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Relationship Between Fasting Plasma Glucagon Level and Renal Function—A Cross-Sectional Study in Individuals With Type 2 Diabetes

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Background and Aim: The kidney is the main site for glucagon clearance. However, a recent study showed that hyperglucagonemia in patients with end-stage renal disease might not be caused by full-length intact glucagon. Additionally, the relationship between glucagon and renal function in early-stage chronic kidney disease (CKD) has not yet been characterized. We studied the association of fasting glucagon with renal function across a wide range of glomerular filtration rates (GFRs) in participants with type 2 diabetes.

Participants and Methods: 326 participants with type 2 diabetes and renal function spanning CKD stage 1 to 5 were included in the present cross-sectional study. Fasting full-length plasma glucagon was quantified using a newly developed ELISA (Mercodia AB, Uppsala, Sweden).

Results: The fasting plasma glucagon level was elevated linearly from CKD stage 1 to 5 [from a median of 2.5 pM (interquartile range, 1.4 to 4.7) in CKD 1 to a median of 8.3 pM (interquartile range, 5.9 to 12.8) in CKD 5; P for trend < 0.0001], from as early as CKD stage 2 compared with that in stage 1 (Bonferroni-corrected $P < 0.0001$). The estimated GFR and homeostatic model of assessment–insulin resistance were the main determinants of the fasting glucagon level. These explained 14.3% and 10.3% of the glucagon variance, respectively. Albuminuria was not associated with fasting glucagon after adjustment for estimated GFR.

Conclusions: Fasting full-length glucagon was elevated linearly with the deterioration in renal function in individuals with type 2 diabetes, even in those with early CKD. In addition to renal function, insulin sensitivity was also a main determinant of glucagon variance.

Freeform/Key Words: glucagon, renal function, chronic kidney disease, type 2 diabetes mellitus

Abbreviations: ACR, albumin/creatinine ratio; BMI, body mass index; CKD, chronic kidney disease; CV, coefficient of variation; DPP4, dipeptidyl peptidase 4; eGFR, estimated glomerular filtration rate; ESRD, end-stage renal disease; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; HOMA2-IR, homeostatic model assessment, version 2, insulin resistance; IQR, interquartile range.

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Glucagon plays important roles in glucose, lipid, and amino acid metabolism. It is also involved in the regulation of energy expenditure, adipose tissue browning, and food intake behavior [1–3]. Hence, study of the determinants of glucagon turnover might gain insight in the pathophysiology of metabolic diseases. However, measurement of plasma glucagon is challenging. This is because the post-translational processing of proglucagon results in a number of peptide hormones. These structurally similar peptides can cross-react with C-terminal glucagon anti-sera, which had been used in most of the immunoassays used in early studies [4, 5]. In contrast, the concentration of circulating glucagon is low. Thus, an assay with high sensitivity is required to accurately quantify plasma glucagon level, especially for the small changes under various pathophysiological status or after pharmacologic interventions [5].

The new ELISA based on monoclonal antibodies on both ends of the glucagon peptide (Mercodia AB, Uppsala, Sweden) offers a reliable tool to quantify the plasma full-length glucagon level with high sensitivity and specificity [6]. Not unexpectedly, measurement using the new ELISA sometimes resulted in outcomes that differed from those using C-terminal glucagon, even from the same study. For example, plasma glucagon measured using the assay from R&D Systems (Minneapolis, MN) showed that 48-week treatment with liraglutide enhanced postchallenge glucagonemia [7]. However, the same plasma samples measured by the assay from Mercodia AB showed no substantial differences in glucagon levels between the liraglutide and placebo arms [8]. In another study, plasma glucagon measured using the Mercodia ELISA and a radioimmunoassay from Merck Millipore (Darmstadt, Germany) also gave different results from the same plasma samples [9], highlighting the importance of assay performance on study outcomes.

The kidney has been considered the main site for glucagon clearance [10, 11]. The concentration of fasting glucagon has been shown to be elevated by two- to fourfold in patients with end-stage renal disease (ESRD) [12, 13]. However, to the best of our knowledge, the relationship between glucagon level and renal function in the early stages of chronic kidney disease (CKD) has not been characterized. In contrast, early studies of the association of glucagonemia with renal function were mainly conducted in nondiabetic populations [12–14]. Data from the diabetic population are still needed because of the overt dysregulation of glucagon secretion in patients with diabetes and the high prevalence of CKD in this population [15–17]. More importantly, a recent study using the new glucagon ELISA showed that hyperglucagonemia in patients with ESRD might not be caused by full-length intact glucagon [6]. These findings support the need to study the association between hyperglucagonemia and renal function impairment further.

