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Diabetic Foot Ulcer as a Cause of Significant Decline in the Renal Function Among South Indian Population With Type 2 Diabetes: Role of TGF-β1 and CCN Family Proteins

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Abstract

In the present study, a total of 428 South Indian subjects were divided into four different groups, consisting of individuals with type 2 diabetes without any other complications (T2DM), T2DM subjects with stage 2 and 3 diabetic kidney disease (CKD), T2DM subjects with grade 2 or 3 diabetic foot ulcer (DFU) and T2DM subjects having both diabetic kidney disease and diabetic foot ulcer (CKDDFU). The study was conducted ambispectively by comparing the changes in renal function among two consecutive periods, i.e., the period prior to the development of grade 2 and 3 diabetic foot ulcer (retrospectively) and after the development of DFU (prospectively). A gradual and uniform reduction of eGFR was observed throughout the study period in the subjects affected with either CKD or DFU alone. Whereas in subjects with both CKD and DFU, there was a sharp decline in the eGFR during the six months prior to the baseline, i.e., the period in which the development of ulcer and its progression to grade 2 or 3 happened. Remarkable elevations in the levels of TGF-β1 and CCN2 (CTGF), as well as a significant reduction in the level of CCN3 (NOV), were observed in the serum of CKDDFU group subjects, compared to the other groups. Increased production of TGF-β1 in response to the inflammatory stimulus from multiple sites in CKDDFU subjects caused a subsequent down-regulation of CCN3, followed by the activation of a large quantity of CCN2.

Keywords

diabetic kidney disease, transforming growth factor, CTGF, NOV

Diabetic foot ulcers (DFUs) and diabetic kidney diseases (DKDs) are the 2 common as well as critical comorbid conditions prevailing in people with diabetes. Individuals who have diabetes were observed to have 12% to 25% chance of developing foot ulcers[1] and nearly 20% to 30% risk of developing kidney diseases[2] in a lifetime. Considering the medical expenses of diabetic patients, those with foot complications were estimated to spend 4 times and those with renal disease spent 3 times more than the subjects without any comorbidities.[3-5] The expenditure may even go higher when these 2 complications coexist.[3]

Earlier studies have reported the higher prevalence of DFU development in type2 diabetes mellitus (T2DM) subjects with existing kidney disease.[6,7] A prospective follow-up study conducted earlier in our hospital, to examine the effect of DFU in declining renal function among the patients with T2DM, revealed that the development of DFU causes a decline in renal function among chronic kidney disease (CKD) patients with DFU and also in DFU patients without DKD.[8] The present study consists of 2 parts in which the first part involves an ambispective study to analyze the association between DFU and DKD by comparing the changes in renal function in South Indian patients with T2DM, DKD, and DFU among 2 consecutive periods, that is, the period prior to the development of grades 2 and 3 DFU (retrospectively) and after the development of DFU (prospectively).

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The main hallmark of CKD is the excessive inflammation leading to interstitial tissue fibrosis, extracellular matrix accumulation, and cell hypertrophy. Transforming growth factor-β (TGF-β) mediates renal cell hypertrophy and stimulates extracellular matrix accumulation causing glomerulosclerosis and tubulointerstitial fibrosis in diabetic patients.[9] The CCN family of matricellular signaling proteins, which act downstream to TGF-β, play a vital role in cell proliferation, angiogenesis, tumorigenesis, and wound healing.[10] The CCN family proteins are emerging as a unique common link across multiple diseases and organs related to injury and repair. CCN family proteins have recently been reported to have involvement in the development of kidney diseases[11]; but its role in the progression of nephropathy in diabetic subjects is not well known. The second part of the current study was an attempt to underpin the mechanism involved in the pathophysiological development of CKD in subjects with diabetic foot infection by measuring the expression levels of TGF-β1 as well as CCN family proteins—CCN2, or connective tissue growth factor (CTGF), and CCN3, or nephroblastoma over expressed (NOV).

