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LCN2 Associated With the Bidirectional Cardio-Kidney Link in Patients With Type 2 Diabetes and Cardiovascular-Kidney-Metabolic Syndrome

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Background: Despite the well-established epidemiological association between diabetic kidney disease (DKD) and coronary artery disease (CAD) in patients with T2DM, which reflects a core feature of cardiovascular-kidney-metabolic (CKM) syndrome, the underlying molecular mechanisms connecting these 2 conditions remain unclear. Within this integrated pathophysiological framework, the potential role of lipocalin-2 (LCN2) was investigated.

Material/Methods: A total of 917 participants with T2DM were stratified into an overall cohort and a BMI-, age-, and sex-matched sub-cohort. Serum LCN2 levels were measured. Immunohistochemistry was performed on cardiac and renal tissues from high-fat diet/streptozotocin-induced diabetic mice. Renal tubular (HK-2) and cardiomyocyte (H9c2) cells were treated with recombinant LCN2 to assess inflammatory responses.

Results: DKD severity was identified as an independent risk factor for CAD in patients with T2DM. Serum LCN2 levels were significantly elevated in patients with DKD or CAD, and were closely correlated with DKD severity and B-type natriuretic peptide levels. Logistic regression analysis further indicated that an elevated serum LCN2 level served as an independent risk factor for the presence of DKD or CAD. Importantly, mediation analysis revealed that increased serum LCN2 may partially mediate the bidirectional association between DKD and CAD. Consistent with these clinical findings, animal experiments demonstrated concurrent upregulation of LCN2 in both the heart and kidney tissues of diabetic mice. Recombinant LCN2 dose-dependently increased interleukin-6 and tumor necrosis factor-α mRNA expression in HK-2 and H9c2 cells.

Conclusions: LCN2 may represent a potential biomarker and partial mediator between DKD and CAD in T2DM, potentially contributing to CKM-related organ crosstalk.

Keywords: biomarkers • coronary disease • diabetes mellitus • kidney diseases • lipocalin-2

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Introduction

Diabetes mellitus (DM) is a major contributor to chronic kidney disease (CKD) and end-stage renal disease (ESRD) on a global scale [1]. Both DM and CKD are well-established risk factors for cardiovascular diseases, and the co-occurrence of DM and CKD synergistically amplifies cardiovascular risk [2,3]. Around 40% of patients with DM develop CKD [4], which is associated with a 3-fold increase in the risk of cardiovascular mortality compared with those with type 2 diabetes mellitus (T2DM) alone [5]. Albuminuria and a reduced estimated glomerular filtration rate (eGFR) are independent predictors of cardiovascular death [6]. Even slight elevations in albuminuria correlate with heightened cardiovascular risk, and when eGFR falls below 45 mL/min per 1.73 m^2, the likelihood of heart failure doubles [7], while the risk of atherosclerotic cardiovascular disease rises linearly [6,8,9]. This underscores the concept of cardiovascular-kidney-metabolic (CKM) syndrome, which highlights the bidirectional and interconnected pathophysiology between the heart, kidney, and metabolic system [10,11]. The link between diabetic kidney disease (DKD) and cardiovascular disease is indeed bidirectional: CKD exacerbates vascular congestion and inflammation, while declining cardiac function impairs renal perfusion [12]. In patients with acute decompensated heart failure, CKD is one of the strongest predictors of mortality and cardiovascular complications. Individuals with cardiac and renal conditions face disproportionately high rates of hospitalization, substantial symptom burden, and elevated mortality [11], underscoring the substantial worldwide impact of this comorbidity and the urgent demand for novel therapeutic strategies.

The development of atherosclerotic cardiovascular disease in individuals with DKD results from an interplay between conventional cardiovascular risk factors, such as metabolic abnormalities, hypertension, and prior cardiovascular events, and non-traditional risk factors, including endothelial dysfunction, chronic inflammation, and oxidative stress [9,13]. Despite the well-established epidemiological association between CKD and coronary artery disease (CAD, a major form of cardiovascular disease) in patients with T2DM, the underlying molecular mechanisms connecting DKD and CAD remain unclear.

Lipocalin-2 (LCN2), also known as neutrophil gelatinase-associated lipocalin [14,15], is a well-established inflammatory mediator and has emerged as a biomarker and potential mediator renal and cardiovascular diseases [16-29]. Previous studies have demonstrated that LCN2 is markedly elevated in acute and chronic kidney injury [16-20] and is associated with the progression of chronic kidney disease [21,22]. In parallel, increased circulating or urinal LCN2 levels have been linked to atherosclerosis [23,24], myocardial infarction [25,26], and cardiac dysfunction [27-29], suggesting a potential role in cardiovascular pathology. Given its involvement in inflammation and tissue injury in both organ systems, LCN2 may represent a biologically plausible link within the CKM framework. However, whether LCN2 contributes to the bidirectional association between DKD and CAD in patients with T2DM has not been clearly established.

Therefore, the aim of this study was to investigate the association between DKD and CAD in patients with T2DM and to evaluate whether serum LCN2 acts as a potential mediator linking these 2 conditions. Our findings demonstrated that (1) DKD severity indices eGFR and urine albumin-to-creatinine ratio (UACR) were independently associated with the presence of CAD in patients with T2DM; (2) serum LCN2 was independently associated with DKD and CAD and statistically partially mediated the relationship between DKD and CAD; and (3) in experimental diabetic models, LCN2 was upregulated in kidney and heart tissues, and exogenous LCN2 induced inflammatory responses in renal tubular cells and cardiomyocytes, providing supportive biological plausibility.

Material and Methods

Study Population and Design

This was a retrospective, single-center observational study conducted at the Shanghai Fifth People's Hospital, Fudan University. From January 2022 to December 2023, a total of 917 patients with T2DM participated in the study. The study protocol was approved by the Ethics Committee of the Shanghai Fifth People's Hospital, Fudan University (2022133), and all participants gave written informed consent. The study was conducted in accordance with the Declaration of Helsinki.

The study process is shown in **Figure 1**. The exclusion criteria were as follows: (1) age < 18 years; (2) nondiabetic kidney disease; (3) recent history of dialysis for acute kidney failure or a kidney transplant; (4) uncontrolled hypertension (defined as systolic blood [SBP] pressure ≥ 150 mm Hg and/or diastolic blood pressure [DBP] ≥ 90 mm Hg despite treatment); (5) acute or chronic infectious disease in the 2 weeks prior to screening blood tests, or a history of anti-inflammatory treatment in the preceding 2 to 4 weeks; (6) type 1 DM and secondary DM; (7) pregnancy; (8) alcohol or drug abuse/dependence; and (9) viral infection or positive carrier status (hepatitis B virus, syphilis, or HIV), liver cirrhosis. These criteria were applied to minimize confounding effects from acute inflammatory conditions and nondiabetic renal diseases.

The diagnosis of T2DM was made according to the 2021 criteria of the American Diabetes Association guidelines [30]. Estimated glomerular filtration rate (eGFR) was determined using the Chronic Kidney Disease Epidemiology Collaboration