In the present cross-sectional study, the primary aim was to examine the relationship between the fasting plasma glucagon level and renal function across a wide spectrum of CKD stages using the newly developed immunoassay for full-length glucagon. The secondary aim was to study the clinical and biochemical determinants of fasting glucagon variance above and beyond renal function in individuals with type 2 diabetes.

1. Participants and Methods

A. Study of Macroangiopathy and Microvascular Reactivity in Type 2 Diabetes Cohort

Participant recruitment for SMART2D (study of macroangiopathy and microvascular reactivity in type 2 diabetes) cohort has been previously described [18]. In brief, 2057 outpatients with type 2 diabetes were recruited from a regional hospital and a primary care polyclinic in the northern region of Singapore from August 2011 to March 2014. All outpatients with type 2 diabetes who were 21 to 90 years old were considered potential candidates for enrollment. The participants were identified by usage of hypoglycemic medications in their medical records or referral from their attending physician. Type 2 diabetes was diagnosed using the following criteria: fasting plasma glucose ≥7.0 mmol/L, random plasma glucose ≥11.1 mmol/L, or hemoglobin A1c (HbA1c) ≥47.5 mmol/mol (6.5%). Ascertainment of type 2 diabetes was mainly determined by exclusion of type 1 diabetes and specific types of

diabetes resulting from other causes. Type 1 diabetes was defined as the requirement for sustainable insulin treatment 1 year after diabetes onset. Potential candidates with autoimmune disease or cancer receiving active treatment, those who were pregnant, those with intake of steroids equivalent to ≥5 mg/d of prednisolone, point-of-care test result for fasting glucose <4.5 mM or >15.0 mM, or HbA1c >108 mmol/mol (12%) after phlebotomy and those who could not fulfill the informed consent requirement were excluded from cohort enrollment.

B. Participant Selection for Study of Fasting Glucagon and Renal Function

Only Chinese participants in SMART2D cohort were included in the present study. Participant selection has been illustrated in an online repository [19]. The CKD stage was defined as ≥90 mL/min/1.73 m^2 for stage 1, 60 to 89 mL/min/1.73 m^2 for stage 2, 30 to 59 mL/min/1.73 m^2 for stage 3, 15 to 29 mL/min/1.73 m^2 for stage 4, and ≤15 mL/min/1.73 m^2 for stage 5. Individuals with renal function in CKD stage 3 (n = 133), stage 4 (n = 36), and stage 5 (n = 22) were all included. Of 22 participants with stage 5 CKD (ESRD), 10 were undergoing hemodialysis, 1 was undergoing peritoneal dialysis, and 11 were not receiving dialysis. Among those with stage 1 (n = 552) and stage 2 (n = 296) CKD, 64 and 71 were randomly selected, respectively. The selected and not selected participants in CKD stage 1 and 2 had a similar clinical profile (data provided in an online repository [19]).

The present study was conducted in compliance with principles laid down by the Declaration of Helsinki as revised in 2008. The Singapore National Health Care Group Domain-Specific review board approved the present study. All participants provided written informed consent.

C. Clinical and Biochemical Variables

Smoking status and diabetes duration were self-reported. Information on medication usage was extracted from the electronic medical records. No participants took sodium-glucose cotransporter 2 inhibitor treatment at enrollment. Two participants in the cohort were receiving glucagon-like peptide 1 receptor agonist liraglutide treatment but none were included in the present study. The participants' weight was measured using a calibrated scale, and their height was measured using a stadiometer. The body mass index (BMI) was calculated as the weight in kilograms divided by the height in square meters. The blood pressure was measured three times with the participant in a seated position with 5-minute intervals in between, and the average of three readings was used.

Blood and spot urine specimens were collected in the morning after the participants had fasted overnight. The blood glucose, total cholesterol, high-density lipoprotein (HDL) cholesterol, and triacylglycerol were quantified using enzymatic methods (Roche Cobas Integra 700, Roche Diagnostics, Basel, Switzerland). Low-density lipoprotein cholesterol was calculated using the Friedewald formula. HbA1c was measured using a point-of-care analyzer (DCA Vantage Analyzer, Siemens, Munich, Germany). Creatinine was measured using an enzymatic method that was traceable to the isotope dilution mass spectrometry reference. The glomerular filtration rate was estimated using the CKD-Epidemiology Collaboration formula. Urinary albumin was quantified using a solid phase competitive chemiluminescent immunoassay (Immulite, DPC, Gwynedd, United Kingdom). The albuminuria level is presented as the albumin/creatinine ratio (ACR).