Materials and Methods

Research Design

A total of 428 subjects (328 males and 100 females) aged between 25 and 70 years, who were admitted in a tertiary care center for diabetes in South India, were recruited for this ambispective study. Subjects were divided into 4 different groups designated as T2DM (group I), CKD (group II), DFU (group III), and CKDDFU (group IV). Group I consisted of individuals having type 2 diabetes without any other complications. Group II consisted of T2DM subjects with stages 2 and 3 DKD as classified based on Kidney Disease Outcomes Quality Initiative guidelines.[12] Group III consisted of T2DM subjects with grade 2 or 3 DFU as categorized based on the classification of the University of Texas Health Science Center. Group IV consisted of individuals having type 2 diabetes with both DKD (stages 2 and 3) and DFUs (grades 2 and 3). Subjects with type 1 diabetes, any acute concurrent illness in the previous 6 months (eg, malignancy and severe gastrointestinal disease), history of nondiabetic or obstructive kidney disease, history of cardiovascular or cerebrovascular diseases, and pregnant women were excluded from the study. The study protocol was approved by the institutional ethics committee (Ref. IEC/N-003/03/2019), and all the methods were performed in accordance with the relevant guidelines and regulations of the institution. Written informed consent was obtained from all the study participants in agreement with the principles of the Declaration of Helsinki.

Analysis of Clinical and Biochemical Parameters

Anthropometric and demographic details such as age, height, weight, body mass index, and duration of diabetes were recorded for all the study subjects. Presence of neuropathy was identified by assessing the vibration perception threshold (a value of ≥25 V was considered as abnormal) using a Biothesiometer (Diabetic Foot Care India Pvt, Ltd, Chennai, India), and the existence of peripheral arterial damage was determined using peripheral vascular Doppler (Versalab). Peripheral blood samples were collected from all the study subjects, and serum/plasma was separated for biochemical analysis. Biochemical parameters such as fasting and postprandial blood glucose (glucose oxidase–peroxidise method), serum triglycerides (glycerol phosphate oxidase–peroxidise–amidopyrine method), serum total cholesterol (cholesterol oxidase–peroxidise amidopyrine method), high-density lipoprotein (HDL; direct method—polyethylene glycol–pretreated enzymes), low-density lipoprotein (LDL; direct method—polyethylene glycol–pretreated enzymes), urea (glutamate dehydrogenase ultraviolet absorption assay), and creatinine (Jaffe's method) were analyzed using Mindray BS400 fully automated biochemistry analyzer. Glycated hemoglobin A1c (HbA1c) was estimated by immunoturbidimetric method (Roche Cobas C 311). Estimated glomerular filtration rate (eGFR) was calculated based on CKD-EPI equation.[13]

Estimation of TGF-β1 and CCN Family Protein Levels in Serum

The serum levels of TGF-β1 and CCN family proteins CTGF and NOV were measured by a quantitative enzyme-linked immunosorbent assay (ELISA) using specific kits designed for TGF-β1 (Human TGF-β1 ELISA Kit, Wuhan USCN Business Co, Ltd, Hubei, China), CTGF (Human CTGF ELISA Kit, Wuhan USCN Business Co, Ltd), and NOV (Human NOV ELISA Kit, Wuhan USCN Business Co, Ltd) in accordance with the manufacturer's instructions. Latent TGF-β1 molecules were preactivated to the immune reactive form using 1 M HCl followed by neutralization with 1.2 M NaOH/0.5 M HEPES. A total of 100 mL of serially diluted standard solutions, as well as prediluted, activated sera were added to the microtiter wells precoated with specific anti-human polyclonal antibodies (TGF-β1/CTGF/NOV) followed by a biotin-conjugated mouse anti-antibodies (TGF-β1/CTGF/NOV) and avidin conjugated to horseradish peroxidase. Color was developed using a 3,3′,5,5′-tetramethylbenzidine substrate and the reaction was terminated with 2 N sulfuric acid. The absorbance was measured at 450 nm using a spectrophotometer. All the samples were determined in duplicate, and respective mean values were calculated. The concentrations of TGF-β1/CTGF/NOV in the samples were then

Table 1. Demographic, Anthropometric, and Biochemical Parameters of the Study Groups[a].