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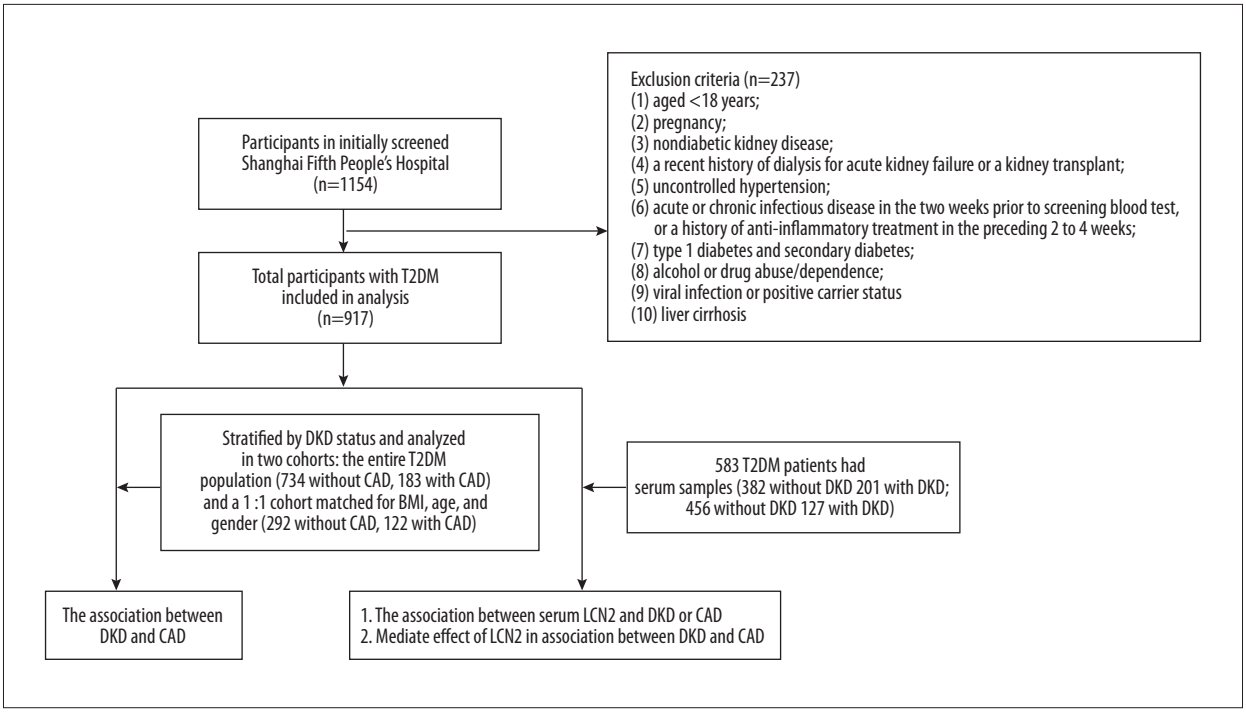

Figure 1. Flowchart of the study.

(CKD-EPI) equation [31]. The definition of DKD was a urine UACR higher than 30 mg/g or eGFR < 60 mL/min per 1.73 m^2 for at least 3 months, as suggested by the statement from the American Diabetes Association [32]. CAD was defined as investigator-reported medical history of myocardial infarction, coronary revascularization, or angiography proven stenosis ≥ 50% in at least 1 major coronary artery [13].

Data Collection and Laboratory Determinations

Clinical and laboratory data were retrospectively collected from electronic medical records at the time of enrollment. All laboratory measurements were obtained after overnight fasting, using standardized protocols. Height, weight, and blood pressure of all patients were measured according to standard protocols. Body mass index (BMI) was calculated by dividing weight in kilograms by height in meters squared. UACR was measured using an automatic biochemical analyzer (Beckman Coulter AU5821) to assess renal function. Venous blood samples were obtained after an overnight fast (at least 10 h) and biochemical parameters (Automatic Biochemical Analyzer, Roche Cobas 8000) were measured. Levels of HbA1C, total cholesterol (TC), triglyceride (TG), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), alanine aminotransferase (ALT), serum creatinine (Scr), uric acid (UA), and B-type natriuretic peptide (BNP) were measured. BNP tertiles were defined based on the distribution of BNP levels in the study population. Cutoff values were determined using sample-based tertile stratification.

Measurement of Serum LCN2 Concentration

Serum was isolated from 1 mL of whole blood by centrifugation, and the LCN2 concentration was quantified using a latex-enhanced immunoturbidimetric assay (Zybio, cat. No: 090110HN01) on a Roche Cobas C702 automatic biochemistry analyzer according to the manufacturer's protocol.

Cell Culture and Treatment

HK-2 (human proximal tubular epithelial cells) and H9c2 cells (rat cardiomyoblasts) were obtained from the Shanghai Institute of Biochemistry and Cell Biology. Both cell lines were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. For the experiments, cells were plated in 12-well plates at a density of 4×10^5 cells/well and subjected to treatment with either vehicle or recombinant LCN2 protein (50 ng/mL, 100 ng/mL and 500 ng/mL) (HY-P70658A) for 48 hours. Cells were harvested after treatment for further analysis.

RNA Isolation and Real-Time Quantitative Polymerase Chain Reaction Analysis

Total RNA of cultured cells was extracted using RNA Extraction Kit (RK30120, ABclonal) followed by cDNA synthesis using a One-step First-strand cDNA synthesis kit (Transgen). SYBR-Green-based quantitative polymerase chain reaction (qPCR) was performed to measure gene expression. The primer sequences

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for qPCR are available from the authors upon request. The mRNA expression levels were normalized to that of β-actin.

Experimental Animals

Eight-week-old male C57BL/6J mice were fed a high-fat diet (HFD) for 8 weeks, followed by intraperitoneal injection of 35 mg/kg of streptozotocin (STZ) for 5 consecutive days. Diabetes was considered successful when random blood glucose levels exceeded 16.7 mmol/L for 2 consecutive days. All mice were housed in the SPF animal facility of the Laboratory Animal Center of Fudan University. The mice were maintained on a HFD for 8 weeks and then killed. Tissues were collected for further analysis. Mouse experimental protocols were approved by the Institutional Animal Care and Use Committee protocols at Fudan University.

Immuno-Histochemistry Staining

The 4-μm sections of paraffin-embedded kidney were deparaffinized, rehydrated, and antigen-retrieved. Slides were incubated in 3% hydrogen peroxide for 15 minutes, then treated with LCN2 (Proteintech, 26991-1-AP, 1: 200) antibody at 4 °C overnight, followed by their corresponding peroxidase-conjugated secondary antibody for 1 hour at room temperature. They were visualized using diaminobenzidine tetrachloride (Dako) for immunohistochemical staining. The sections were then photographed using a computer-assisted video imaging system (NIKON DP controller, Japan). A total of 5 random fields of each stained specimen (magnification, 200×) was used to calculate the mean integrated optical density of LCN2 using Image-Pro Plus 6.0 software (Media Cybernetics, Inc, Rockville, MD, USA). All slides were evaluated independently by 2 investigators blinded to patient identity and clinical outcomes. Paraffin-embedded heart tissues were processed using the same protocol as kidney tissues, including deparaffinization, antigen retrieval, and incubation with the same anti-LCN2 antibody. Imaging and quantification of LCN2 expression in heart tissues were consistent with those from the kidney analysis.

Statistical Analysis

All data were analyzed using SPSS 29.0 software (IBM Corp) and R (version 4.5.1, R Foundation for Statistical Computing). A 2-tailed P value <0.05 was considered statistically significance. Continuous variables are expressed as mean ± standard error (SE) or median (quartile range), and categorical variables as a number (percent). To eliminate potential bias due to uneven distribution of covariates between T2DM with or without DKD, a case-control matching method was applied to match variables, including pre-pregnancy BMI, age, and sex. Matching tolerance was 2, 2, and 0, respectively. Normality was assessed using the Shapiro-Wilk test. Multicollinearity was evaluated using variance inflation factors. Comparisons between 2 groups were performed using an independent-sample t test or the nonparametric Mann-Whitney U test, as appropriate. For comparisons among multiple groups, 1-way analysis of variance (ANOVA) was conducted, followed by Tukey or Dunnett post hoc tests with adjustment for multiple comparisons. Categorical variables were analyzed by the chi-square test. Spearman rank correlation was used to evaluate the relationship between 2 continuous variables. To determine risk factors for development of CAD in T2DM, logistic regression analysis was performed. Multivariate linear regression analysis was used to analyze the factors associated with LCN2. Missing data were minimal (< 5%) and were handled using complete-case analysis.

Mediation Analysis

To investigate whether serum LCN2 mediated (n = 583) the association between DKD and CAD, causal mediation analysis was performed within a counterfactual framework. For the DKD to LCN2 to CAD model, DKD was treated as the exposure, LCN2 as the mediator, and CAD as the outcome. For the reverse model, CAD was treated as the exposure and DKD as the outcome. Linear regression was used for the mediator model, and logistic regression for the outcome model. All models were adjusted for age, sex, DM duration, BP, BMI, HbA1C, UA, LDL-C, and TG. The average causal mediation effect, average direct effect, and total effect were estimated using the "mediation" package in R. Statistical inference was performed using nonparametric bootstrap resampling (1000 iterations) to generate 95% confidence intervals. The proportion mediated was calculated as the ratio of indirect effect to total effect.