Blood samples for the present study were collected in EDTA-coated tubes and kept at 4°C for <30 minutes before being transferred to the laboratory. The plasma was separated by centrifuge and stored in small aliquots at −80°C. The plasma β- hydroxybutyrate level was measured using a colorimetric assay kit (Cayman Chemical, Ann Arbor, MI) with an intra- and interassay coefficient of variation (CV) of 3.1% and 3.9%, respectively. Plasma c-peptide was measured using an ELISA from Mercodia AB (Uppsala, Sweden) [20]. The intra- and interassay CVs were <5%. Insulin resistance was estimated from the fasting c-peptide and glucose using the homeostatic model assessment, version 2, insulin resistance (HOMA2-IR; available at: www.dtu.ox.ac.uk) [21].

Fasting full-length glucagon was measured in plasma without a previous freeze-thaw cycle. Compared with early assays, which were based on C-terminal anti-sera, the new ELISA (catalog no. 10-1271-01; Mercodia AB) showed only minimal cross-reactivity with glicentin (<0.8%), oxyntomodulin (<4.4%), glucagon-like peptide-1 and its derivatives (<0.1% for all) [6, 22]. The reported sensitivity of this assay is 1 pM, with a reported 4.0% intra-assay CV and 8.3% interassay CV.

D. Statistical Analysis

Data are presented as the mean ± SD, median [interquartile range (IQR)], or proportion, as appropriate. Plasma glucagon, glucagon/glucose ratio, HOMA2-IR, plasma triacylglycerol, β-hydroxybutyrate, and urine ACR were natural log-transformed before further analyses because of their right-skewed distributions.

Differences in the fasting plasma glucagon levels between the male and female participants and those between dialytic and nondialytic ESRD participants were compared using Student *t* tests. Differences in the clinical and biochemical variables across the tertiles of fasting glucagon levels (Table 1) and across the five CKD stages (Table 2) were compared using one-way ANOVA or χ^2 tests, as appropriate. The trend of linearity of the glucagon levels across CKD stages was examined by linear regression, in which the CKD stage was coded as a continuous variable and entered as a dependent variable. The median glucagon level in the participants at each CKD stage was entered as an independent variable. The trend of linearity for the glucagon/glucose ratio across the CKD stages was examined using the same approach.

Table 1. Participant Characteristics Stratified by Fasting Glucagon Tertiles

Characteristic	Tertile 1 (n = 109)	Tertile 2 (n = 109)	Tertile 3 (n = 108)	*P* Value[a]
Glucagon, pmol/L	2.25 (1.55–2.92)	4.62 (3.95–5.32)	9.46 (7.64–13.2)	By design
Fasting blood glucose, mM	7.5 ± 2.3	7.9 ± 2.5	8.5 ± 2.8	0.02[b]
Age, y	63.8 ± 10.0	62.9 ± 10.2	62.4 ± 10.1	0.61
Male sex, %	47.7	60.6	70.4	0.003[b]
Current smoker, %	9.2	10.2	5.6	0.43
Diabetes duration, y	14.5 ± 11.7	17.2 ± 10.4	16.1 ± 8.6	0.16
BMI, kg/m^2	26.5 ± 5.2	26.1 ± 4.4	28.0 ± 5.0	0.01[b]
HbA1c, %	7.3 ± 1.0	7.7 ± 1.2	8.0 ± 1.4	<0.0001[b]
HbA1c, mmol/mol	56.3 ± 7.7	60.7 ± 9.5	63.9 ± 11.2	<0.0001[b]
Blood pressure, mm Hg				
Systolic	145 ± 21	146 ± 21	152 ± 24	0.04[b]
Diastolic	78 ± 10	78 ± 10	80 ± 11	0.21
Lipid profile, mM				
HDL cholesterol	1.36 ± 0.34	1.27 ± 0.35	1.22 ± 0.32	0.01[b]
LDL cholesterol	2.61 ± 0.72	2.73 ± 0.95	2.65 ± 0.90	0.56
Triacylglycerol	1.34 (1.04–1.88)	1.53 (1.15–2.12)	1.85 (1.27–2.63)	<0.0001[b]
HOMA2-IR[c]	1.3 (1.0–2.1)	1.8 (1.2–2.6)	2.3 (1.4–3.3)	<0.0001[b]
eGFR, mL/min/1.73 m^2	73 ± 27	57 ± 27	45 ± 26	<0.0001[b]
ACR, mg/g	26 (6–110)	76 (21–387)	229 (38–1347)	<0.0001[b]
Use of medication, %				
Metformin	78.0	70.4	60.2	0.02[b]
Insulin	26.9	37.6	39.8	0.10
DPP4 inhibitor	5.6	9.2	23.1	<0.001[b]
Sulfonylurea	40.7	46.8	54.6	0.12
Statin	82.6	87.2	84.3	0.64
RAS blocker	64.8	69.7	78.7	0.07