Parameters/Groups	T2DM	CKD	DFU	CKDDFU
Age (years)	49.76 ± 9.09	57. 89 ± 8.98***	57.32 ± 11.19***	60.02 ± 9.40***
Body mass index (kg/m^2)	26.89 ± 4.47	27.71 ± 6.07	27.94 ± 7.23	26.94 ± 5.12
Systolic BP (mm Hg)	123.68 ± 12.49	133.38 ± 19.31	123.08 ± 15.97	128.28 ± 18.35
Diastolic BP (mm Hg)	77.27 ± 8.56	80.96 ± 8.60	76.88 ± 8.07	78.82 ± 9.47
Duration of diabetes (years)	8.09 ± 5.03	13.55 ± 7.87*	12.83 ± 8.75	14.38 ± 8.49**
Fasting plasma glucose (mg/dL)	184.4 ± 67.35	168.53 ± 67.52	167.15 ± 72.48	178.33 ± 96.54
Postprandial glucose (mg/dL)	278.55 ± 94.67	263.73 ± 99.76	245.16 ± 87.30**	265.03 ± 102.40
HbA1c (%)	8.64 ± 1.74	8.54 ± 1.95	9.74 ± 2.08	10.21 ± 5.72
Urea (mg/dL)	21.59 ± 7.65	35.05 ± 15.62**	25.56 ± 10.01	42.07 ± 25.05***¥¥¥
Creatinine (mg/dL)	1.01 ± 0.13	1.44 ± 0.53	1.00 ± 0.15	1.60 ± 0.72
eGFR (mL/min/1.73 m^2)	79.85 ± 10.52	54.67 ± 17.71***	77.12 ± 17.80###	55.23 ± 18.59***¥¥¥
Albumin-creatinine ratio (mg/g)	13.31 ± 6.46	67.12 ± 44.02***	21.50 ± 10.24###	75.77 ± 46.49***¥¥¥
Total cholesterol (mg/dL)	167.12 ± 43.76	168.76 ± 48.80	150.85 ± 46.63	141.24 ± 48.32*#
Triglycerides (mg/dL)	142.54 ± 100.72	142.30 ± 73.18	122.99 ± 57.51	146.71 ± 108.84
HDL (mg/dL)	44.57 ± 33.49	40.28 ± 9.58	34.95 ± 11.96	29.7 ± 11.35
LDL (mg/dL)	95.94 ± 29.84	99.06 ± 32.32	90.81 ± 30.96	85.58 ± 31.98
VLDL (mg/dL)	29.54 ± 15.09	32.89 ± 21.63	26.28 ± 11.51	27.7 ± 14.49
Non HDL (mg/dL)	125.75 ± 42.31	130.59 ± 45.49	116.84 ± 41.39	112.88 ± 43.30
Neuropathy (%)	—	43.56	88.46###	91.13###
Retinopathy (%)	—	40.48	55.00	75.00###

Abbreviations: T2DM, type 2 diabetes mellitus; CKD, chronic kidney disease; DFU, diabetic foot ulcer; BP, blood pressure; HbA1c, hemoglobin A1c; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein.
[a]Values are mean ± SD.
*$P < .05$, **$P < .01$, ***$P < .001$, versus T2DM group; #$P < .05$, ##$P < .01$, ###$P < .001$, versus CKD group; and ¥$P < .05$, ¥¥$P < .01$, ¥¥¥$P < .001$, versus DFU group.

calculated by comparing the optical density of the samples with the standard curve.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Version 20.0 software. Mean and standard deviation for continuous variables and percentages for categorical variables were reported accordingly. T test and χ^2 test were performed as applicable for comparing the variables between different groups, and a P value $<.05$ was considered to be statistically significant.

