



Associations of Plasma Glucagon Levels with Estimated Glomerular Filtration Rate, Albuminuria and Diabetic Kidney Disease in Patients with Type 2 Diabetes Mellitus

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Background: Type 2 diabetes mellitus (T2DM) is characterized by elevated fasting glucagon and impaired suppression of postprandial glucagon secretion, which may participate in diabetic complications. Therefore, we investigated the associations of plasma glucagon with estimated glomerular filtration rate (eGFR), albuminuria and diabetic kidney disease (DKD) in T2DM patients.

Methods: Fasting glucagon and postchallenge glucagon (assessed by area under the glucagon curve [AUC_{glu}]) levels were determined during oral glucose tolerance tests. Patients with an eGFR <60 mL/min/1.73 m² and/or a urinary albumin-to-creatinine ratio (UACR) ≥30 mg/g who presented with diabetic retinopathy were identified as having DKD.

Results: Of the 2,436 recruited patients, fasting glucagon was correlated with eGFR and UACR ($r=-0.112$ and $r=0.157$, respectively; $P<0.001$), and AUC_{glu} was also correlated with eGFR and UACR ($r=-0.267$ and $r=0.234$, respectively; $P<0.001$). Moreover, 31.7% ($n=771$) presented with DKD; the prevalence of DKD was 27.3%, 27.6%, 32.5%, and 39.2% in the first (Q1), second (Q2), third (Q3), and fourth quartile (Q4) of fasting glucagon, respectively; and the corresponding prevalence for AUC_{glu} was 25.9%, 22.7%, 33.7%, and 44.4%, respectively. Furthermore, after adjusting for other clinical covariates, the adjusted odds ratios (ORs; 95% confidence intervals) for DKD in Q2, Q3, and Q4 versus Q1 of fasting glucagon were 0.946 (0.697 to 1.284), 1.209 (0.895 to 1.634), and 1.521 (1.129 to 2.049), respectively; the corresponding ORs of AUC_{glu} were 0.825 (0.611 to 1.114), 1.323 (0.989 to 1.769), and 2.066 (1.546 to 2.760), respectively. Additionally, when we restricted our analysis in patients with glycosylated hemoglobin <7.0% ($n=471$), we found fasting glucagon and AUC_{glu} were still independently associated with DKD.

Conclusion: Both increased fasting and postchallenge glucagon levels were independently associated with DKD in T2DM patients.

Keywords: Diabetes mellitus, type 2; Diabetic nephropathies; Glucagon

INTRODUCTION

Diabetic kidney disease (DKD), with a decreased glomerular filtration rate and increased albuminuria as its main clinical

characteristics [1], is the most prevalent chronic microvascular disease associated with diabetes [2]. DKD is the major cause of end-stage renal disease (ESRD) worldwide [3], and patients with DKD have a significantly increased risk of cardiovascular

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and all-cause mortality before they reach ESRD [4]. Moreover, the presence of DKD at baseline can independently predict major adverse cardiovascular events and other microvascular complications in patients with type 2 diabetes mellitus (T2DM) [5]. Thus, ongoing studies worldwide are attempting to investigate how pathological factors and conditions affect DKD to interfere with its incidence and progression in patients with T2DM.

Patients with T2DM manifest an impaired regulation of glucagon secretion, which leads, importantly, to hyperglycaemia. Specifically, T2DM is characterized by elevated fasting plasma glucagon levels and impaired suppression of glucagon secretion in α -cells following oral source of hyperglycaemia or even paradoxically elevated [6-8]. Abnormally elevated plasma glucagon levels, also termed hyperglucagonemia, may contribute to the progression of diabetes by increasing hepatic glucose production and exacerbating systemic hyperglycaemia, as well as inhibiting the insulin signalling pathway [9,10]. These pathophysiological changes due to hyperglucagonemia may, in turn, take part in the occurrence and progression of diabetes-related complications, such as DKD. Li et al. [11] demonstrated that long-term exposure to hyperglucagonemia could induce early kidney injury phenotypes of T2DM in mice. Wang et al. [12] showed that T2DM patients with nephropathy exhibited increased plasma glucagon levels. However, there is a lack of comprehensive studies on the associations of plasma glucagon levels with kidney function and DKD with large sample sizes of T2DM patients.

In the present study, we comprehensively investigated the associations of fasting plasma glucagon levels and postchallenge plasma glucagon levels with estimated glomerular filtration rate (eGFR), albuminuria and the presence of DKD in a large-scale sample of patients with T2DM.

METHODS

Patient recruitment

Patients with T2DM who visited the outpatient department or were admitted as inpatients to the Department of Endocrinology, Affiliated Hospital 2 of Nantong University between 2014 and 2020 were consecutively recruited in this study. The study flowchart is displayed in Supplementary Fig. 1. During the recruitment stage, the major inclusion criteria were as follows: (1) met the T2DM diagnostic criteria released by the American Diabetes Association in 2011 [13]; (2) aged 20 to 75 years; and (3) underwent serum creatinine and cystatin C measurement