Results

DKD Was an Independent Risk Factor for CAD in Patients with T2DM

A total of 298 (18.77%) patients were diagnosed with DKD. Compared with patients without DKD, those with DKD were older and had a longer duration of DM, a higher percentage of hypertension history, a greater proportion of angiotensin II receptor blocker or angiotensin-converting enzyme inhibitor use, and a higher percentage of CAD (all $P < 0.001$, **Table 1 and Figure 2A**). Moreover, patients with DKD had significantly higher levels of HbA1c, UA, UACR, Scr, and BNP, as well as significantly lower eGFR levels than those without DKD (all $P < 0.001$, **Table 1**). There were no significant differences in sex distribution, the proportion of patients with a smoking history, history of cerebral infarction, use of sodium-glucose cotransporter 2 inhibitor, or levels of BP, BMI, left ventricular ejection fraction, TG, TC, HDL-C, and LDL-C (all $P > 0.05$; **Table 1**). After

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Table 1. Characteristics of patients with and without DKD in all T2DM and matched case-control study.

	All patients			Matched case-control		
	Patients without DKD	Patients with DKD	*P*	Patients without DKD	Patients with DKD	*P*
n	619	298		207	207	
Anthropometric parameters						
Age (years)	62.24 ± 0.54	67.14 ± 0.72	< 0.001	66.47 ± 0.62	66.59 ± 0.63	0.896
Sex						
Male	364 (58.80)	176 (59.06)	0.941	129 (62.32)	129 (62.32)	1.000
Female	255 (41.20)	122 (40.94)		78 (37.68)	78 (37.68)	
History of smoking	291 (47.01)	129 (43.29)	0.289	103 (49.76)	92 (44.44)	0.279
History of hypertension	226 (51.13)	194 (73.76)	< 0.001	60 (58.82)	135 (72.97)	0.014
History of cerebral infarction	128 (36.16)	107 (38.21)	0.595	58 (48.33)	74 (38.35)	0.082
Duration of diabetes (years)	9.37 ± 0.35	13.25 ± 0.59	< 0.001	10.51 ± 0.66	12.92 ± 0.68	0.011
Antihypertension drugs						
ARB or ACEI	148 (33.48)	125 (47.53)	< 0.001	41 (40.20)	87 (47.03)	0.093
Non-ARB and non-ACEI	59 (13.35)	58 (22.05)		15 (14.71)	38 (20.54)	
SGLT2 inhibitor	73 (16.52)	45 (17.11)	0.838	16 (15.69)	34 (18.38)	0.565
SBP (mm Hg)	132.31 ± 0.65	134.15 ± 1.02	0.131	132.80 ± 1.16	133.67 ± 1.17	0.600
DBP (mm Hg)	79.40 ± 0.43	78.53 ± 0.64	0.246	77.96 ± 0.69	78.09 ± 0.72	0.896
BMI (kg/m^2)	24.61 ± 0.15	24.56 ± 0.22	0.849	24.24 ± 0.21	24.19 ± 0.21	0.882
Parameters						
HbA1C (%)	9.02 ± 0.09	9.51 ± 0.14	0.003	8.86 ± 0.16	9.52 ± 0.17	0.006
ALT (U/L)	30.06 ± 1.76	21.15 ± 1.02	< 0.001	25.40 ± 1.75	21.10 ± 1.15	0.020
Scr (μmol/L)	67.41 ± 0.62	102.72 ± 2.86	< 0.001	68.08 ± 1.06	100.85 ± 3.33	< 0.001
eGFR (mL/min per 1.73 m^2)	99.64 ± 0.97	73.60 ± 2.16	< 0.001	94.66 ± 1.36	74.70 ± 2.39	< 0.001
UACR (mg/g)	7.00 (4.00, 13.50)	98.50 (44.50, 304.50)	< 0.001	7.00 (4.00, 12.00)	92.00 (44.00, 286.00)	< 0.001
UA (μmol/L)	300.77 ± 3.45	347.65 ± 6.46	< 0.001	291.92 ± 6.01	349.37 ± 7.96	< 0.001
BNP (pg/mL)	28.10 (20.00, 60.00)	135.5 (54.48, 337.25)	< 0.001	44.60 (20.00, 69.25)	109.00 (47.25, 304.50)	< 0.001
LVEF (%)	65.13 ± 0.25	64.57 ± 0.32	0.089	64.80 ± 0.57	64.92 ± 0.38	0.885
TG (mmol/L)	1.87 ± 0.07	2.18 ± 0.14	0.051	1.66 ± 0.10	2.03 ± 0.16	0.044
TC (mmol/L)	4.35 ± 0.05	4.51 ± 0.09	0.135	4.19 ± 0.99	4.53 ± 0.10	0.016
HDL-C (mmol/L)	1.12 ± 0.01	1.15 ± 0.04	0.603	1.09 ± 0.02	1.16 ± 0.06	0.226
LDL-C (mmol/L)	2.67 ± 0.04	2.66 ± 0.07	0.969	2.58 ± 0.06	2.72 ± 0.08	0.151

Data are presented as mean ± SE, or median (25th-75th percentile) or n (%), Continuous variables were compared using independent *t* tests. Categorical variables were compared using chi-square. Abbreviations: T2DM, type 2 diabetes mellitus; DKD, diabetes kidney disease; BMI, body mass index; ARB, angiotensin II receptor blocker; ACEI, angiotensin-converting enzyme inhibitor; SGLT2 inhibitor, sodium-glucose cotransporter 2 inhibitor; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1C, glycated hemoglobin; ALT, alanine aminotransferase; Scr, serum creatinine; UA, uric acid; eGFR, estimated glomerular filtration rate; UACR, urine albumin-to-creatinine ratio; BNP, B-type natriuretic peptide; LVEF, left ventricular ejection fraction; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

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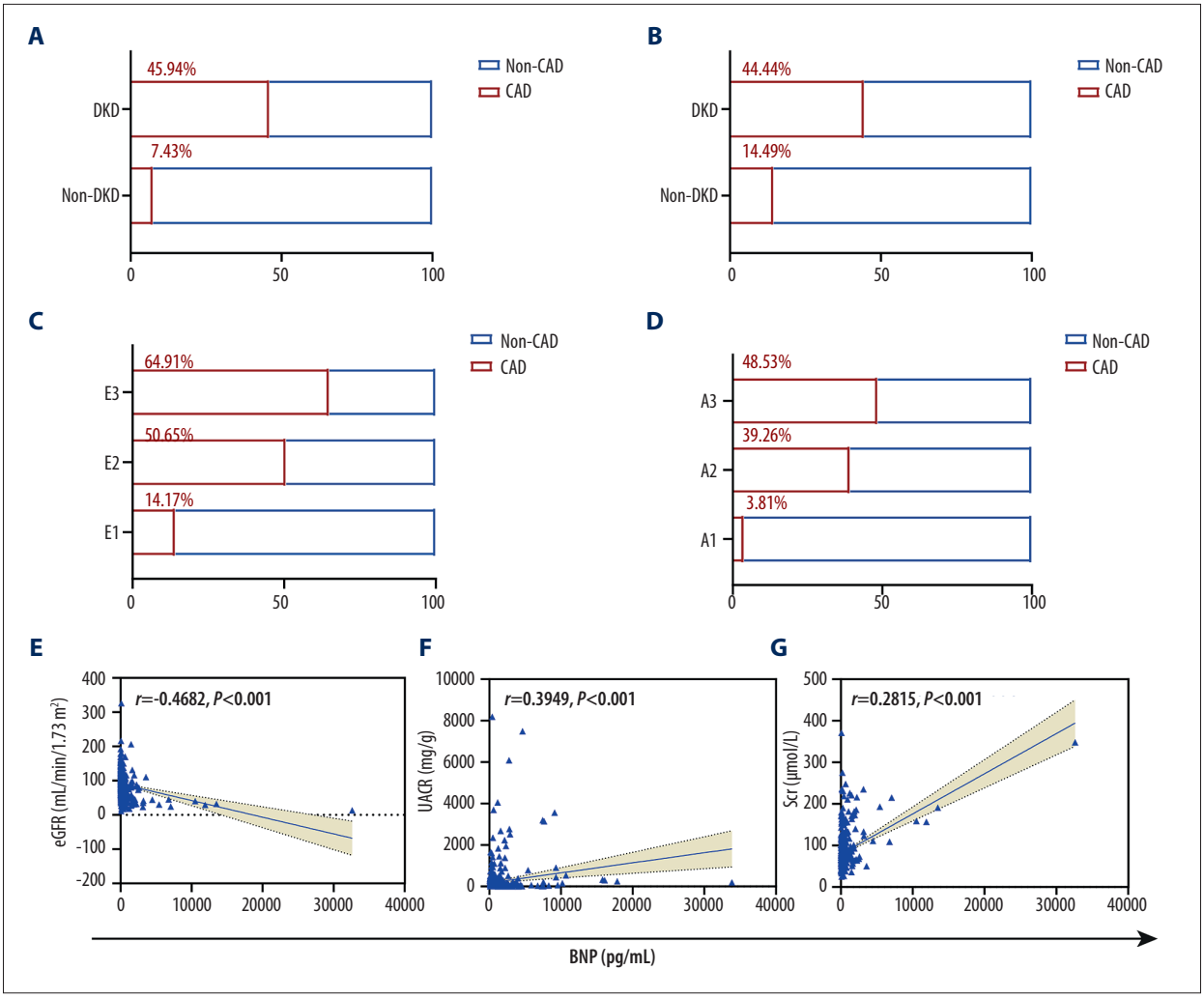