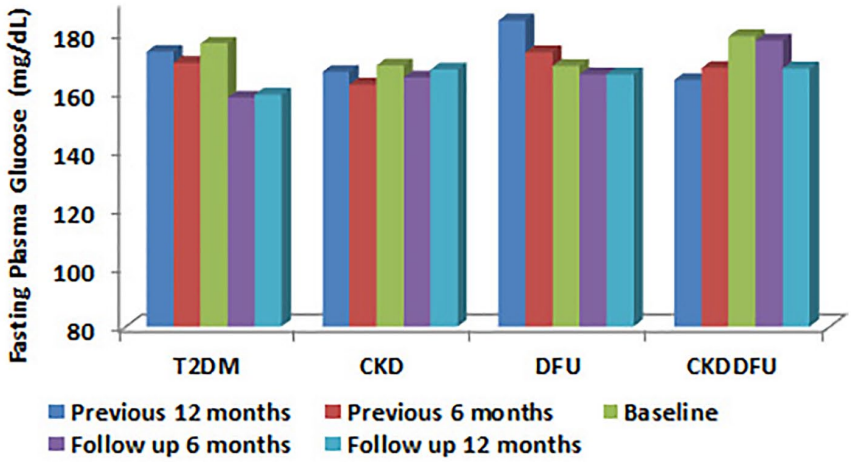
Results

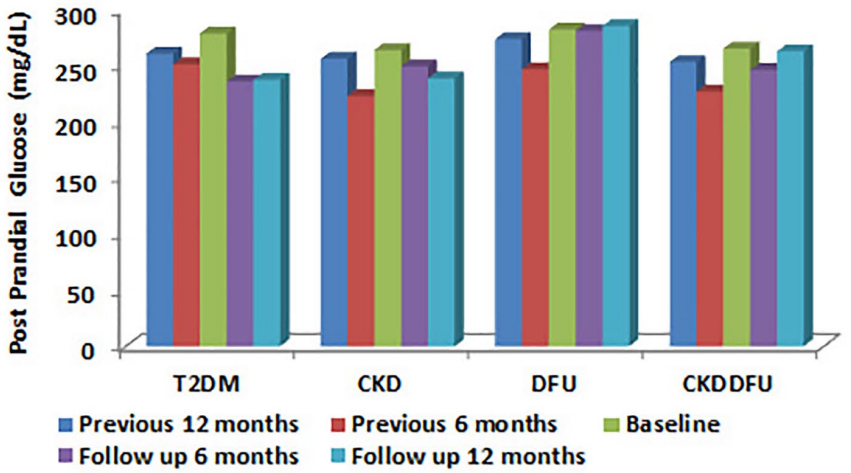
Analysis of Clinical and Biochemical Parameters

The anthropometric and biochemical parameters analyzed for different study groups at baseline are given in Table 1. T2DM group subjects were younger and had less duration of diabetes compared with the other study groups. CKDDFU group subjects had a longer duration of diabetes, followed by CKD group subjects. The body mass indexes of all the 4 study groups can be categorized as overweight (25-30 kg/m^2) according to the World Health Organization recommendations. The systolic and diastolic blood pressure of CKD group was higher compared with the other groups. Fasting and postprandial blood glucose levels of all the study groups were considerably elevated, indicating poor glycemic control. HbA1c levels of CKDDFU group subjects were remarkably higher than T2DM and CKD groups.

Urea and creatinine levels were elevated, and the eGFR was drastically reduced in the CKD and CKDDFU groups compared with the T2DM and DFU groups. Albumin-creatinine (A/C) ratio of CKD and CKDDFU groups was considerably higher than the T2DM and DFU groups. But there was no significant variation observed in the urea, creatinine, A/C ratio, and eGFR values among T2DM and DFU groups. Total cholesterol, triglycerides, LDL, VLDL, and non-HDL values for all the groups were found to be within the normal range. However, HDL values of DFU and CKDDFU groups were lower than the recommended healthy range. Presence of neuropathy and retinopathy was higher in the CKDDFU group followed by the DFU group.

Results of comparative analysis of fasting and postprandial blood glucose and HbA1c levels for the study groups over the period is given in Figure 1. There was a gradual decrease in the HbA1c values during the follow-up period in all the groups. Figure 2 represents the pattern of change in the urea and creatinine values of all the study groups over the period. There was a substantial increase in the levels of creatinine and urea during the follow-up period in the CKD,





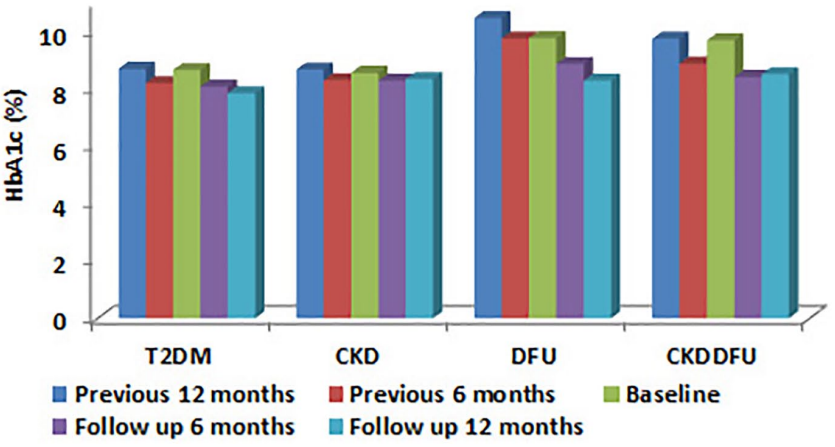


Figure 1. Trend analysis of blood glucose and HbA1c values for the study groups over the period.