and urinary albumin-to-creatinine ratio (UACR) evaluation. We also excluded patients with any of the following conditions: (1) type 1 diabetes mellitus, including testing positive for diabetes-associated autoantibodies; (2) history of cancer, especially in the urinary system; (3) chronic viral hepatitis and hepatic cirrhosis; (4) abnormal thyroid function; (5) connective tissue diseases; (6) recent administration of steroid hormones; (7) surgeries associated with the kidneys, urinary bladder and excretory passages; (8) primary glomerular disease such as glomerulonephritis; (9) urinary tract infection, such as pyelonephritis; (10) severe cardiovascular and cerebrovascular diseases, such as myocardial infarction requiring revascularization, heart failure and stroke with sequelae; (11) the use of sodium-glucose cotransporter-2 inhibitors (SGLT-2Is); and (12) ESRD requiring renal replacement therapy. Finally, a total of 2,436 T2DM patients with complete data were pooled for analysis. The performance of our study complied with the principles of the Declaration of Helsinki. Our study received ethics approval from the Medical Ethics Committee at the Affiliated Hospital 2 of Nantong University (2014YX012), with written informed consent received from all patients.

Data collection

When the patients visited the Department of Endocrinology, they were interviewed and examined to collect clinical data, including data on medical histories, diabetic duration, age, sex, height, weight, body mass index (BMI), systolic/diastolic blood pressure (SBP/DBP), and prescribed medications. The contemporaneous biochemical and imaging data were extracted from the medical records in the hospital information platform. The medication prescriptions focused on glucose-lowering therapies such as intervention by lifestyle alone, insulin injections, insulin secretagogues, α -glucosidase inhibitors (AGIs), metformin, thiazolidinedione (i.e., pioglitazone), glucagon-like peptide-1 receptor agonists (GLP-1RAs), dipeptidyl peptidase-4 inhibitors (DPP-4Is), and SGLT-2Is, and other recent medications such as antihypertensive drugs, anticoagulants, statins, and steroid hormones. Past medical histories included other self-reported types of diabetes, hypertension history, chronic hepatitis, and hepatic cirrhosis (i.e., hepatitis B or C virus infection), inflammation in the urinary system (i.e., pyelonephritis and cystitis), surgeries in the urinary system (kidneys, urinary bladder, and excretory passages), a history of cancer (especially in the urinary system), severe cardiovascular and cerebrovascular diseases (i.e., myocardial infarction requiring revascular-

ization, heart failure of New York Heart Association functional class III or IV, and stroke with sequelae), thyroid dysfunction (i.e., hyperthyroidism and hypothyroidism), etc.

DKD assessment

Fasting venous blood samples were also drawn for the measurement of serum creatinine and cystatin C, and eGFR was calculated by the combined creatinine-cystatin C equation from Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) (2012) [14]. Urinary albumin and creatinine were determined to calculate the UACR. Patients with an eGFR <60 mL/min/1.73 m² and/or a UACR ≥30 mg/g [15] who presented with diabetic retinopathy [16] were identified as having DKD. Diabetic retinopathy was identified by one of the following: (1) a history of diabetic retinopathy; (2) previously received photocoagulation or vitrectomy therapy for diabetic retinopathy; and (3) received nonmydriatic digital fundus photographs to screen for diabetic retinopathy (TOPCON, TRC-NW400). The photographs were analysed by experienced ophthalmologists to identify diabetic retinopathy, and fluorescein angiography was performed on the equivocal image.

Evaluation of plasma glucagon levels and other biomarkers during the oral glucose tolerance test

All patients were guided to consume their usual stable diet for three days before the oral glucose tolerance test (OGTT) and to suspend all glucose-lowering therapies one day before the OGTT. In the early morning on the test day, venous blood samples were obtained at fasting (0 minute) and at 30, 60, 120, and 180 minutes after 75-g glucose loading for the synchronous detection of glucose, C-peptide, and glucagon levels. Insulin sensitivity was derived from the homeostasis model assessment of insulin resistance using C-peptide ($IS_{HOMA_{CP}} = 22.5 / (\text{fasting glucose} \times \text{fasting C-peptide})$) [17]. Endogenous β -cell function was assessed by the area under the C-peptide curve (AUC_{CP}) [18,19]. Postchallenge glucose levels were assessed by the area under the glucose curve (AUC_{glu}). Fasting plasma glucagon levels were determined, and postchallenge plasma glucagon levels were assessed by the area under the glucagon curve (AUC_{gla}) during the OGTT.

Laboratory tests

The level of urinary albumin was detected with the immunoturbidimetry method (Image 800; Beckman Coulter, Brea, CA, USA), and urinary creatinine was detected with the enzymatic method (Model 7600; Hitachi, Tokyo, Japan).

Regarding the biochemical parameters during the OGTT, glucose levels were detected with the oxidase method (Model 7600; Hitachi), serum C-peptide levels were detected with the chemiluminescence method (DxI 800; Beckman Coulter), and plasma glucagon levels were detected with the radioimmunoassay method in an automated γ -counter (GC-1200; USTC Zonkia, Hefei, China). On the test day, fasting venous blood samples were also obtained from all patients for the detection of clinical biochemical indices. Serum creatinine (using the enzymatic method), cystatin C (using the latex-enhanced immunoturbidimetric assay method), uric acid (UA), triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were detected with an automated biochemical analyser (Model 7600; Hitachi). The glycosylated hemoglobin (HbA1c) levels were detected with an ion exchange-based HPLC method (D-10; Bio-Rad, Hercules, CA, USA).