Figure 2. The association between DKD and CAD. (**A, B**) Comparison of CAD percentage between patients with DKD and without DKD in all T2DM patients (**A**) and matched case-control study (**B**). (**C**) Comparison of CAD percentage among patients with T2DM with E1 (eGFR P ≥ 60 mL/min/1.73 m²), E2 (eGFR 45-60 mL/min/1.73 m²), and E3 (eGFR < 45 mL/min/1.73 m²). (**D**) Comparison of CAD percentage among patients with T2DM with A1 (UACR < 30 mg/g), A2 (30 mg/g ≤ UACR ≤ 300 mg/g) to A3 (UACR > 300 mg/g). (**E**) The relationship between eGFR and BNP. (**F**) The relationship between UACR and BNP. (**G**) The relationship between Scr and BNP. Data were analyzed using the chi-square test (**A-D**). Correlation was analyzed using Spearman's rank correlation analysis (**E-G**). Abbreviations: T2DM, type 2 diabetes mellitus; DKD, diabetes kidney disease; CAD, coronary artery disease; Scr, serum creatinine; eGFR, estimated glomerular filtration rate; UACR, urine albumin-to-creatinine ratio.

being matched for age, sex, and BMI, patients with DKD still showed a significantly longer duration of DM, higher percentage of CAD, higher levels of HbA1C, UA, UACR, Scr, and BNP, and lower eGFR levels (all $P < 0.001$; **Table 1 and Figure 2B**).

The cohort was stratified by eGFR into 3 groups: E1 (≥ 60 mL/min/1.73 m²), E2 (45-60 mL/min/1.73 m²), and E3 (< 45 mL/min/1.73 m²). The cohort was also stratified by UACR into 3 groups: A1 (normal albuminuria, < 30 mg/g), A2 (microalbuminuria, 30-300 mg/g), and A3 (macroalbuminuria, > 300 mg/g). Lower eGFR was associated with a stepwise increase in CAD percentage ($P < 0.001$, **Figure 2C**). Similarly, higher albuminuria demonstrated a stepwise increase with increased CAD percentage ($P < 0.001$, **Figure 2D**). Spearman correlation analyses showed that eGFR was negatively correlated with BNP levels (r = -0.4682, $P < 0.001$; **Figure 2E**), while UACR (r = 0.3949, $P < 0.001$; **Figure 2F**) and Scr (r = 0.2815, $P < 0.001$; **Figure 2G**) were positively correlated with BNP.

To identify independent risk factors for CAD in patients with T2DM, logistic regression analysis was performed. DKD was a strong risk factor in both the unadjusted (OR,8.726; 95% CI, 5.949-12.800; $P < 0.001$) and adjusted models (OR,7.379; 95% CI, 4.835-11.263, $P < 0.001$) (**Table 2**). The adjusted model

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Table 2. Logistic regression analysis to determine risk factors for development of CAD in patients with T2DM.

Model		OR (95% CI)	P
Model 1	DKD	8.726 (5.949-12.800)	< 0.001
Model 2	DKD	7.229 (4.865-10.743)	< 0.001
	Age (years)	1.045 (1.025-1.065)	< 0.001
Model 3	DKD	7.379 (4.835-11.263)	< 0.001
	Age (years)	1.041 (1.020-1.062)	0.045
Model 1	eGFR ≥ 60 mL/min/1.73 m^2	Reference	
	45 ≤ eGFR < 60 mL/min/1.73 m^2	5.299 (3.218-8.725)	< 0.001
	eGFR < 45 mL/min/1.73 m^2	9.796 (5.456-17.588)	< 0.001
Model 2	eGFR ≥ 60 mL/min/1.73 m^2	Reference	
	45 ≤ eGFR < 60 mL/min/1.73 m^2	3.802 (2.250-6.425)	< 0.001
	eGFR < 45 mL/min/1.73 m^2	7.418 (4.053-13.579)	< 0.001
	Age (years)	1.041 (1.022-1.061)	< 0.001
Model 3	eGFR ≥ 60 mL/min/1.73 m^2	Reference	
	45 ≤ eGFR < 60 mL/min/1.73 m^2	3.515 (2.025-6.103)	< 0.001
	eGFR < 45 mL/min/1.73 m^2	6.604 (3.413-12.779)	< 0.001
	Age (years)	1.041 (1.021-1.062)	< 0.001
Model 1	UACR < 30 mg/g	Reference	
	30 ≤ UACR ≤ 300 mg/g	13.987 (7.821-25.012)	< 0.001
	UACR > 300 mg/g	20.007 (10.108-39.598)	< 0.001
Model 2	UACR < 30 mg/g	Reference	
	30 ≤ UACR ≤ 300 mg/g	11.984 (6.556-21.905)	< 0.001
	UACR > 300 mg/g	19.548 (9.517-40.153)	< 0.001
	Age (years)	1.047 (1.021-1.074)	< 0.001
Model 3	UACR <-30 mg/g	Reference	
	30 ≤ UACR ≤ 300 mg/g	12.182 (6.579-22.559)	< 0.001
	UACR > 300 mg/g	20.027 (9.552-41.991)	< 0.001
	Age (years)	1.044 (1.017-1.072)	< 0.001

Model 1 was unadjusted for confounding factors. Model 2 included DKD, age, sex, duration of diabetes, BMI, SBP, and DBP. Model 3 included DKD, age, sex, duration of diabetes, BMI, SBP, DBP, HbA1C, UA, LDL-C, and TG. Abbreviations: CAD, coronary artery disease; T2DM, type 2 diabetes mellitus; DKD, diabetes kidney disease; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1C, glycated hemoglobin; UA, uric acid; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; OR, odds ratio.

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included age, sex, DM duration, BMI, BP, HbA1C, UA, LDL-C, and TG. Subsequently, when stratified according to eGFR value, both E2 and E3 were independent risk factors for CAD (OR,3.515; 95% CI, 2.025-6.103; $P < 0.001$; OR,6.604; 95% CI, 3.413-12.779; $P < 0.001$, respectively) (**Table 2**). When stratified according to UACR level, microalbuminuria and macroalbuminuria (OR,12.182; 95% CI, 6.579-22.559; $P < 0.001$; OR,20.027; 95% CI, 9.552-41.991; $P < 0.001$) (**Table 2**) were independent risk factors for CAD development.

Elevated Serum LCN2 Was Closely Associated With and Served as an Independent Risk Factor for DKD in Patients With T2DM

LCN2, a biomarker of kidney injury [16-18], has also been implicated in the pathogenesis of CAD [23,24,33,34]. To explore the potential link between DKD and CAD, we measured serum LCN2 levels in patients with T2DM. As expected, serum LCN2 levels were significantly higher in patients with DKD than in those without DKD, both in the overall cohort and after adjusting for age, sex, and BMI (all $P < 0.001$; **Figure 3A, 3B**). Furthermore, a clear stepwise increase in serum LCN2 levels was observed in parallel with declining renal function. LCN2 levels rose significantly across worsening eGFR categories (from E1 to E3; $P < 0.001$; **Figure 3C**) and with increasing albuminuria severity (from normo- to macroalbuminuria; $P < 0.001$; **Figure 3D**). Serum LCN2 levels were negatively correlated with eGFR ($r = -0.3466$, $P < 0.001$; **Figure 3E**), and positively correlated with Scr ($r = 0.3664$, $P < 0.001$; **Figure 3F**) and UACR ($r = 0.2525$, $P < 0.001$; **Figure 3G**). These correlations remained significant after adjusting for potential confounders (eGFR: $\beta = -0.454$, $P < 0.001$; Scr: $\beta = 0.572$; $P < 0.001$; UACR: $\beta = 0.247$; $P < 0.001$) (**Table 3**).