DFU, and CKDDFU groups. A gradual decrease in eGFR was observed in the DFU and T2DM groups, whereas in the CKD and CKDDFU groups the rate of decline in eGFR was considerably high (Figure 3). The average reduction of eGFR during the previous and follow-up period within the group is given in Table 2. A uniform reduction of eGFR was

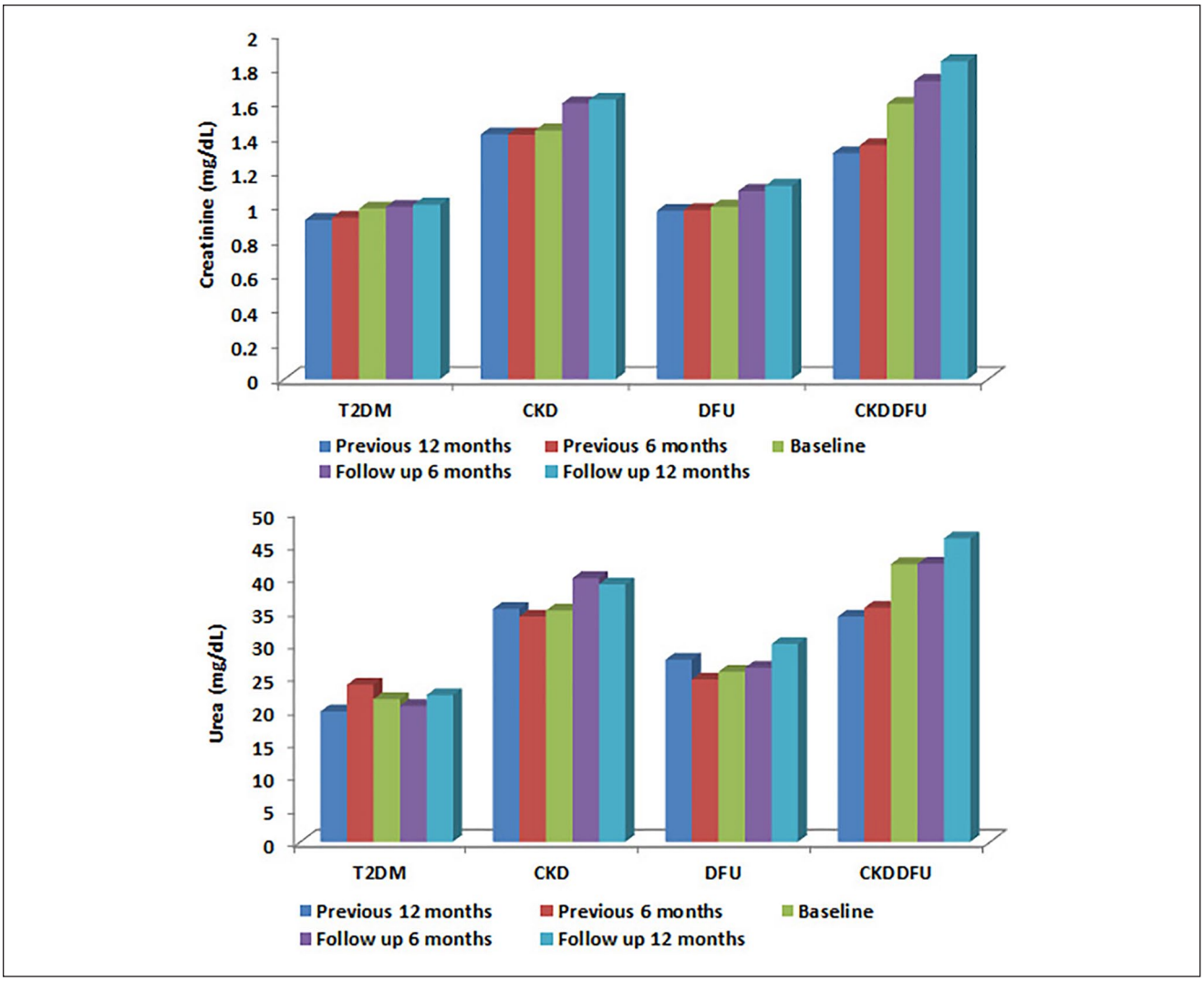


Figure 2. Trend analysis of creatinine and urea levels for the study groups over the period.

observed before and after the development of foot ulcer in the CKD as well as DFU groups. Whereas in CKDDFU group there was a sharp decline in eGFR during the 6 months prior to the baseline, that is, the period in which the development of ulcer and its progression to grade 2 or 3 happened (Figure 3 and Table 2).