Data presented as mean ± SD or median (IQR).
Abbreviations: LDL, low-density lipoprotein; RAS, renin-angiotensin system.
[a]One-way ANOVA or χ^2 test for among-group comparisons, as appropriate.
[b]Variables with statistically significant differences across glucagon tertiles.
[c]HOMA2-IR was calculated based on fasting glucose and fasting c-peptide levels.

Table 2. Participant Characteristics Stratified by CKD Stage (n = 326)

Characteristic	CKD 1 (n = 64)	CKD 2 (n = 71)	CKD 3 (n = 133)	CKD 4 (n = 36)	CKD 5 (n = 22)	*P* Value[a]
Glucagon, pmol/L	2.52 (1.38–4.66)	3.80 (2.82–6.46)	5.11 (3.55–7.70)	6.18 (3.47–11.7)	8.26 (5.80–12.8)	<0.0001[b]
Age, y	57.5 ± 9.2	62.5 ± 9.9	66.2 ± 9.7	65.3 ± 8.7	58.2 ± 9.7	<0.0001[b]
Male sex, %	46.9	56.3	63.2	69.4	68.2	0.12
Current smoker, %	14.1	8.5	7.6	2.8	4.5	0.32
Diabetes duration, y	10.4 ± 7.8	14.9 ± 11.2	17.5 ± 10.0	22.0 ± 9.7	16.4 ± 9.6	<0.0001[b]
BMI, kg/m^2	26.9 ± 5.4	27.0 ± 4.7	26.7 ± 5.1	26.8 ± 3.8	27.2 ± 4.8	0.98
Fasting glucose, mM	7.8 ± 2.0	7.4 ± 2.4	8.1 ± 2.9	8.5 ± 2.6	8.3 ± 2.7	0.21
HbA1c, %	7.4 ± 1.1	7.6 ± 1.2	7.6 ± 1.2	8.3 ± 1.5	7.7 ± 1.4	0.04[b]
HbA1c, mmol/mol	57.4 ± 8.5	59.6 ± 9.4	59.6 ± 9.4	67.2 ± 12.1	60.7 ± 11.0	0.04[b]
Blood pressure, mm Hg						
Systolic	140 ± 17	146 ± 12	147 ± 20	157 ± 21	167 ± 31	<0.0001[b]
Diastolic	79 ± 9	80 ± 10	77 ± 10	77 ± 9	81 ± 17	0.29
Lipid profile, mM						
HDL cholesterol	1.36 ± 0.35	1.32 ± 0.37	1.25 ± 0.32	1.25 ± 0.33	1.20 ± 0.34	0.17
LDL cholesterol	2.75 ± 0.88	2.46 ± 0.60	2.60 ± 0.79	2.96 ± 1.12	2.99 ± 1.22	0.01[b]
Triacylglycerol	1.35 (0.96–1.73)	1.35 (1.04–2.06)	1.73 (1.20–2.25)	1.63 (1.14–2.76)	2.16 (1.28–2.99)	0.005[b]
HOMA2-IR[c]	1.5 (1.1–2.1)	1.8 (1.3–2.4)	1.9 (1.2–3.0)	1.9 (1.0–3.0)	NA	0.13
eGFR, mL/min/1.73 m^2	101 ± 8	76 ± 9	47 ± 9	22 ± 5	9 ± 4	By design
ACR, mg/g	17 (3–66)	24 (7–98)	114 (25–409)	948 (159–1667)	2376 (1352–5456)	<0.0001[b]
Use of medication, %						
Metformin	87.5	91.5	75.9	11.4	0	<0.0001[b]
Sulfonylurea	48.4	49.3	50.8	30.6	45.5	0.32
Insulin	10.9	25.4	38.3	80.0	40.9	<0.0001[b]
DPP4 inhibitor	9.4	7.0	13.6	33.3	0	0.01[b]
Statin	79.7	83.1	84.2	94.4	90.9	0.32
RAS blocker	53.1	64.3	83.5	80.6	54.5	<0.0001[b]

Data presented as mean ± SD or median (IQR).
Abbreviations: LDL, low-density lipoprotein; RAS, renin-angiotensin system.
[a]One-way ANOVA or χ^2 test for among-group comparisons.
[b]Variables with statistically significant differences across glucagon tertiles.
[c]HOMA2-IR was calculated based on fasting glucose and fasting c-peptide levels.