Logistic regression analyses were performed to assess whether serum LCN2 was independently associated with DKD. Serum LCN2 was analyzed as a continuous variable in regression models. Higher serum LCN2 levels were associated with an increased risk of DKD in the unadjusted model (OR, 5.921; 95% CI 3.887-9.019; $P < 0.001$). The association remained significant after adjusting for CAD, age, sex, DM duration, BMI, BP, HbA1c, UA, LDL-C, and TG (OR, 3.772; 95% CI 2.325-6.120; $P < 0.001$).

Elevated Serum LCN2 Was Closely Associated With and Served as an Independent Risk Factor for CAD in Patients With T2DM

Similarly, serum LCN2 levels were significantly higher in patients with CAD than in those without CAD, both in the overall T2DM cohort and case-control study (all $P < 0.001$; **Figure 3H, 3I**). When patients were stratified by BNP tertiles, serum LCN2 levels were significantly higher in the highest tertile (> 161.00 pg/mL) than in the middle (41.60-161.00 pg/mL) and lowest (< 41.60 pg/mL) tertiles ($P < 0.001$; **Figure 3J**). Spearman correlation analysis demonstrated a positive association between serum LCN2 and BNP levels ($r = 0.3212$, $P < 0.001$; **Figure 3K**), which remained significant after multivariable adjustment ($\beta = 0.671$, $P < 0.001$; **Table 3**). Logistic regression analyses with CAD as the outcome showed that elevated serum LCN2 levels were significantly associated with an increased risk of CAD in the unadjusted model (OR, 5.710; 95% CI, 3.671-8.881; $P < 0.001$). This association remained significant after adjusting for DKD and other covariates (OR, 3.188; 95% CI, 1.975-5.144; $P < 0.001$; **Table 4**).

Elevated Serum LCN2 Was Associated With a Partial Mediation Effect in the Bidirectional Relationship Between DKD and CAD in Patients With T2DM

A cohort with available serum LCN2 measurements (n = 583) was stratified according to the presence or absence of DKD and CAD. We first evaluated whether serum LCN2 mediated the association between DKD and CAD. The indirect, direct, and total effect with corresponding 95% CIs are presented in **Figure 4**. Logistic regression analysis showed that DKD was a significant independent risk factor for CAD in the unadjusted model (OR, 9.802; 95% CI, 6.215-15.462; $P < 0.001$; **Figure 4A**). This association remained significant after adjusting for age, sex, DM duration, BP, BMI, HbA1C, UA, LDL-C, and TG (OR, 7.570; 95% CI, 4.496-12.746, $P < 0.001$; **Figure 4B**). Mediation analysis indicated that serum LCN2 may partially mediate the association between DKD and CAD, accounting for 29.77% (95% CI, 18.12%-44.27%; $P < 0.001$) of the total effect in the unadjusted model (**Figure 4A**) and 29.79% (95% CI, 17.03%-46.53%; $P < 0.001$) after adjusting for covariates (**Figure 4B**).

We next performed a complementary mediation analysis to assess the reverse pathway, examining whether serum LCN2 was estimated to partially mediate the association between CAD and DKD. CAD was a strong independent risk factor for DKD both in unadjusted analyses (OR, 9.802; 95% CI, 6.215-15.462, $P < 0.001$; **Figure 4C**) and full adjusted models (OR, 7.124; 95% CI, 4.250-11.939; $P < 0.001$; **Figure 4D**). Serum LCN2 partially mediated this association, accounting for 38.78% (95% CI, 25.38%-53.86%; $P < 0.001$) of the total effect in the unadjusted model (**Figure 4C**) and 37.97% (95% CI, 23.28%-55.77%; $P < 0.001$) after adjustment for covariates (**Figure 4D**).

To further confirm the potential role of LCN2 in linking DKD and CAD, LCN2 expression was examined in kidney and heart tissues from HFD-STZ–induced diabetic mice and control mice. Consistent with our human data, LCN2 expression was significantly increased in both the kidney and heart tissues of HFD-STZ mice compared with controls (**Figure 5A, 5B**). In addition, given the established role of LCN2 in inflammation, HK-2 and H9c2 cells were treated with increased concentration of

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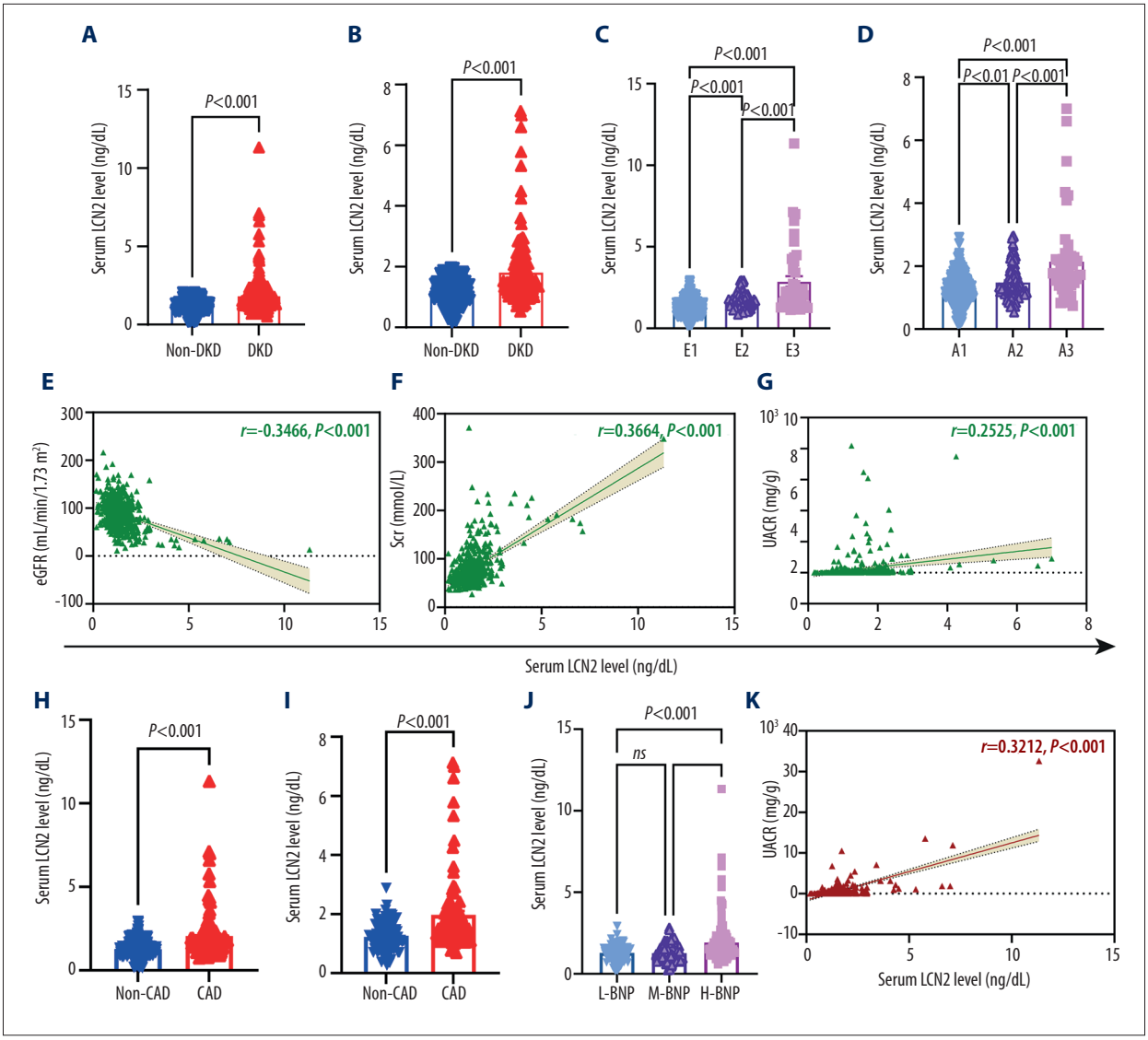