Statistical analyses

Clinical variables are displayed for all patients with T2DM and for two subgroups, those with and without DKD, and are expressed as mean \pm standard deviation for normally distributed data, as the medians (interquartile ranges) for skewed continuous data and as the frequencies (percentages) for categorical data. If a variable was skewed, it would receive a logarithm transformation for further statistical analysis.

We used Student's *t*-tests and the chi-squared test to detect differences in continuous data and categorical data, respectively, between the two subgroups with and without DKD. Corresponding test statistics, including *F* values and χ^2 values, were also provided.

Bivariate correlations of plasma glucagon levels with eGFR and UACR were analysed by means of Pearson's correlation tests, and scatter plots for these correlations are graphically displayed. Moreover, to show the degree of influence of plasma glucagon levels on eGFR and UACR, we used multivariable linear regression analyses to detect the mean differences (B; 95% confidence interval [CI]) in eGFR or UACR among the patients in different plasma glucagon level quartiles, with the first quartile (Q1) set as the reference value.

Furthermore, to investigate the impact of fasting or postchallenge glucagon levels on the risk of DKD in patients with T2DM, we used two multivariate logistic regression analyses to explore the odds ratios (ORs [95% CIs]) for DKD in different

levels of fasting or postchallenge glucagon. The first regression model explored the associations of the second (Q2), third (Q3), and fourth quartiles (Q4) of fasting plasma glucagon levels with DKD relative to that of the first quartile (Q1). The second regression model explored the associations of Q2, Q3, and Q4 of postchallenge glucagon levels (AUC_{glu}) with DKD relative to Q1. The potential confounding factors were adjusted in the models. SPSS for Windows standard version 19.0 (IBM Co., Armonk, NY, USA) was used to input and analyse the data, and statistical significance was identified when the P value was less than 0.05.

RESULTS

Table 1 summarizes the clinical characteristics of the recruited patients for all patients and for the two subgroups with and without DKD. Of the recruited patients with T2DM, 31.7% ($n=771$) presented with DKD, and patients with DKD were characterized by increased fasting plasma glucagon, AUC_{glu} and AUC_{cp} and decreased IS_{HOMAcp} when compared to patients without DKD, but AUC_{cp} values were comparable between the two subgroups. Clinical variables such as age, the proportion of females, BMI, SBP, DBP, diabetic duration, the prevalence of hypertension, statin use, TG, TC, UA, HbA1c, and postchallenge 2-hour glucose were higher in patients with DKD than in patients without DKD, while HDL-C, serum albumin and haemoglobin were lower in patients with DKD than in patients without DKD. LDL-C did not exhibit any difference between the subgroups with and without DKD. Comparisons of the use of glucose-lowering therapies showed that patients with DKD tended to have a higher frequency of insulin treatments and a lower frequency of metformin use than patients without DKD, whereas the use of lifestyle intervention, insulin secretagogues, pioglitazone, AGIs, DPP-4Is, and GLP-1RAs was comparable between the two subgroups.

Pearson's correlation tests showed that fasting plasma glucagon was negatively correlated with eGFR ($r=-0.112$, $P<0.001$) and positively correlated with UACR ($r=0.157$, $P<0.001$), and AUC_{glu} was also negatively correlated with eGFR ($r=-0.267$, $P<0.001$) and positively correlated with UACR ($r=0.234$, $P<0.001$). Scatter plots for these correlations are graphically displayed in Figs 1 and 2. Moreover, we used multivariable linear regression analyses to show the degree of the influence of plasma glucagon levels on eGFR and UACR in all T2DM patients. After adjusting for other clinical risk factors, the adjusted mean

changes (B) in eGFR and $\log_{10}UACR$ of the patients in Q4 versus Q1 of fasting plasma glucagon were -5.087 mL/min/ 1.73 m² (95% CI, -7.383 to -2.790) and 0.124 mg/g (95% CI, 0.076 to 0.171), respectively (Table 2). The corresponding mean changes in eGFR and $\log_{10}UACR$ of the patients in Q4 versus Q1 of AUC_{glu} were -13.89 mL/min/ 1.73 m² (95% CI, -16.18 to -11.60) and 0.188 mg/g (95% CI, 0.140 to 0.236), respectively (Table 3).

Of the recruited patients with T2DM, 31.7% ($n=771$) presented with DKD, and the prevalence of DKD increased from 27.3%, 27.6%, and 32.5% to 39.2% from Q1, Q2, and Q3 to Q4 for fasting plasma glucagon (P for trend <0.001) (Table 4). Compared with the patients in Q1 of fasting plasma glucagon, those in Q2, Q3, and Q4 of fasting plasma glucagon experienced ORs of DKD of 1.016 (95% CI, 0.789 to 1.308), 1.283 (95% CI, 1.003 to 1.640), and 1.722 (95% CI, 1.353 to 2.191), respectively (P for trend <0.001) (Table 4). Moreover, after adjusting for glycaemic status and other clinical risk factors via multivariate logistic regression analysis, the corresponding ORs for DKD of patients in Q2, Q3, and Q4 versus those in Q1 of fasting plasma glucagon were 0.946 (95% CI, 0.697 to 1.284), 1.209 (95% CI, 0.895 to 1.634), and 1.521 (95% CI, 1.129 to 2.049), respectively (P for trend <0.001) (Table 2).