Stepwise multivariable linear regression model was fitted to study which clinical and biochemical variables were independently associated with the fasting glucagon level. Glucagon (log-transformed) was entered as a dependent variable in the model. Inclusion of the candidate covariates for selection was determined by biological plausibility. Age, sex, BMI, fasting glucose, HbA1c, diabetes duration, systolic blood pressure, HDL cholesterol, triacylglycerol, HOMA2-IR, estimated glomerular filtration rate (eGFR), urinary ACR, sulfonylurea (binary), dipeptidyl peptidase 4 (DPP4) inhibitor (binary), and exogenous insulin (binary) treatment were entered as independent variables. Variables with probability of $F \leq 0.05$ were entered and those with a probability of $F > 0.1$ were removed in the stepping method. The proportion of variance in fasting glucagon explained by the independent variables was estimated using adjusted R^2 (coefficient of determination).

Given that male participants had a substantially greater fasting glucagon level in the present study, which had also been reported in an early study [23], the product of sex × CKD stage was entered as a covariate to examine whether sex modulated the association of fasting glucagon with renal function.

Statistical analysis was performed using SPSS, version 22 (IBM Corp., Armonk, NY). A two-sided $P < 0.05$ level was considered to indicate statistical significance.

2. Results

A. Participant Characteristics

The participants with fasting glucagon levels in the higher tertiles had higher levels of fasting blood glucose, HbA1c, BMI, systolic blood pressure, and triacylglycerol and a lower level of HDL cholesterol. HOMA2-IR, an indicator of insulin sensitivity, was elevated across the

tertiles of glucagon levels. Participants with a higher level of fasting glucagon had a lower eGFR and a higher ACR (Table 1). Also, they were more likely to be receiving DPP4 inhibitor therapy but were less likely to be receiving metformin.

The participants were also stratified by CKD stage to visualize the clinical and biochemical characteristics (Table 2). Those with a lower eGFR were older and had a longer duration of diabetes in general. They had poorer glycemic control and higher levels of systolic blood pressure, low-density lipoprotein cholesterol, and triacylglycerol. The proportion of male sex and the levels of BMI, HDL cholesterol, and HOMA2-IR did not differ significantly across the CKD stages.

B. Fasting Plasma Glucagon Level in Participants with Type 2 Diabetes and CKD

The 5th and 95th percentiles of fasting glucagon concentrations in the participants in the present study were 1.2 and 15.7 pM, respectively. The level of fasting glucagon in the participants with stage 5 CKD (median, 8.3 pM; IQR, 5.8 to 12.8) was 3.2-fold greater than that in those with stage 1 CKD (median, 2.5 pM; IQR, 1.4 to 4.7). No statistically significant differences were found in the fasting glucagon levels between dialytic and nondialytic participants with stage 5 CKD (median, 11.4 pM; IQR, 5.9 to 15.8; vs median, 7.6 pM; IQR, 5.4 to 11.4; $P = 0.17$).

Consistent with early reports [23], male participants had a significantly greater level of fasting glucagon than did the female participants (median, 5.0 pM; IQR, 3.4 to 8.3; vs median, 3.8 pM; IQR, 2.5 to 6.1; $P = 0.004$). Sex did not modulate the association of fasting glucagon with renal function ($P_{\text{interaction}} = 0.10$).

The fasting plasma glucagon level was elevated linearly from CKD stage 1 to 5 (both P_{ANOVA} and $P_{\text{trend of linearity}} < 0.001$; Table 2 and Fig. 1A). *Post hoc* analysis showed that the elevation of fasting glucagon was statistically significant from as early as CKD stage 2 compared with CKD stage 1 (Bonferroni-corrected $P < 0.0001$). The linear trend of glucagon elevation across the CKD stages did not change materially after normalization of the glucagon level to the fasting glucose level (Fig. 1B).

C. Determinants of Fasting Glucagon Variance in Individuals With Type 2 Diabetes

We studied the clinical and biochemical determinants of fasting glucagon variance in participants with CKD stage 1 to 4 (n = 304) because (i) catabolism of c-peptide in the kidney with stage 5 CKD was overtly different from those with stage 1 to 4 CKD and HOMA2-IR was derived from c-peptide in the present study; and (ii) the metabolic dysregulation in participants with stage 5 CKD might be highly heterogeneous owing to the dialysis status.