Estimation of TGF-β1 and CCN Family Protein Levels in the Serum

Serum levels of TGF-β1, CTGF, and NOV were analyzed using the ELISA method, and the results are given in Figure 4. TGF-β1 levels were found to be significantly elevated in the CKDDFU group compared with the other groups. CKD and DFU groups also showed a moderate increase in the levels of TGF-β1 compared with the T2DM group. A similar trend was observed in the serum levels of CTGF as well. CKDDFU group showed a significant increase and DFU and CKD groups showed a moderate increase in the level of CTGF. The serum level of NOV was found to be significantly reduced in the CKDDFU group compared with the other groups. CKD and DFU groups also showed a moderate decrease in the level of NOV compared with the T2DM group. The change in the TGF-β1, CTGF, and NOV levels in the serum of CKDDFU subjects are considerably high than the subjects with CKD or DFU alone.

Discussion

DKD is one of the principal reasons for kidney failure in persons with diabetes. Development of foot ulcer is another critical and challenging complication among people with diabetes, and if proper care is not given, it may eventually lead to amputation. The coexistence of both these conditions

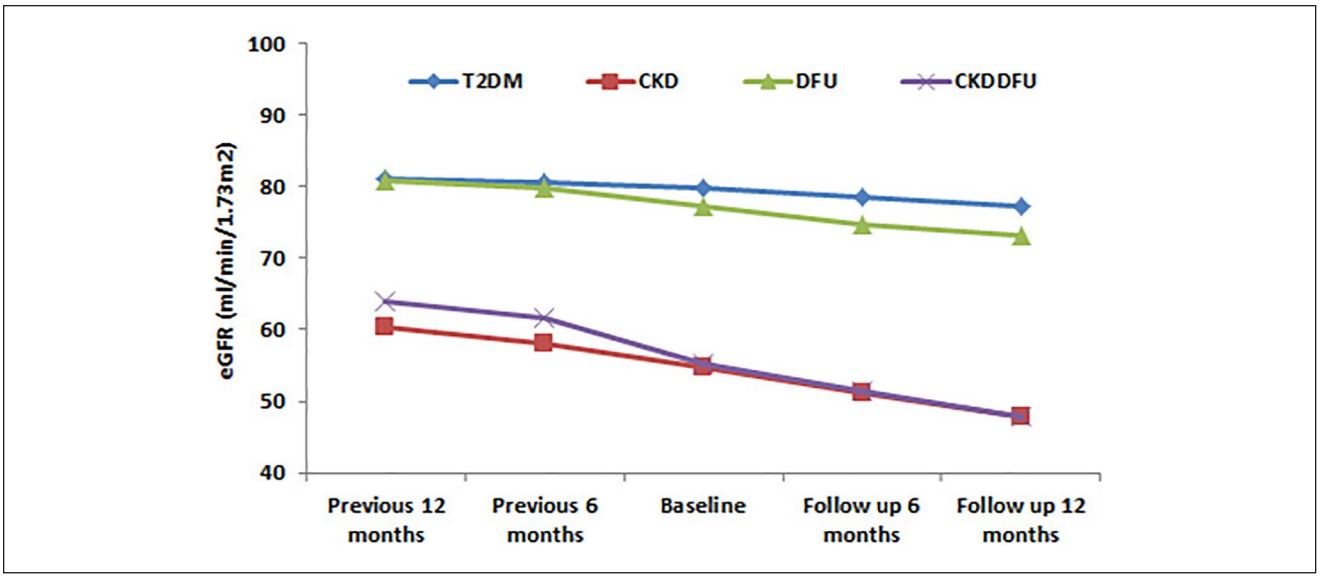


Figure 3. Trend analysis of eGFR for the study groups over the period.