Furthermore, of the recruited patients with T2DM, the prevalence of DKD increased from 25.9%, 22.7%, and 33.7% to 44.4% from Q1, Q2, and Q3 to Q4 of AUC_{glu} (P for trend <0.001) (Table 4). Compared with the patients in Q1 of AUC_{glu} , those in Q2, Q3, and Q4 of AUC_{glu} experienced ORs of DKD of 0.841 (95% CI, 0.647 to 1.093), 1.454 (95% CI, 1.134 to 1.863), and 2.283 (95% CI, 1.791 to 2.910), respectively (P for trend <0.001) (Table 4). Moreover, after adjusting for glycaemic status and other clinical risk factors via multivariate logistic regression analysis, the corresponding ORs for DKD of patients in Q2, Q3, and Q4 versus those in Q1 of AUC_{glu} were 0.825 (95% CI, 0.611 to 1.114), 1.323 (95% CI, 0.989 to 1.769), and 2.066 (95% CI, 1.546 to 2.760), respectively (P for trend <0.001) (Table 4).

Additionally, when we restricted our analysis to the recruited patients with HbA1c $<7.0\%$ ($n=471$), we found that fasting glucagon and AUC_{glu} levels were still independently associated with kidney damage indices (eGFR and UACR), and increased fasting glucagon and AUC_{glu} levels were still risk factors for DKD in these patients with well-controlled T2DM (Supplementary Tables 1-4, Supplementary Figs. 2 and 3).

Table 1. Clinical characteristics of all patients with and without DKD

Variable	Type 2 diabetes mellitus			<i>F</i> / <i>x</i> ² value	<i>P</i> value
	Total	Without DKD	With DKD		
Number	2,436	1,665	771	-	-
Age, yr	55.7±11.4	54.7±11.3	57.8±11.4	6.298	<0.001
Female sex	1,078 (44.3)	705 (42.3)	373 (48.4)	7.784	0.005
BMI, kg/m ²	25.44±3.63	25.18±3.56	26.01±3.71	5.299	<0.001
SBP, mm Hg	135.2±17.2	133.0±16.3	139.9±18.3	9.351	<0.001
DBP, mm Hg	79.4±10.5	79.1±10.1	80.3±11.0	2.691	0.007
Diabetic duration, yr	5.57±4.45	5.07±4.08	6.64±5.01	8.189	<0.001
Glucose-lowering therapies					
Diabetes drug-free	257 (10.6)	185 (11.1)	72 (9.3)	1.755	0.185
Insulin treatments	611 (25.1)	388 (23.3)	223 (28.9)	8.858	0.003
Insulin-secretagogues	1,226 (50.3)	842 (50.6)	384 (49.8)	0.123	0.725
Metformin	1,335 (54.8)	943 (56.6)	392 (50.8)	7.141	0.008
Pioglitazone	733 (30.1)	514 (30.9)	219 (28.4)	1.524	0.217
AGIs	584 (24.0)	385 (23.1)	199 (25.8)	2.088	0.148
DPP-4Is	642 (26.4)	423 (25.4)	219 (28.4)	2.442	0.118
GLP-1RAs	65 (2.7)	42 (2.5)	23 (3.0)	0.431	0.512
Hypertension	1,089 (44.7)	636 (38.2)	453 (58.8)	90.086	<0.001
Statin medication	954 (39.2)	618 (37.1)	336 (43.6)	9.238	0.002
TG, mmol/L	1.71 (1.11–2.77)	1.61 (1.04–2.59)	1.94 (1.29–3.18)	5.197	<0.001
TC, mmol/L	4.46±1.11	4.40±0.95	4.57±1.38	3.187	<0.001
HDL-C, mmol/L	1.09±0.29	1.10±0.29	1.06±0.29	-2.408	0.016
LDL-C, mmol/L	2.59±0.79	2.57±0.77	2.63±0.84	1.509	0.131
Serum UA, μmol/L	300.4±96.5	289.7±91.6	323.7±102.5	8.207	<0.001
Serum albumin, g/L	39.2±4.1	39.4±3.9	38.7±4.4	-3.779	<0.001
Haemoglobin, g/L	137.5±16.1	138.4±15.4	135.7±17.0	-4.272	<0.001
IS _{HOMAcp}	4.08 (2.30–8.05)	4.22 (2.44–8.76)	3.77 (2.04–7.10)	-4.057	<0.001
AUC _{cp} , ng/mL·hr	12.4±5.9	12.3±5.7	12.6±6.3	1.494	0.135
AUC _{glu} , mmol/L·hr	41.8±9.2	41.4±9.3	42.5±8.9	2.624	0.009
HbA1c, %	8.73±1.34	8.68±1.37	8.82±1.28	2.189	0.029
Postchallenge 2-hr glucose, mmol/L	15.51±1.74	15.33±1.75	15.90±1.65	7.610	<0.001
Fasting glucagon, pg/mL	126.5±30.2	124.3±28.6	131.4±32.7	5.446	<0.001
AUC _{glu} , pg/mL·hr	403.7±175.0	380.2±149.4	454.5±211.7	9.939	<0.001
eGFR, mL/min/1.73 m ²	100.8±24.6	104.6±21.4	92.7±28.8	-11.358	<0.001
UACR, mg/g	20.1 (14.5–37.0)	16.2 (13.0–20.9)	60.6 (39.3–140.0)	19.799	<0.001
Log ₁₀ UACR, mg/g	1.44±0.44	1.21±0.16	1.93±0.46	57.842	<0.001

Values are presented as mean ± standard deviation, number (%), or median (interquartile range). Student's *t*-test and chi-square test were applied to detect differences in their corresponding type of data, and corresponding test statistics (*F* and *x*² values) were also provided.