Figure 3. The association between serum LCN2 levels and DKD or CAD. (**A, B**) Comparison of serum LCN2 levels between patients with DKD and without DKD in all T2DM patients (**A**) and matched case-control study (**B**). (**C**) Comparison of serum LCN2 levels among patients with T2DM with E1 (eGFR ≥ 60 mL/min/1.73 m²), E2 (eGFR 45-60 mL/min/1.73 m²), and E3 (eGFR < 45 mL/min/1.73 m²). (**D**) Comparison of serum LCN2 levels among Patients with T2DM with A1 (UACR < 30 mg/g), A2 (30 mg/g ≤ UACR ≤ 300 mg/g) to A3 (UACR > 300 mg/g). (**E**) The relationship between serum LCN2 levels and eGFR. (**F**) The relationship between serum LCN2 levels and Scr. (**G**) The relationship between serum LCN2 levels and UACR. (**H, I**) Comparison of serum LCN2 levels between patients with CAD and without CAD in all T2DM subjects (**H**) and matched case-control study (**I**). (**J**) Comparison of serum LCN2 levels across tertiles of BNP level (L-BNP, BNP < 41.60 pg/mL; M-BNP, 41.60-161.00 pg/mL; H-BNP, BNP > 161.00 pg/mL); BNP tertiles were derived based on the distribution within the study population, with cut points defined using sample-based tertile stratification. (**K**) The relationship between serum LCN2 levels and BNP level. Data were analyzed using independent sample *t* test (**A, B, H, I**) or a 1-way ANOVA followed by Tukey's post hoc analysis (**C, D, J**). Correlation was analyzed using Spearman's rank correlation analysis (**E-G, K**). Abbreviations: T2DM, type 2 diabetes mellitus; DKD, diabetes kidney disease; CAD, coronary artery disease; LCN2, lipocalin-2; Scr, serum creatinine; eGFR, estimated glomerular filtration rate; UACR, urine albumin-to-creatinine ratio; L-BNP, the low BNP group (lower tercile, < 41.60 pg/mL); M-BNP, the middle BNP group (median tercile, 41.60-161.00 pg/mL); H-BNP, the high BNP group (upper tercile, > 161.00 pg/mL).

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Table 3. Multivariate linear regression analysis to determine the factors associated with LCN2 in patients with T2DM.

Variables	Unstandardized B-coefficient	SE	Standardized B-coefficient	*P*
LCN2 (ng/dL) (n = 562) (1)				
eGFR (mL/min per 1.73 m^2)	-0.013	0.001	-0.454	< 0.001
Age (y)	-0.007	0.003	-0.106	0.046
Male	0.124	0.070	0.072	0.074
Duration of diabetes (y)	0.002	0.004	0.018	0.658
SBP (mm Hg)	0.001	0.003	0.024	0.633
DBP (mm Hg)	-0.004	0.004	-0.050	0.342
BMI (kg/m^2)	-0.004	0.010	-0.019	0.643
HbA1C (%)	0.036	0.015	0.099	0.017
UA (mmol/L)	0.000	0.000	0.030	0.503
LDL-C (mmol/L)	-0.076	0.034	-0.090	0.025
TG (mmol/L)	-0.025	0.020	-0.050	0.227
LCN2 (ng/dL) (n = 565) (1)				
Scr (μmol)	0.013	0.001	0.572	< 0.001
Age (y)	-0.001	0.003	-0.023	0.605
Male	-0.055	0.064	-0.032	0.391
Duration of diabetes (y)	0.002	0.003	0.017	0.648
SBP (mm Hg)	0.002	0.002	0.032	0.472
DBP (mm Hg)	-0.004	0.004	-0.048	0.319
BMI (kg/m^2)	-0.004	0.009	-0.018	0.637
HbA1C (%)	0.022	0.014	0.059	0.113
UA (mmol/L)	-8.568E-05	0.000	-0.010	0.804
LDL-C (mmol/L)	-0.058	0.031	-0.068	0.063
TG (mmol/L)	-0.034	0.019	-0.069	0.069
LCN2 (ng/dL) (n = 403) (1)				
UACR (mg/g)	0.000	0.000	0.247	< 0.001
Age (y)	0.004	0.003	0.079	0.189
Male	0.130	0.073	0.090	0.076
Duration of diabetes (y)	0.002	0.004	0.020	0.716
SBP (mm Hg)	-0.002	0.003	-0.047	0.461
DBP (mm Hg)	-0.003	0.005	-0.045	0.515
BMI (kg/m^2)	-0.005	0.010	-0.024	0.654
HbA1C (%)	0.017	0.015	0.057	0.270
UA (mmol/L)	0.001	0.000	0.195	0.000
LDL-C (mmol/L)	-0.060	0.035	-0.085	0.086
TG (mmol/L)	-0.026	0.020	-0.069	0.184
LCN2 (ng/dL) (n = 417) (1)				
BNP (pg/mL)	0.000	0.000	0.671	< 0.001
Age (y)	0.000	0.003	0.003	0.939

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Table 3 continued. Multivariate linear regression analysis to determine the factors associated with LCN2 in patients with T2DM.

Variables	Unstandardized B-coefficient	SE	Standardized B-coefficient	*P*
Male	0.131	0.072	0.068	0.070
Duration of diabetes (y)	0.002	0.004	0.023	0.545
SBP (mm Hg)	0.002	0.003	0.030	0.497
DBP (mm Hg)	-0.006	0.004	-0.069	0.133
BMI (kg/m^2)	-0.004	0.009	-0.016	0.678
HbA1C (%)	0.034	0.015	0.084	0.024
UA (mmol/L)	0.001	0.000	0.118	0.002
LDL-C (mmol/L)	-0.052	0.033	-0.057	0.117
TG (mmol/L)	-0.020	0.020	-0.038	0.319

Enter method was used for multivariate linear regression analysis. (1): Adjusted for age, sex, duration of diabetes, BP, BMI, HbA1C, UA, LDL-C and TG. Abbreviations: T2DM, type 2 diabetes mellitus; LCN2, lipocalin-2; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1C, glycated hemoglobin; Scr, serum creatinine; UA, uric acid; eGFR, estimated glomerular filtration rate; UACR, urine albumin-to-creatinine ratio; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; BNP, B-type natriuretic peptide.

Table 4. Logistic regression analysis to determine elevated serum LCN2 was an independent risk factor for DKD or CAD in patients with T2DM.

	Model 1	Model 2
Outcome variable: DKD	/	/
OR (95% CI)	5.921 (3.887 to 9.019)	3.772 (2.325 to 6.120)
P	< 0.001	< 0.001
Outcome variable: CAD	/	/
OR (95% CI)	5.710 (3.671 to 8.881)	3.188 (1.975 to 5.144)
P	< 0.001	< 0.001

Model 1 was unadjusted for confounding factors. Model 2 was adjusted for age, sex, duration of diabetes, BMI, SBP, DBP, HbA1C, UA, LDL-C, and TG, with CAD and DKD included reciprocally as covariates depending on the outcome. Serum LCN2 was analyzed as a continuous variable in regression models. ORs were expressed per unit increase in serum LCN2 concentration (ng/dL).
Abbreviations: T2DM, type 2 diabetes mellitus; DKD, diabetes kidney disease; CAD, coronary artery disease; LCN2, lipocalin-2; OR, odds ratio; CI, confidence interval.

recombinant LCN2 protein. LCN2 treatment resulted in a concentration-dependent increase in interleukin (IL)-6 and tumor necrosis factor (TNF)-α mRNA expression in both cell types (**Figure 5C, 5D**).

Discussion

To date, a number of studies have explored the association between DKD and CAD [2,7,9,13,35], while the underlying mechanisms remain unclear. To our knowledge, this is the first study to investigate a potential mechanistic link between these conditions using a noninvasive clinical index in a T2DM cohort. This investigation is particularly relevant within the framework of CKM syndrome, which highlights the interconnected pathophysiology linking the heart, kidney, and metabolic system.