Table 2. Average Reduction of eGFR According to Time Within the Group.

	eGFR Reduction (mL/min/1.73 m^2)			
Group	Previous 12th Month Versus Previous Sixth Month	Previous Sixth Month Versus Baseline	Baseline Versus Follow-up Sixth Month	Follow-up Sixth Month Versus Follow-up 12th Month
T2DM	0.534	0.646	1.436	1.181
CKD	2.381	3.330	3.643	3.186
DFU	1.125	2.634	2.533	1.333
CKDDFU	2.342	6.398	3.870	3.462

Abbreviations: eGFR, estimated glomerular filtration rate; T2DM, type 2 diabetes mellitus; CKD, chronic kidney disease; DFU, diabetic foot ulcer.

adversely affects the physical quality of life in patients with diabetes, making them more susceptible to other critical health issues.[14-16] Studies carried out earlier in diabetic subjects revealed a potential relationship between DKDs and the development of DFU.[6,7] In an observational study conducted earlier to analyze the association of CKD and DFU, it was revealed that patients with CKD even in the initial stages showed a high threat for developing DFU leading to lower extremity amputation DFU.[6] Another retrospective cohort study performed at a single tertiary university center also established the strong association between the degree of renal function impairment and DFS in both type 1 and type 2 diabetes subjects with DFU.[7]

The present study investigated the effect of diabetic foot infection on the renal function of subjects with and without CKD before and after the onset of DFUs. There was a significant decline in eGFR among CKD patients who developed foot ulcers than the CKD patients with no evidence of DFU. The reduction of eGFR in the DFU patients with no signs of CKD earlier was also slow and gradual compared with those with existing CKD. Thus, the results revealed that the existence of both the conditions in the patients with diabetes accelerated the progression of renal damage to a greater extent. A sharp decline in eGFR during the 6 months prior to the baseline in the CKDDFU group indicates that the damaging effect was more during the initial period of development of ulcer and its progression to grade 2 or 3.

The development of DKDs involves several phases and shares multiple mechanisms. Even though DKD is considered a microvascular complication of diabetes, recent evidence also indicates the role of inflammation in the podocyte loss and epithelial dysfunction, which are the hallmarks of end-stage renal disease. Normal wound healing process consists of a short inflammatory phase followed by a proliferative phase and a remodeling phase that are required to provide sufficient wound strength and closure of wound at an appropriate time.[17] Prolonged inflammation leads to the activation of regeneration pathways that contributes to the development and progression of renal diseases.[18,19] TGF-β1 plays a major role in the maintenance of tissue homeostasis and is overexpressed in disease states and in inflammation. The CCN proteins are a complex family of multifunctional proteins playing