DKD, diabetic kidney disease; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; AGI, α-glucosidase inhibitor; DPP-4I, dipeptidyl peptidase-4 inhibitor; GLP-1RA, glucagon-like peptide-1 receptor agonist; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; UA, uric acid; IS_{HOMAcp}, homeostasis model assessment of insulin resistance using C-peptide; AUC_{cp}, area under the C-peptide curve; AUC_{glu}, area under the glucose curve; HbA1c, glycosylated hemoglobin; AUC_{glu}, area under the glucagon curve; eGFR, estimated glomerular filtration rate; UACR, urinary albumin-to-creatinine ratio.

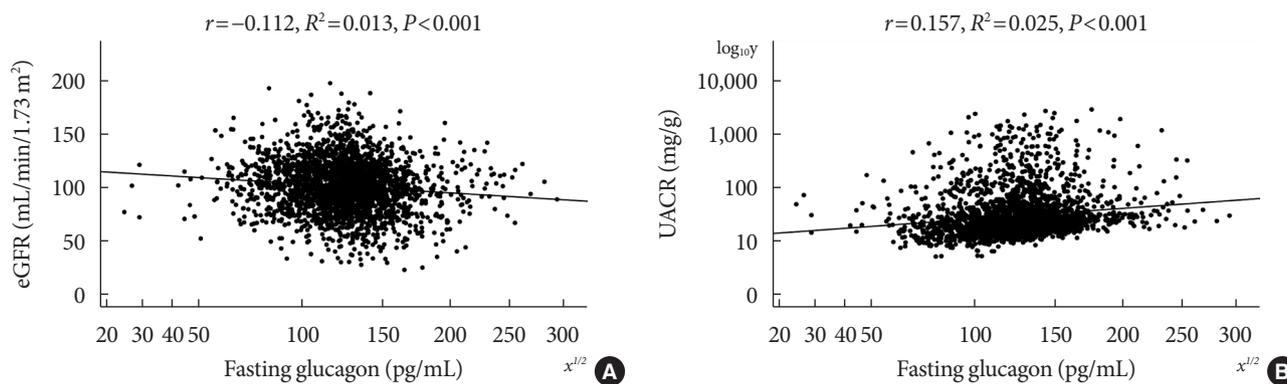


Fig. 1. Scatter plot for the correlations of fasting glucagon with kidney damage indices in all type 2 diabetes mellitus patients. (A) Estimated glomerular filtration rate (eGFR). (B) Urinary albumin-to-creatinine ratio (UACR).

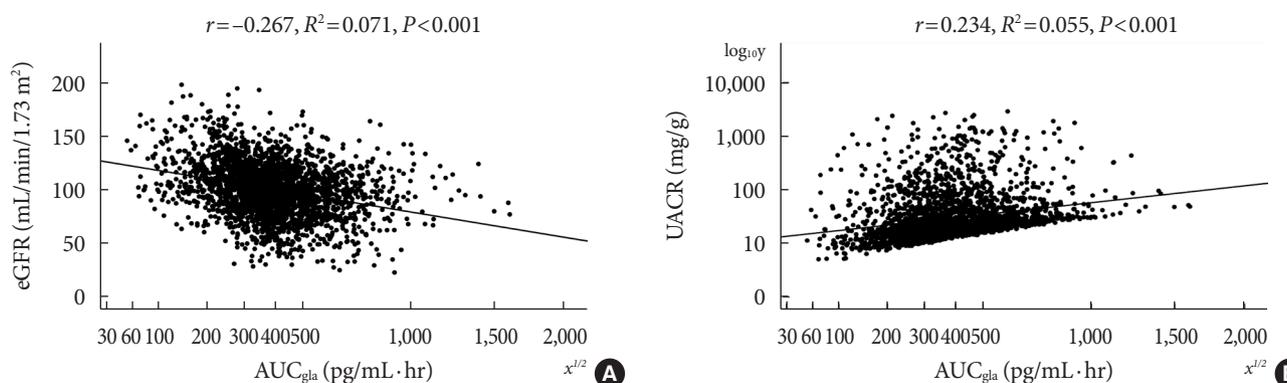


Fig. 2. Scatter plot for the correlations of area under the glucagon curve (AUC_{gla}) with kidney damage indices in all type 2 diabetes mellitus patients. (A) Estimated glomerular filtration rate (eGFR). (B) Urinary albumin-to-creatinine ratio (UACR).

