Metabion* (Germany). The thermal cycling conditions of the RT-PCR program started with an initial activation/denaturation stage of 95°C (15 minutes), followed by 40 cycles of denaturation at 95°C (15 seconds), and combined annealing/extension at 60°C (60 seconds). All qRT-PCR analyses were performed in duplicate. The 2-ΔΔCT method was used to calculate the relative changes in gene expression determined from the real-time PCR analysis (*Livak KJ*, Schmittgen TD).

Statistical analysis was done with *IBM SPSS Statistics for Windows, version 22.0* software (*IBM Corp.*, Armonk, NY, USA). Chi-square test was used to analyze histopathological parameters, while Kruskal-Wallis test, Friedman variance analysis, and post-hoc Dunn's test were used to analyze differences in urinalysis parameters between the study groups. Data were expressed as mean [standard deviation (SD)], range, and percentages (%) where appropriate. Results with $P<0.05$ were considered to be statistically significant.

Results

Urinary protein/creatinine ratio was not significantly different from Day 0 to Day 7 in the CON group. On Day 4, PAN group had significantly higher UPCR compared to the other groups ($P<0.05$). On Day 7, no significant difference was found between the groups ($P=0.337$). PAN group showed a significant increase in UPCR between Day 0 and Day 7 ($P=0.015$) (**Table 1**).

On Day 0, median UPCR was significantly lower in the PAN-C group [1.00 (range 0.70-1.40)] compared to the CON [1.75 (range 1.40-1.90), ($P<0.05$)] and PAN [1.80 (range 1.50-1.90), ($P<0.01$)] groups. On Day 4, the UPCR was significantly lower in both the PAN-C [1.45 (1.20-1.80)] and PAN-P [1.40 (1.10-1.80)] groups compared to the PAN group [2.15 (2.00-2.40)] ($P<0.01$ for each), while the PAN group had significantly higher UPCR than the CON group [1.75 (1.40-2.00), ($P<0.05$)]. No significant difference in UPCR was noted among groups on Day 7 (**Table 1**).

No significant difference in nephrin mRNA

expression was noted among study groups, whereas median podocin mRNA expression was significantly higher in the PAN-P group than in the PAN group [22.55 (22.42-23.02) grain/μm^2 *vs.* 22.06 (21.81-22.06) grain/μm^2, respectively, (P<0.01) (**Table 2**).

Histopathological analysis revealed normal renal histology in the CON group, glomerular hypercellularity in the PAN group, and minimal hypercellularity in the PAN-C and PAN-P groups (**Figure 1**).

No significant difference was noted among study groups in terms of nephrin or podocin immunoreactivity. Albeit not statistically significant, fewer rats had moderate immunoreactivity to podocin and nephrin in the PAN (16.7% for each), PAN-C (33.3% and 50.0%, respectively), and PAN-P (33.3% and 50.0%, respectively) groups than in the CON group (66.7% and 83.3%, respectively) (**Table 3**). Furthermore, immunohistochemical analysis revealed that moderate degree expression was higher in the PAN-C and PAN-P groups than in the PAN group (**Figure 2**).

Discussion

Our findings in a rat model of PAN-induced nephrotic syndrome revealed that UPCR was significantly higher in the PAN than in the CON group, and UPCR was significantly lower in the PAN-C and PAN-P groups than in the PAN group. In accordance with the induction of NS in the PAN group, it is likely that calcitriol and paricalcitol supplementation ameliorated the proteinuria in rats with PAN-induced NS.

Likewise, calcitriol was reported to be associated with preservation of the number of glomerular podocytes and amelioration of albuminuria in a rat model of subtotal nephrectomy,[4] and with amelioration of nephrosis in a rat model of PAN-induced nephrotic syndrome.[23] In addition, paricalcitol was reported to improve proteinuria in human with diabetic nephropathy[24] as well as CKD.[14]

Our findings indicate similar efficacy of vitamin D analogues on reduction of proteinuria in NS, while also providing evidence of the potential utility of not only calcitriol, but also paricalcitol in reduction of nephrotic-range proteinuria. Notably, in a study of kidney transplant patients, the lowest rate of

Table 1. Urinary protein/creatinine ratio on Days 0, 4, and 7 among groups

Variables	Median UPCR (range)			
	Day 0	Day 4	Day 7	P value[a]
CON	1.75 (1.40-1.90)	1.75 (1.40-2.00)	1.65 (1.40-2.00)	0.957
PAN	1.80 (1.50-1.90)	2.15 (2.00-2.40)^{*,w}	2.15 (1.80-2.50)	0.015
PAN-C	1.00 (0.70-1.40)^{w,q}	1.45 (1.20-1.80)^{q}	1.85 (1.60-2.00)*	0.030
PAN-P	1.25 (0.90-1.60)	1.40 (1.10-1.80)^{q}	1.90 (1.70-2.30)**	0.008
P value[b]	0.028	0.010	0.337	