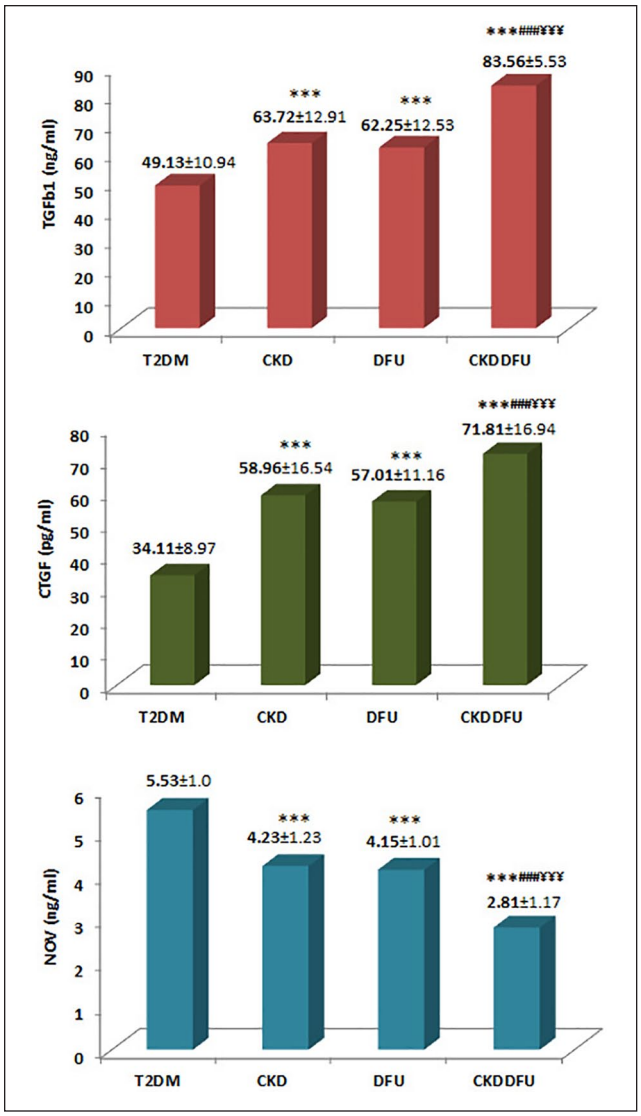


Figure 4. Serum levels of TGF-β1, CTGF, and NOV among the study groups.
*$P < .05$, **$P < .01$, ***$P < .001$, versus T2DM group; #$P < .05$, ##$P < .01$, ###$P < .001$, versus CKD group; and ¥$P < .05$, ¥¥$P < .01$, ¥¥¥$P < .001$, versus DFU group.

diverse roles in regulating cellular function and tissue pathology.[20,21] The involvement of CCN family proteins in the process of wound healing as well as in the development of fibrosis has been explored recently by many researchers. CCN2 (CTGF) and CCN3 (NOV) have been identified as playing a critical role in fibrosis development.[11,22] Increased TGF-β1 production and activity by the cells as an early response to injury results in a downregulation of CCN3. CCN3 act as an endogenous negative regulator of extracellular matrix (ECM) and fibrosis. The reduced CCN3 allows for the increased transcription and translation of CCN2 mRNA, leading to its increased activity. CCN2 functions downstream of TGF, driving increased ECM accumulation and fibrosis. The increase in CCN2 signaling then causes a phenotypic change in mesangial cells making it a myofibroblastic cell type associated with increased metabolism, leading to the deposition of ECM.[11] Downregulation of CCN3 also results in increased expression of podocyte CCN2 along with a loss in cell number and function.[11]

The present study analyzed the involvement of TGF-β1 and CCN family proteins (CCN2 and CCN3) in the development and progression of DKD in subjects with active foot ulcers. The levels of TGF-β1 were found to be elevated in the CKDDFU group followed by the CKD and DFU groups when compared with the T2DM group. Results indicate that high levels of glucose along with enhanced inflammatory stimulus might have induced a high amount of TGF-β1 in DFU patients with existing DKD. The elevation of TGF-β1 was found to be higher in subjects with both CKD and DFU compared with those with DFU or CKD alone, indicating the larger influence of inflammatory stimulus from multiple sites. As a result of increased TGF-β1, the levels of CCN3 were declined, and subsequently, CCN2 levels were markedly elevated in the subjects with both DKD and foot ulcer.[23,24] CCN2 or CTGF is considered an essential factor in the development of diabetic nephropathy. Even though the biological actions of TGF-β1 are complex and affect many different cell types, CTGF act more specifically in the processes involving connective tissue formation during wound repair or fibrotic disorders.[25] The upregulation of CCN2 or CTGF functions as a downstream mediator of TGF-β1 action on connective tissue cells, where it stimulates cell proliferation and extracellular matrix synthesis leading to glomerulosclerosis and interstitial fibrosis, the common defects happening in diabetic renal disease. Observations of the earlier studies also suggested the critical involvement of CTGF in inducing structural and functional damage in diabetic nephropathy in both type 1 and type 2 diabetic patients.[26-28] Plasma CTGF levels were found to be elevated in type 1 diabetic patients with nephropathy and perceived to be correlated with proteinuria and creatinine clearance.[27]

In conclusion, the outcomes of the present study revealed that there was a marked increase in the creatinine levels and a sharp decline in eGFR among CKD patients who developed foot ulcers than the CKD patients with no evidence of DFU. The findings also provided a better understanding of the molecular mechanism underlining the deterioration of renal function in T2DM patients with CKD and DFU by addressing the role of CCN family proteins (CCN2 and CCN3) in the pathogenesis. Findings of the study point out to the development of agents that regulate CCN family proteins, especially those that inhibit CTGF levels, as a new clinical approach in the prevention and progression of fibrotic renal diseases. However, the study has a few limitations. The sample size was small, and further studies in large populations are required to confirm the effects. Moreover, the levels of TGF-β1 and CCN family proteins were determined only in

the baseline period and analysis of these marker levels in different stages of disease development, within each group, is critical for their therapeutic clinical application.

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Declaration of Conflicting Interests

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