We first demonstrated that the presence of DKD significantly increased the risk of CAD. By further assessing DKD severity using eGFR and UACR, we confirmed that both the occurrence and increased severity of DKD were independent risk factors for CAD, consistent with prior epidemiological observations [6,36,37]. A key finding was that serum LCN2 levels were significantly elevated in T2DM patients with either DKD

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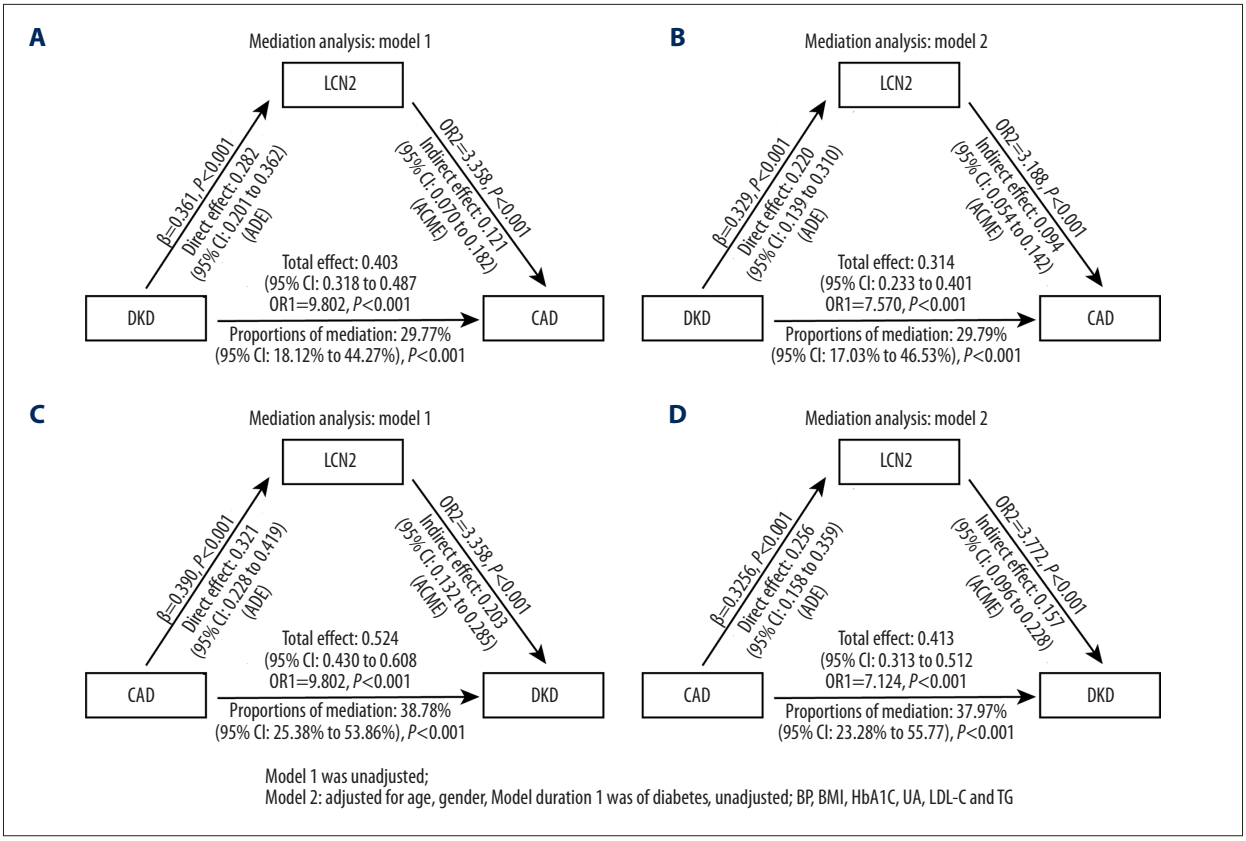

Figure 4. The potential mediated role of LCN2 in association between DKD and CAD. (**A, B**) Mediate effect of serum LCN2 in association between DKD and CAD after unadjusted (**A**) and adjusted confounding factors (**B**). (**C, D**) Reverse mediation analysis evaluating the role of serum LCN2 in the association between CAD and DKD after unadjusted (**C**) and adjusted confounding factors (**D**). The average causal mediation effect (ACME), direct effect (ADE), and total effect are presented. The proportion mediated (%) represents the percentage of the total effect explained by LCN2.The mediator model was fitted using linear regression, and the outcome model using logistic regression. Adjustments included age, gender, duration of diabetes, BP, BMI, HbA1C, UA, LDL-C, and TG. Abbreviations: DKD, diabetes kidney disease; CAD, coronary artery disease; LCN2, lipocalin-2.

or CAD and showed close correlation with DKD severity and BNP level. This aligns with previous reports implying the roles of LCN2 in acute kidney injury and membranous nephropathy [18], as well as in coronary artery disease and myocardial infarction [14,38]. Importantly, elevated serum LCN2 was identified as an independent risk factor for both DKD and CAD. Mediation analysis suggested that increased serum LCN2 levels may partially mediate the bidirectional association between DKD and CAD, accounting for approximately 30% to 38% of this relationship. This finding provides a potential clue for understanding the bidirectional organ crosstalk central to CKM syndrome. Animal and cell experiments supported these clinical results, demonstrating concurrent upregulation of LCN2 in the cardiac and renal tissues of diabetic mice. Furthermore, recombinant LCN2 increased the mRNA expression of pro-inflammatory factors in H9c2 and HK-2 cells. Our findings may extend the current understanding of the cardiorenal axis in DM by suggesting that LCN2 may be involved as a potential linking factor between renal and cardiovascular complications in patients with T2DM, thereby contributing to the pathophysiological model of CKM syndrome.

As mentioned above, the development of CAD in patients with T2DM and DKD is driven in part by shared cardiometabolic risk factors, including hypertension, diabetes, and dyslipidemia [9,13]. Furthermore, several hypothesized mechanisms link DKD and CAD, leading to characteristic changes in the vasculature and the heart. These include inflammation, oxidative stress, activation of the renin-angiotensin-aldosterone system, mineral and bone disorders, and accumulation of uremic toxins [39]. Our study provides another possible mechanism that links DKD and CAD within the integrated framework of CKM syndrome.

Previous studies have partially clarified the relationship between LCN2 and CKD. For example, in patients with CKD, renal expression as well as urinary and serum LCN2 levels were elevated [17,18,20,40]. Studies demonstrated that genetic deletion of LCN2 delayed CKD progression in mice, suggesting that LCN2