[a]days; [b]study groups; CON=control group; PAN=NS group; PAN-C= NS + calcitriol group; PAN-P= NS + paricalcitol group.
*P<0.05 and **P<0.01; compared to day 0 levels in the same group. ^{w}P<0.05 compared to CON group; ^{q}P<0.01 compared to PAN group

Table 2. Real-time PCR analysis for nephrin and podocin mRNA expression

Variables	Median nephrin expression, grain/μm^2 (range)	P value	Median podocin expression, grain/μm^2 (range)	P value
CON	24.24 (23.48-26.11)	0.721	22.48 (21.89-22.97)	0.007
PAN	24.41 (22.94-25.30)		22.06 (21.81-22.06)	
PAN-C	24.40 (22.77-25.14)		22.07 (21.91-22.54)	
PAN-P	25.06 (24.07-26.42)		22.55 (22.42-23.02)*	

*P<0.01, compared to PAN group

Table 3. Immunoreactivity against nephrin and podocin primary antibodies

Variables	Podocin immunoreactivity, n (%)			Nephrin immunoreactivity, n (%)		
	Mild	Moderate	P value	Mild	Moderate	P value
CON	2(33.3)	4(66.7)	0.852	1(16.7)	5(83.3)	0.511
PAN	5(83.3)	1(16.7)		5(83.3)	1(16.7)	
PAN-C	4(66.7)	2(33.3)		3(50.0)	3(50.0)	
PAN-P	4(66.7)	2(33.3)		3(50.0)	3(50.0)	

Chi-square test

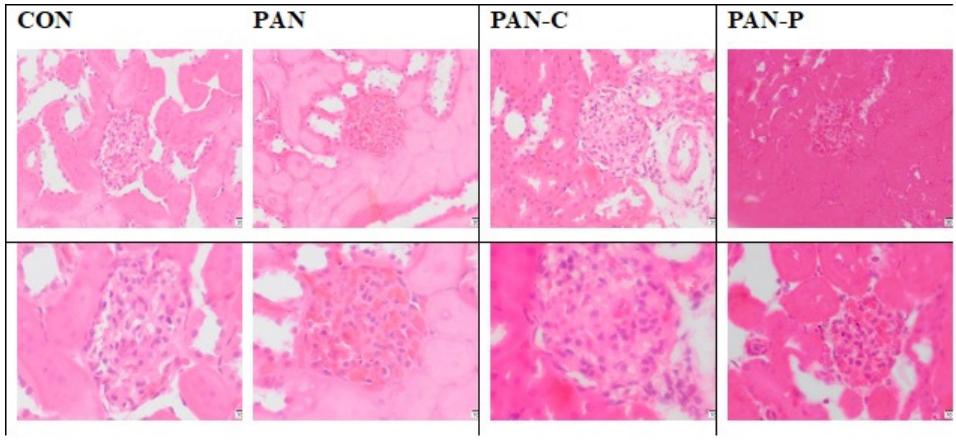

Figure 1. Hematoxylin-eosin staining for histopathological analysis in study groups

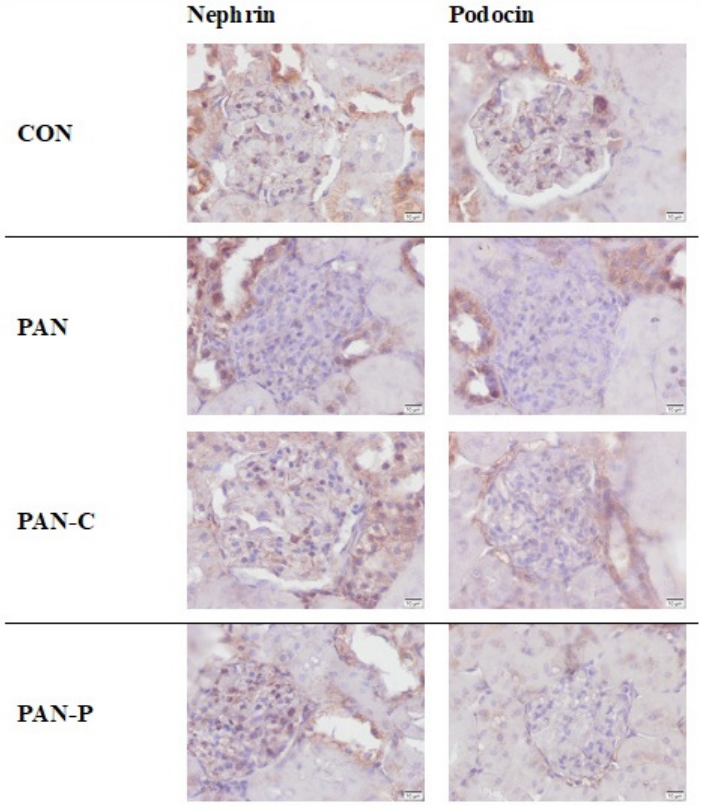

Figure 2. Immunohistochemical analysis for immunoreactivity against nephrin and podocin primary antibodies

proteinuria was reported in patients who were treated with paricalcitol and who had concomitantly the highest level of vitamin D.[25]