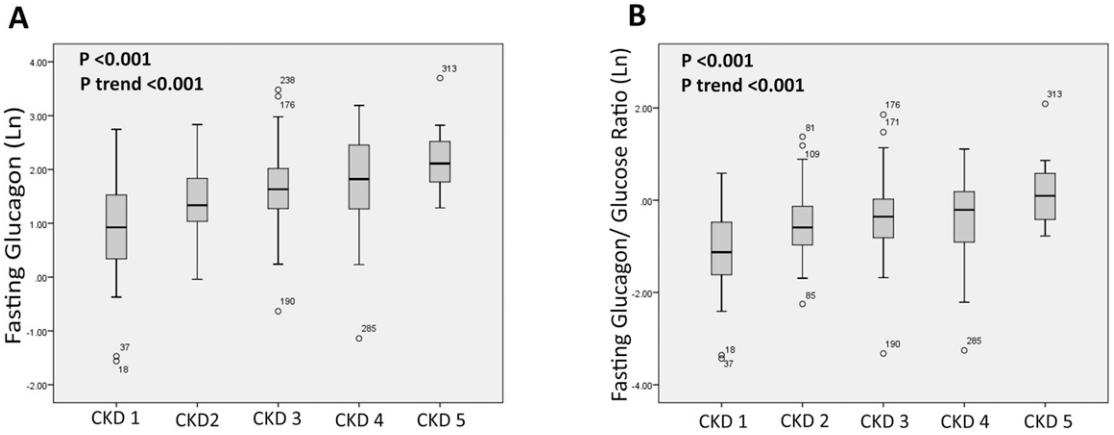

Figure 1. Box plots of (A) fasting glucagon level (natural log-transformed) across CKD stage 1 to 5 and (B) fasting glucagon/glucose ratio across CKD stage 1 to 5.

Stepwise multivariable linear regression model revealed that eGFR, HOMA2-IR, fasting triacylglycerol, sex, and the usage of the DPP4 inhibitor were independently associated with the fasting plasma glucagon level in individuals with type 2 diabetes. These five variables, in combination, might explain 27.5% of the variance in fasting glucagon. DPP4 inhibitor usage was independently associated with a greater level of fasting glucagon. However, the ACR level was not independently associated with the fasting glucagon level after adjustment for the eGFR.

The eGFR and HOMA2-IR values explained 14.3% and 10.3% of the variance in fasting glucagon, individually (Fig. 2). When combined, they explained 22.8% of the glucagon variance. The fasting triacylglycerol level explained 7.7% of the glucagon variance individually. However, it only added 2.1% to the explanation of the glucagon variance compared with eGFR and HOMA2-IR. Furthermore, including DPP4 inhibitor usage and sex in the linear regression model explained an additional 1.8% and 0.8% of the glucagon variance, respectively.

D. Fasting Glucagon Level Correlated With Ketone Bodies in Individuals With Type 2 Diabetes

In consideration that a greater glucagon level might stimulate ketone body production [15, 24], we next examined the association of fasting glucagon with β-hydroxybutyrate, the main component of ketone bodies. Fasting glucagon correlated positively with β-hydroxybutyrate (Pearson $r = 0.19$, $P = 0.001$). On multivariable analysis, β-hydroxybutyrate level was associated with fasting glucagon after adjustment for eGFR and HOMA2-IR ($P = 0.002$). The association was attenuated after further adjustment for the plasma triacylglycerol level ($P = 0.09$).

E. Sensitivity Analysis

The linear trend of glucagon elevation across the CKD stages did not materially change after excluding participants with DPP4 inhibitor usage [19]. Including participants with kidney