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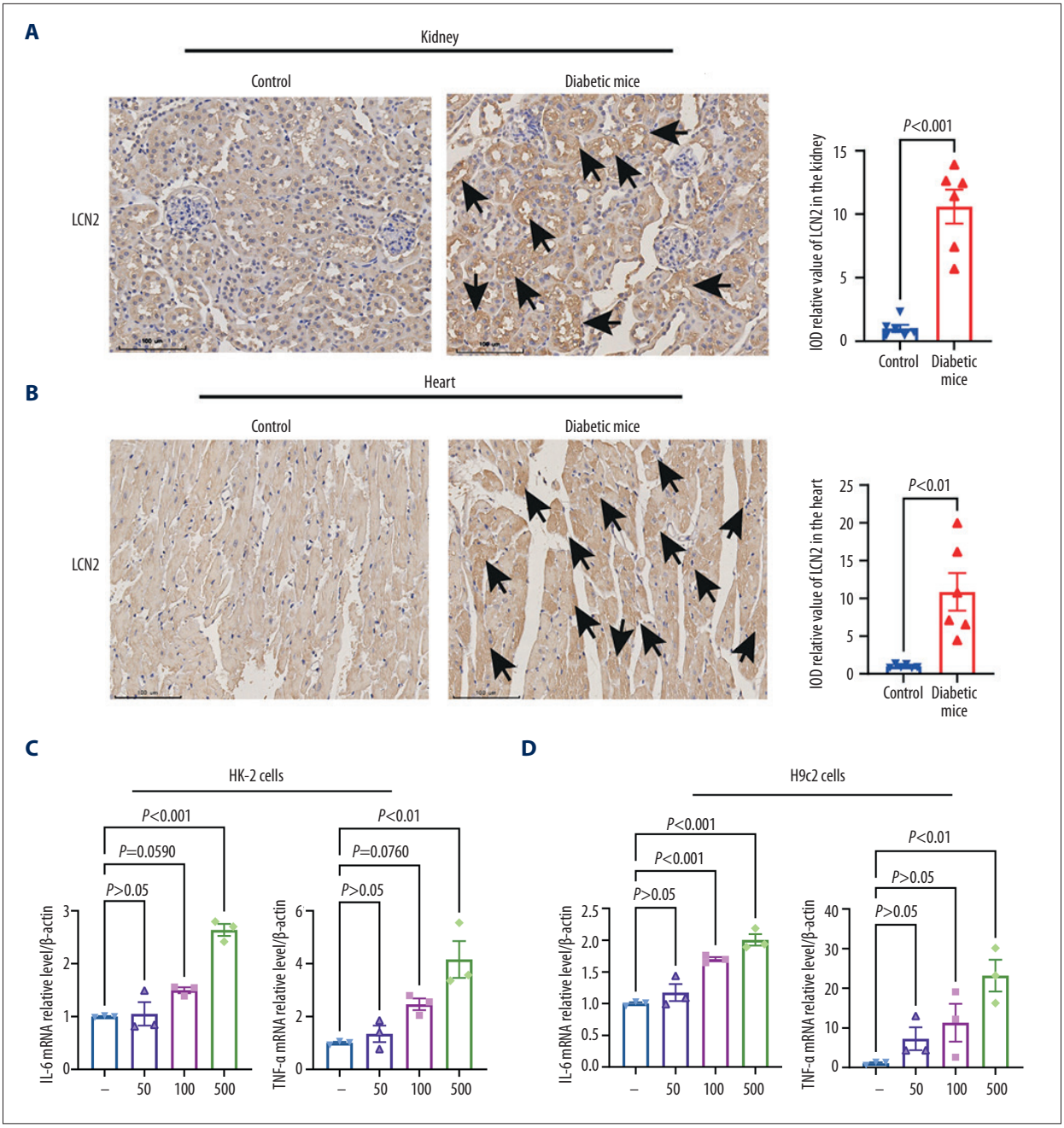

Figure 5. The association between LCN2 and enhanced pro-inflammatory responses in cardiomyocytes and renal tubular cells. (**A**) Comparison of renal LCN2 expression between control and HFD-STZ-induced diabetic mice (n = 6 for each group). Scale bar, 100 μm. (**B**) Comparison of cardiac LCN2 expression between control and HFD-STZ-induced diabetic mice (n = 6 for each group). Scale bar, 100 μm. (**C, D**) Effect of varying concentrations of LCN2 protein on inflammatory factor mRNA levels in HK-2 (**C**) and H9c2 (**D**) cells (n = 3 for each group). Data were analyzed using independent sample *t* test (**A, B**) a one-way ANOVA followed by Dunnett's post hoc analysis (**C, D**). Abbreviations: LCN2, lipocalin-2; HFD, high fat diet; STZ, streptozotocin.

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was not merely a biomarker but a potential driver of CKD progression [21,22]. Beyond the kidney, LCN2 has been suggested to be involved in cardiovascular disease, a core component of CKM syndrome. First, Hemdahl et al [33] reported that LCN2 can form a complex with matrix metalloproteinase-9 (MMP-9), thereby inhibiting MMP-9 degradation and enhancing its proteolytic activity, which contributed to the development of vulnerable atherosclerotic plaques. Sung et al [41] observed a marked upregulation of myocardial LCN2 in mouse ischemic hearts compared with sham-operated controls, and administration of recombinant LCN2 protein exacerbated cardiomyocyte death and myocardial dysfunction. In line with these findings, Song et al [42] showed that holo-Lcn2 enhanced mitochondrial reactive oxygen species generation and impaired mitochondrial oxidative phosphorylation in cultured adult rat cardiomyocytes. More recently, Xu et al [29] revealed that LCN2 expression was significantly elevated in the hearts of diabetic mice, and its overexpression aggravated palmitic acid–induced apoptosis and inflammatory responses in H9c2 cells. All of this suggests that LCN2 may play roles in the development of both CKD and cardiovascular diseases.

Consistent with previous studies, we found that serum LCN2 levels were significantly elevated and served as an independent risk factor in patients with T2DM complicated with DKD or CAD. Notably, bidirectional mediation analysis revealed that increased serum LCN2 partially mediated the statistical association between DKD and CAD in these patients, offering a quantifiable link within the CKM continuum. Supporting these clinical observations, animal experiments demonstrated concurrent upregulation of LCN2 in the cardiac and renal tissues of diabetic mice. In vitro, recombinant LCN2 protein upregulated the mRNA expression of IL-6 and TNF-α in HK-2 and H9c2 cells, suggesting that LCN2 may be associated with the pro-inflammatory link between renal and cardiac complications, a key pathophysiological feature of CKM syndrome [10,11]. We therefore speculate that kidney-derived LCN2 in the circulation may be associated with pro-inflammatory effects on cardiac tissues; conversely, cardiac-derived LCN2 may also be associated with renal inflammatory responses. Although these clinical associations are compelling, the present study does not fully establish a mechanistic basis for LCN2 in bridging DKD and CAD. Therefore, further investigation using relevant rodent models or primary cell cultures is warranted to clarify the precise mediating role of LCN2 in the context of CKM syndrome.

This study has several limitations. First, all participants were recruited from a single clinical center, which may limit the generalizability of our findings and could introduce selection bias. The study was exploratory in nature and no formal a priori power calculation was performed. Second, mediation analysis in a cross-sectional design is based on a counterfactual statistical framework and does not establish causal pathways. Furthermore, direct assessment of local LCN2 concentrations in coronary and renal arteries was not feasible due to the difficulty in obtaining arterial blood samples from these vascular beds. Taken together, given the observational nature of this study, all findings should be interpreted as associative rather than causal. Although our experimental findings provide supportive biological context, they do not establish that LCN2 is necessary or sufficient to drive cardiorenal interactions in vivo. Future studies, particularly prospective cohort studies and mechanistic investigations using primary cardiomyocytes or renal proximal tubular epithelial cells in combination with well-established rodent models of CKM syndrome, are warranted to validate these findings and to clarify the role of LCN2 in cardiorenal interactions.

Conclusions

Our findings demonstrate that LCN2 may represent a clinically relevant biomarker and a potential mediator linking DKD and CAD in T2DM. Clinically, serum LCN2 elevation is an independent risk factor for both conditions and statistically mediates their bidirectional relationship. Experimentally, LCN2 is concurrently upregulated in heart and kidney tissues under diabetic stress and was associated with increased pro-inflammatory responses in cardiomyocytes and renal tubular cells. This study thus suggests that LCN2 may represent a shared pathological factor, providing potential insights into the organ crosstalk underlying CKM syndrome, and may serve as a candidate for cardiorenal risk stratification and a potential target for future therapeutic investigation.

Data Availability

The datasets generated and/or analyzed during the present study are not publicly available but are available from the corresponding author on reasonable request.

Acknowledgements

We acknowledge all medical staff involved in the diagnosis and treatment of type 2 diabetes mellitus in participating centers, and all the patients involved in this study.

Ethics statement

The study protocol was approved by the Ethics Committee of the Shanghai Fifth People's Hospital, Fudan University (2022133) and all participants gave written informed consents. The study was conducted in accordance with the Declaration of Helsinki.

Declaration of Figures' Authenticity

All figures submitted have been created by the authors who confirm that the images are original with no duplication and have not been previously published in whole or in part.

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Abbreviations

T2DM, type 2 diabetes mellitus; **DKD**, diabetes kidney disease; **CAD**, coronary artery disease; **HFD**, high fat diet; **STZ**, streptozotocin; **LCN2**, lipocalin-2; **BMI**, body mass index; **BP**, blood pressure; **HbA1C**, glycated hemoglobin; **ALT**, alanine aminotransferase; **Scr**, serum creatinine; **UA**, uric acid; **eGFR**, estimated glomerular filtration rate; **UACR**, urine albumin-to-creatinine ratio; **BNP**, B-type natriuretic peptide; **TG**, triglyceride; **TC**, total cholesterol; **HDL-C**, high-density lipoprotein cholesterol; **LDL-C**, low-density lipoprotein cholesterol

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