DISCUSSION

In the present study, we explored the associations of fasting and postchallenge plasma glucagon levels with the presence of DKD in a large sample of patients with T2DM ($n=2,436$). The strengths of our study are as follows: first, both increased fasting plasma glucagon levels and postchallenge plasma glucagon levels are revealed to be mildly correlated with decreased eGFR and increased UACR; second, 31.7% ($n=771$) of the recruited patients presented with DKD, and the prevalence of DKD showed an increasing trend with increasing quartiles of either fasting plasma glucagon levels or postchallenge plasma glucagon levels; third, patients from Q4 of fasting plasma glucagon levels exhibited a 1.521-fold (95% CI, 1.129 to 2.049) increased DKD risk compared with those in Q1 after adjustment for other confounders, and the corresponding adjusted DKD risk for patients from Q4 of postchallenge plasma glucagon levels was

2.066-fold (95% CI, 1.546 to 2.760); and fourth, when we restricted our analysis to the recruited patients with HbA1c <7.0% ($n=471$), we found fasting and postchallenge plasma glucagon levels were still independently associated with DKD. Collectively, hyperglucagonemia in the top quartile of fasting or postchallenge glucagon levels is associated with an increased risk of DKD in patients with T2DM independent of glucose levels.

Ongoing work worldwide is seeking markers of and risk factors for DKD. The incidence and progression of DKD is in the background of diabetes; therefore, glycaemic disorders may be the initiator and driving force for DKD, and other various cardiovascular risks may be the promoters for DKD [20]. The irreversible risk factors included older age, sex, ethnicity, a family history of DKD and a long duration of diabetes, and the reversible factors included smoking, hypertension, dyslipidaemia, hypercoagulation, insulin resistance, albuminuria, anaemia,

Table 2. Mean differences (B [95% CI]) in kidney damage indices among the quartiles of fasting glucagon in all T2DM patients

Model	Q1	Q2	Q3	Q4	P value for trend
Fasting glucagon, pg/mL	25.0–108.2	108.3–125.5	125.6–141.7	141.8–209.3	-
Number	609	602	613	612	-
eGFR, mL/min/1.73 m ²	104.27 ± 23.58	102.91 ± 25.68	99.96 ± 24.85	96.30 ± 23.64	<0.001
Model 0	0-reference	-1.359 (-4.114 to -1.397)	-4.310 (-7.053 to -1.567)	-7.968 (-10.71 to -5.224)	<0.001
Model 1	0-reference	-2.340 (-4.703 to -0.006)	-3.871 (-6.193 to -1.549)	-7.618 (-9.947 to -5.289)	<0.001
Model 2	0-reference	-1.245 (-3.527 to 1.036)	-2.133 (-4.424 to 0.158)	-5.204 (-7.517 to -2.891)	<0.001
Model 3	0-reference	-1.167 (-3.435 to 1.101)	-1.984 (-4.259 to 0.290)	-5.087 (-7.383 to -2.790)	<0.001
Log ₁₀ UACR, mg/g	1.365 ± 0.424	1.401 ± 0.442	1.443 ± 0.435	1.545 ± 0.445	<0.001
Model 0	0-reference	0.035 (-0.014 to 0.085)	0.078 (0.029 to 0.127)	0.180 (0.131 to 0.229)	<0.001
Model 1	0-reference	0.037 (-0.010 to 0.083)	0.076 (0.030 to 0.123)	0.174 (0.127 to 0.220)	<0.001
Model 2	0-reference	0.023 (-0.024 to 0.070)	0.065 (0.018 to 0.112)	0.125 (0.078 to 0.173)	<0.001
Model 3	0-reference	0.021 (-0.026 to 0.068)	0.063 (0.016 to 0.110)	0.124 (0.076 to 0.171)	<0.001

Values are presented as range, number, mean ± standard deviation, or mean difference (95% confidence interval).

Model 0: crude; Model 1: adjusted for age, sex, diabetic duration, body mass index, systolic blood pressure, diastolic blood pressure, statins medication, and hypertension; Model 2: additionally adjusted for lipid profiles, serum uric acid, serum albumin, hemoglobin, homeostasis model assessment of insulin resistance using C-peptide, area under the C-peptide curve, area under the glucose curve, postchallenge 2-hour glucose, and glycosylated hemoglobin; Model 3: additionally adjusted for glucose-lowering therapies.

CI, confidence interval; T2DM, type 2 diabetes mellitus; eGFR, estimated glomerular filtration rate; UACR, urinary albumin-to-creatinine ratio.

Table 3. Mean differences (B [95% CI]) in kidney damage indices among the quartiles of AUC_{glu} in all T2DM patients

Model	Q1	Q2	Q3	Q4	P value for trend
AUC _{glu} , pg/mL·hr	53–289	290–372	373–484	485–1,603	-
Number	599	617	609	611	-
eGFR, mL/min/1.73 m ²	113.95 ± 25.85	100.70 ± 20.72	96.09 ± 23.57	92.90 ± 22.89	<0.001
Model 0	0-reference	-13.26 (-15.89 to -10.64)	-17.85 (-20.49 to -15.22)	-21.05 (-23.68 to -18.42)	<0.001
Model 1	0-reference	-10.87 (-13.11 to -8.629)	-14.73 (-16.99 to -12.47)	-16.69 (-18.95 to -14.22)	<0.001
Model 2	0-reference	-9.698 (-11.91 to -7.490)	-12.63 (-14.87 to -10.38)	-13.93 (-16.22 to -11.63)	<0.001
Model 3	0-reference	-9.918 (-11.21 to -7.718)	-12.58 (-14.82 to -10.35)	-13.89 (-16.18 to -11.60)	<0.001
Log ₁₀ UACR, mg/g	1.341 ± 0.461	1.344 ± 0.372	1.495 ± 0.473	1.575 ± 0.409	<0.001
Model 0	0-reference	0.004 (-0.045 to 0.052)	0.154 (0.106 to 0.203)	0.234 (0.186 to 0.283)	<0.001
Model 1	0-reference	0.007 (-0.040 to 0.053)	0.149 (0.102 to 0.195)	0.221 (0.174 to 0.268)	<0.001
Model 2	0-reference	0.008 (-0.038 to 0.054)	0.121 (0.074 to 0.168)	0.190 (0.141 to 0.238)	<0.001
Model 3	0-reference	0.009 (-0.038 to 0.055)	0.119 (0.072 to 0.167)	0.188 (0.140 to 0.236)	<0.001

Values are presented as range, number, mean ± standard deviation, or mean difference (95% confidence interval).