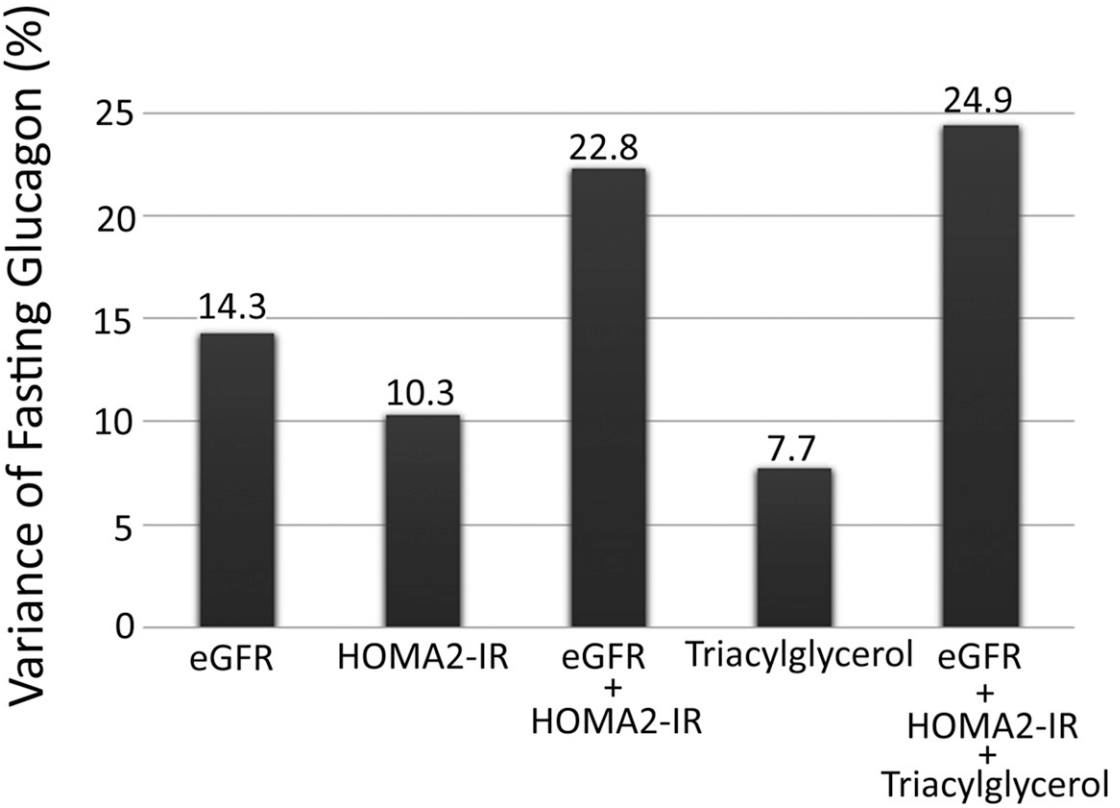

Figure 2. Proportion of variance in fasting glucagon explained by eGFR, HOMA2-IR, and triacylglycerol individually and combined.

function in CKD stage 5 in the multivariable linear regression models also did not materially change the outcomes.

The fasting glucagon level did not correlate with the sample storage time (Pearson $r = -0.01$; $P = 0.84$). Adjustment for the plasma storage time did not alter the associations of the fasting glucagon levels with the clinical and biochemical variables.

3. Discussion

Using the new immunoassay with a high specificity for full-length glucagon, we observed that the fasting glucagon level was elevated linearly across the five CKD stages in individuals with type 2 diabetes, from as early as CKD stage 2. Furthermore, we found that insulin sensitivity was another major determinant of glucagon variance above and beyond renal function in individuals with type 2 diabetes. To the best of our knowledge, the present study might be the first to systematically examine the relationship between the fasting glucagon level and renal function across a wide spectrum of CKD stages.

In our participants with type 2 diabetes and ESRD (CKD stage 5), the absolute concentration of intact glucagon (median, 8.3 pM) was comparable to that observed by Wewer Albrechtsen *et al.* (6) in the nondiabetic ESRD population (~12 pM). The level of fasting glucagon in the ESRD participants in the present study was 3.2-fold greater than that in those with stage 1 CKD. The magnitude of elevation was in agreement with previous studies in the 1970s, which reported a two- to fourfold increase in the fasting glucagon level in ESRD [12, 13]. Therefore, our work using the new immunoassay on full-length intact glucagon has reconfirmed that hyperglucagonemia is a prominent feature of ESRD. Although we could not compare the level of intact glucagon and C-terminal glucagon head to head, it would seem that the absolute concentration of fasting intact glucagon in ESRD participant will be lower than their C-terminal glucagon, which was reported to be ~30 pM [6]. In contrast, we found that the level of fasting glucagon is elevated in a linear pattern across CKD stages 1 to 5, with a substantial increase from as early as CKD stage 2. These data suggest that any future studies of glucagon pathophysiology should consider renal impairment, especially among patients with diabetes owing to the high prevalence of CKD in this population [17].