However, despite the significant increase in UPCR in the PAN-C and PAN-P groups as compared to Day 0 levels for each group, the lack of significant difference of UPCR among study groups on Day 7 revealed a potentially temporary effect of both Vit D analogues on reduction amelioration of proteinuria in our rat model of PAN-induced NS.

Our findings were consistent with an experimental study which reported low calcitriol blood levels in control rats and calcitriol-treated nephrotic rats for 21 days.[5] Likewise, serum 25(OH) D level was shown to increase and was correlated with serum albumin concentration in NS patients with successful treatment and clinical remission, whereas serum level of 1,25(OH)2D remained low even after remission and had no correlation with serum albumin concentration.[26] Notably, presence of reduced viable nephrons and impaired activation of 1α-hydroxylase resulting in a decreased amount of substrate 25(OH) D or impaired renal synthesis of 1,25 (OH) 2D were associated with decreased free 1,25 (OH) 2D levels in nephrotic patients.[6,26-28]

In our study, histopathological analysis revealed similarly milder glomerular hypercellularity in PAN-C and PAN-P groups as compared to the PAN group. In addition, calcitriol and paricalcitol showed similar efficacy in terms of amelioration of proteinuria with similar levels of UPCR on Days 0, 4, and 7 between the PAN-C and PAN-P groups. However, the PAN-C group of rats had significantly lower baseline UPCR than the CON rats, emphasizing the likelihood of higher baseline vulnerability to NS and related urinary changes.

Decreased nephrin level reported in human NS[29-31] has been associated with dysfunction of the filtration barrier due to failure to form functional complexes in the slit diaphragm, along with the loss of nephrin.[2] While reduced mRNA expression of nephrin and podocin have been reported in various proteinuric renal diseases including NS,[14,21,32] our findings revealed no decrease in nephrin and podocin expression in the PAN group compared to the CON group, whereas significantly higher podocin expression was noted in paricalcitol-treated vs. not-treated NS rats. Moreover, while no significant increase was noted in immunoreactivity for podocin and nephrin in the PAN, PAN-C, and PAN-P groups compared to the CON group, the PAN-C and PAN-P groups tended to have higher podocin and nephrin immunoreactivity than the PAN group in our study. This finding seems to also support the previously reported association of 1,25-dihydroxyvitamin D3 with activation of the nephrin gene promoter in a dose-dependent manner, leading to induced expression of nephrin mRNA.[2] Thus, calcitriol has been suggested to exert its anti-proteinuric effect by means of induction or preservation of nephrin expression in podocytes.[2]

Our findings support the association of Vit D analogues with decrease in nephrotic range-proteinuria,[16] whereas given the lack of a significant difference between the CON and PAN groups in terms of podocin and nephrin expression, the reduction of proteinuria by calcitriol and paricalcitol seems unlikely to be due to direct upregulation of nephrin and podocin in podocytes. The higher podocin expression in the PAN-P than PAN group seems also to not translate into a more remarkable reduction of proteinuria in paricalcitol-treated rats compared to calcitriol-treated rats.

Nephrotic syndrome was induced via a widely used a single PAN injection method[33-36] that results in histological abnormalities of glomerular epithelial and endothelial cells accompanied by proteinuria and in concordance with features of the clinical syndrome.[36-39] Moreover, previous studies have indicated the likelihood of proteinuria secondary to age-dependent increase in renal glomerulosclerosis in Wistar Albino rats after a 6-month life span.[40] The Wistar Albino rats in our study were 6-8 weeks of age.

In conclusion, our findings reveal similar efficacy of 7-day calcitriol and paricalcitol supplementation in a rat model of PAN-induced NS, in terms of temporary amelioration of proteinuria, non-significant tendency of higher nephrin and podocin immunoreactivity, and similar nephrin mRNA expression compared to the PAN group, along with significantly higher podocin mRNA expression in paricalcitol-treated vs. untreated rats with NS. Our findings seem to indicate similar efficacy of the vitamin D analogues calcitriol and paricalcitol in time-limited reduction of proteinuria in NS, yet suggest the likelihood of mechanisms other than direct upregulation of nephrin and podocin in podocytes underlie the renoprotective effects of

vitamin D analogues.

Conflict of interest

The financial support of our work was financed by the coordination unit of the scientific projects of Manisa Celal Bayar University. The project number was 2016-103.

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