Model 0: crude; Model 1: adjusted for age, sex, diabetic duration, body mass index, systolic blood pressure, diastolic blood pressure, statins medication, and hypertension; Model 2: additionally adjusted for lipid profiles, serum uric acid, serum albumin, hemoglobin, homeostasis model assessment of insulin resistance using C-peptide, area under the C-peptide curve, area under the glucose curve, postchallenge 2-hour glucose, and glycosylated hemoglobin; Model 3: additionally adjusted for glucose-lowering therapies.

CI, confidence interval; AUC_{glu}, area under the glucagon curve; T2DM, type 2 diabetes mellitus; eGFR, estimated glomerular filtration rate; UACR, urinary albumin-to-creatinine ratio.

Table 4. ORs (95% CIs) of DKD according to the quartiles of plasma glucagon levels in all T2DM patients

Variable	Q1	Q2	Q3	Q4	P value for trend
Fasting glucagon, pg/mL	25.0–108.2	108.3–125.5	125.6–141.7	141.8–209.3	-
Number	609	602	613	612	-
DKD	166 (27.3)	166 (27.6)	199 (32.5)	240 (39.2)	<0.001
Model 0	1–reference	1.016 (0.789–1.308)	1.283 (1.003–1.640)	1.722 (1.353–2.191)	<0.001
Model 1	1–reference	1.029 (0.791–1.340)	1.305 (1.010–1.686)	1.770 (1.375–2.278)	<0.001
Model 2	1–reference	0.957 (0.706–1.298)	1.218 (0.902–1.644)	1.532 (1.138–2.061)	0.001
Model 3	1–reference	0.946 (0.697–1.284)	1.209 (0.895–1.634)	1.521 (1.129–2.049)	0.001
AUC _{glu} , pg/mL · hr	53–289	290–372	373–484	485–1603	-
Number	599	617	609	611	-
DKD	155 (25.9)	140 (22.7)	205 (33.7)	271 (44.4)	<0.001
Model 0	1–reference	0.841 (0.647–1.093)	1.454 (1.134–1.863)	2.283 (1.791–2.910)	<0.001
Model 1	1–reference	0.849 (0.645–1.116)	1.476 (1.137–1.915)	2.290 (1.772–2.959)	<0.001
Model 2	1–reference	0.823 (0.611–1.110)	1.331 (0.996–1.778)	2.071 (1.552–2.763)	<0.001
Model 3	1–reference	0.825 (0.611–1.114)	1.323 (0.989–1.769)	2.066 (1.546–2.760)	<0.001

Values are presented as range, number (%), or odds ratio (95% confidence interval).

Model 0: crude; Model 1: adjusted for age, sex, diabetic duration, body mass index, systolic blood pressure, diastolic blood pressure, statins medication, and hypertension; Model 2: additionally adjusted for lipid profiles, serum uric acid, serum albumin, hemoglobin, homeostasis model assessment of insulin resistance using C-peptide, area under the C-peptide curve, area under the glucose curve, postchallenge 2-hour glucose, and glycosylated hemoglobin; Model 3: additionally adjusted for glucose-lowering therapies.

OR, odds ratio; CI, confidence interval; DKD, diabetic kidney disease; T2DM, type 2 diabetes mellitus; AUC_{glu}, area under the glucagon curve.

mia, etc. [21-23]. These risk factors subsequently may account for the immune-inflammatory responses in the progression of DKD. In addition, in the immune-inflammatory response pathways, innate immune cells (mast cells, etc.), proinflammatory cytokines (interleukin-1, -6, -18, and tumour necrosis factor- α , etc.), chemokines (monocyte chemoattractant protein-1, etc.), cell adhesion molecules, and growth factor (transforming growth factor- β , etc.) have been implicated in the pathogenesis of DKD [24-26]. These responses ultimately may lead to irreversible changes in the function and structure of DKD, such as interstitial fibrosis and glomerulosclerosis in the kidney [27]. Moreover, impaired regulation of glucagon secretion and islet β -cell dysfunction are associated with DKD. Deficiency in endogenous β -cell function assessed by AUC_{cp} is linked to an increased prevalence of nephropathy in the population with T2DM [18], and T2DM patients with nephropathy exhibited abnormally elevated glucagon levels [12]. In our present study, hyperglucagonemia at both fasting status and postchallenge status was found to be associated with an increased prevalence of DKD in patients with T2DM, independent of glycaemic controls and other clinical risk factors.