We found that HOMA2-IR might explain ~9% of the fasting glucagon variance independently of renal function (Fig. 2). The concept of insulin resistance has been extended to α-cells [23]. Mice with α-cell–specific insulin receptor knockout exhibited an enhanced glucagon secretion in response to hypoglycemia and L-arginine stimulation [25], suggesting a role for insulin signaling in the regulation of α-cell glucagon secretion. Two recent independent clinical studies have shown that insulin resistance is accompanied by increased fasting glucagon and delayed glucagon suppression in individuals with normal and impaired glucose regulation [26, 27]. Our observation in participants with type 2 diabetes is agreeable with these early findings in the nondiabetic population. We believe that a greater HOMA2-IR might be a proxy for α-cell insulin resistance. Therefore, the positive association of HOMA2-IR with a fasting glucagon level might indicate the impaired repression of glucagon secretion owing to insulin resistance in α-cells. Additionally, we found that plasma triacylglycerol is associated with fasting glucagon, independent of the eGFR and HOMA2-IR, suggesting that lipid metabolism might, at least, in part, contribute to hyperglucagonemia in patients with type 2 diabetes. Also, insulin resistance occurs in patients with CKD at different stages of kidney impairment [28, 29]. However, the site of insulin resistance secondary to CKD is mainly localized to the skeletal muscle. We could not specifically address the interrelationship among renal function impairment, muscle insulin resistance, and hyperglucagonenima in the present study.

The finding of a greater level of fasting glucagon in those using a DPP4 inhibitor is intriguing. DPP4 can cleave glucagon *in vitro* [30]. However, glucagon does not appear to be a good substrate for DPP4 *in vivo* [6, 31]. In clinical studies, the effect of DPP4 inhibitor intake on fasting glucagon has been inconclusive. In some studies, DPP4 inhibitor usage showed no

effect on fasting glucagon [32]. In another study, the α-cell response to hypoglycemia might even be enhanced by treatment with a DPP4 inhibitor [33]. However, the assay performances were not carefully characterized in these early studies. The discrepancies might also be partly explained by differences in the participant characteristics and study designs. Further studies are warranted to confirm our findings and elucidate the underlying mechanisms.

Hyperglucagonemia in patients with type 2 diabetes and CKD could have clinical implications. A greater glucagon level might prompt the body to access stored nutrients, increase hepatic glucose production from glycogen and amino acids, and promote lipolysis [32, 34]. Therefore, hyperglucagonemia might adversely affect glycemic control in patients with type 2 diabetes and CKD owing to enhanced gluconeogenesis in the liver. In addition, excessive glucagon might have potential adverse effects on amino acid turnover, which contributes to muscle wasting by favoring amino acid catabolism in patients with diabetes and CKD [10, 35, 36]. In addition to its metabolic actions, glucagon also affects kidney function. A greater level of glucagon increases the GFR [37–39], influences solute and fluid handling in the renal tubule [40, 41], and regulates the acid-base balance [42, 43]. In an animal model, long-term infusion of glucagon induced kidney hypertrophy, mesangial expansion, and extracellular matrix deposition [44]. Therefore, the role of hyperglucagonemia in kidney disease development and progression warrants further studies.

The strengths of the present study included employment of a newly developed glucagon immunoassay with high sensitivity and specificity and a well-phenotyped study population with renal function encompassing all five stages of CKD. Nevertheless, our data should be interpreted in the context of several weaknesses. First, this was a cross-sectional study. Therefore, we could not infer causality. Second, we focused on the association of CKD and fasting glucagon in individuals with type 2 diabetes. This group of patients exhibit, not only fasting hyperglucagonemia, but also postprandial glucagon nonsuppression [45]. The relationship among renal function, insulin resistance, and postprandial glucagonemia remains to be elucidated. Also, the findings from our present study might not be readily extrapolated to the populations with type 1 diabetes and those without diabetes; Third, we used HOMA2-IR as a surrogate for insulin sensitivity and eGFR to estimate renal function. Although often used in epidemiological studies, these are not reference standard measurements. Fourth, given the focus of the present study, several other important factors in the regulation of glucagon turnover, such as plasma amino acids, long-chain free fatty acid, and gut hormones were not addressed in the present work and await further studies [32, 46]. Fifth, we did not measure the C-terminal glucagon level and compare the outcomes from new and old assays directly in the same participant. Additionally, although our findings from the participants with type 2 diabetes and CKD were generally in agreement with those from early studies of nondiabetic populations, we could not compare the glucagon levels in the participants with CKD with and without type 2 diabetes.

4. Conclusions

The fasting glucagon level was elevated linearly across the spectrum of CKD stages in individuals with type 2 diabetes, even in those with early CKD. The role of hyperglucagonemia in CKD development and progression and the associated metabolic dysregulation warrants further studies.

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