Hyperglucagonemia is at least partially responsible for disorders of glucose, lipid and amino acid metabolism, which, in turn, take part in the pathophysiology of diabetes, obesity and nonalcoholic fatty liver disease [28] and ultimately lead to various adverse health outcomes. Relative hyperglucagonemia, under the condition of deficiency in insulin secretion, is involved in the pathogenesis of glycaemic variability measured by continuous glucose monitoring and serum hyperglycaemia in diabetes [29-31]. These glycaemic disorders are closely associated with the morbidity and mortality of cardiovascular diseases [32,33]. Hyperglucagonemia could also contribute to adverse cardiovascular outcomes through lipid metabolism pathways. Ali et al. [34] demonstrated that glucagon administration could increase long-chain acylcarnitines by triggering an increase in β -oxidation in ischaemic mouse hearts, which, in turn, promoted cardiac ischaemic injury in mice and led to cardiomyocyte apoptosis. In contrast, Gao et al. [35] showed that the blockade of the glucagon receptor (GCGR) can ameliorate cardiac hypertrophy and fibrotic remodelling and attenuate exacerbation in heart failure after myocardial infarction in mice. In addition, the blockade of GCGR-mediated improvement in AMP-activated protein kinase (AMPK)-induced lipid oxidation and protection against cardiac dysfunction is independent of the correction of glycaemia [36]. Moreover, elevat-

ed levels of glucagon were correlated with serum levels of total amino acids and several non-branched-chain amino acids, such as glutamine and asparagine [37]. These abnormal changes in glutamine and asparagine are likely metabolic markers for chronic kidney disease [38,39]. Additionally, acute elevation of plasma glucagon has been documented to directly induce glomerular hyperfiltration—a hallmark of early glomerular injury in T2DM [40], and long-term exposure to hyperglucagonemia could also induce kidney injury phenotypes of T2DM [11]. In population-based studies, T2DM patients with nephropathy exhibited increased glucagon levels at different times after glucose loading [12], and fasting glucagon levels were elevated linearly with the progression of kidney dysfunction in patients with T2DM [41]. In our present study of 2,436 patients with T2DM, we used diabetic retinopathy to aid in the diagnosis of DKD on the basis of decreased eGFR and/or increased UACR, which may avoid the limitations of eGFR and albuminuria. We found that increased fasting and postchallenge glucagon levels were not only associated with the kidney dysfunction index—a decline in eGFR—but also associated with damage to the glomerular filtration barrier—increased albuminuria. Patients from the upper quartile of fasting glucagon levels or postchallenge glucagon levels experienced an increased risk for DKD compared to those from the lower quartile. Hyperglucagonemia in the upper quartile of fasting or postchallenge glucagon levels accounts for an increased risk of DKD in patients with T2DM.

Some underlying mechanisms have been suggested in the relationship between hyperglucagonemia and DKD. In the context of diabetes, disorder in glycaemic metabolism is a critical and initiating risk factor for DKD. Elevated fasting glucagon levels contribute to fasting hyperglycaemia by increasing glycolysis and gluconeogenesis in the liver, and impaired suppression of glucagon secretion may contribute to postprandial hyperglycaemia [30]. The subsequent fluctuations between fasting and postprandial hyperglycaemia may contribute to glycaemic variability throughout the day. All these features in glycaemic disorders can lead to the incidence and progression of DKD [42,43]. Moreover, hyperglucagonemia can also cause kidney damage in a glucose-independent manner. Increased plasma glucagon levels, via direct overactivation of GCGR-mediated signalling pathways, induce the phosphorylation of extracellular signal-regulated kinase 1/2 (Erk1/2), finally stimulating mesangial cell (MC) growth and hypertrophy and resulting in glomerular mesangial expansion and extracellular matrix deposition

[44]. Glucagon can induce the phosphorylation of ERK1/2 in MCs by three signalling pathways, including the cAMP-dependent protein kinase A (PKA) pathway, the phospholipase C (PLC)/inositol 1,4,5-trisphosphate (IP3)/Ca²⁺ pathways [45], and the pathway that stimulates angiotensin II (Ang II) formation and interacts with the Ang II receptor [46]. Furthermore, GCGR antagonists are expected to block the metabolic and growth effects of hyperglucagonemia in T2DM [44,47].

Several limitations of the study must be addressed. First, this study is based on the analysis of cross-sectional data, which may not necessarily indicate the existence of a causal link between increased glucagon levels and DKD. A follow-up study is needed to compensate for this limitation. Second, our present study is influenced by the heterogeneities of T2DM, in that the levels of insulin and glucagon may be influenced by glucose-lowering therapies. We adjusted glucose-lowering therapies during the analyses and tried to compensate for the limitation as much as possible. Third, the gold standard of the diagnosis of DKD is based on renal biopsy, but it is too labour-intensive and expensive to be applied in large clinical studies. Fourth, we assessed only glucagon secretion of α -cells in response to oral glucose, and postprandial glucagon levels may be different when responding to the different nutrients.

In conclusion, both increased fasting plasma glucagon levels and postchallenge plasma glucagon levels following an OGTT were independently associated with DKD in patients with T2DM.

SUPPLEMENTARY MATERIALS

Supplementary materials related to this article can be found online at <https://doi.org/10.4093/dmj.2020.0149>.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

AUTHOR CONTRIBUTIONS

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Acquisition, analysis, or interpretation of data: L.L.S., H.Y.H., L.H.Z., F.X., D.M.Z., X.L.Z., T.C., X.Q.W.

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