ORIGINAL ARTICLE

The association of glucagon with disease severity and progression in patients with autosomal dominant polycystic kidney disease: an observational cohort study

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ABSTRACT

Background. Mammalian target of rapamycin (mTOR) inhibitors and ketogenesis have been shown to ameliorate disease progression in experimental autosomal dominant polycystic kidney disease (ADPKD). Glucagon is known to lower mTOR activity and stimulate ketogenesis. We hypothesized that in ADPKD patients, higher endogenous glucagon is associated with less disease severity and progression.

Methods. Data were analysed from 664 Dutch ADPKD patients participating in the Developing Intervention Strategies to Halt Progression of ADPKD observational cohort, including patients ≥18 years of age with an estimated glomerular filtration rate (eGFR) ≥15 mL/min/1.73 m² and excluding patients with concomitant diseases or medication use that may impact the natural course of ADPKD. The association between glucagon and disease severity and progression was tested using multivariate linear regression and mixed modelling, respectively.

Results. The median glucagon concentration was 5.0 pmol/L [interquartile range (IQR) 3.4–7.2] and differed significantly between females and males [4.3 pmol/L (IQR 2.9–6.0) and 6.6 (4.5–9.5), P < 0.001, respectively]. Intrasubject stability of glucagon in 30 patients showed a strong correlation (Pearson’s correlation coefficient 0.893; P < 0.001). Moreover, glucagon showed significant associations with known determinants (sex, body mass index and copeptin; all P < 0.01) and known downstream effects (glucose, haemoglobin A1c and cholesterol; all P < 0.05), suggesting that glucagon was measured reliably. Cross-sectionally, glucagon was associated with eGFR and height-adjusted total kidney volume, but in the opposite direction of our hypothesis, and these lost significance after adjustment for confounders. Glucagon was not associated with an annual decline in kidney function or growth in kidney volume.
Conclusions. These data do not provide evidence for a role of endogenous glucagon as a protective hormone in ADPKD. Intervention studies are needed to determine the relation between glucagon and ADPKD.

Keywords: ADPKD, eGFR, estimated glomerular filtration rate, glucagon, PKD, polycystic kidney disease, TKV, total kidney volume

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is the most common kidney hereditary disorder, with a prevalence of ~1:2500 in the general population [1]. ADPKD is characterized by cyst development and growth in both kidneys, leading to kidney function decline, resulting in kidney failure in the majority of patients before the age of 60 years [1]. Currently there are limited treatment options for ADPKD patients.

Recently it was discovered that one of the key features of ADPKD is a dysregulated glucose metabolism [2]. PKD cells favour aerobic glycolysis, similar to the Warburg effect in cancer cells [2]. These metabolic alterations in PKD cells are mediated by the activation of mammalian target of rapamycin (mTOR) [2]. mTOR inhibitors, such as sirolimus and everolimus, have previously been shown to be renoprotective in experimental PKD [3]. However, the doses needed led to extrarenal toxicity, whereas the use of lower doses in clinical trials was not effective [4]. Alternative treatments aimed at inhibition of mTOR, such as being on a ketogenic and food-restricted diet, also ameliorated disease progression in two animal models of PKD [5, 6].

Glucagon is known to be increased during food restriction, stimulating ketogenesis [7] and inhibiting mTOR [8]. Therefore we hypothesized that glucagon might be protective in ADPKD and that a higher endogenous glucagon concentration will be associated with less disease severity at baseline and less disease progression during follow-up. We tested these associations in a large observational cohort of ADPKD patients.

MATERIALS AND METHODS

Study design and study population

For this study we used the data of the Developing Intervention Strategies to Halt Progression of ADPKD (DIPAK) observational cohort study that was designed to investigate the natural course of ADPKD. Data were collected at the University Medical Centres of Groningen, Leiden, Nijmegen and Rotterdam. The DIPAK cohort consists of 664 ADPKD patients, with currently a median follow-up of 4 years. Inclusion is still ongoing. Inclusion criteria were having ADPKD according to the modified Ravine criteria [9], age ≥18 years and an estimated glomerular filtration rate (eGFR) ≥15 ml/min/1.73 m². Exclusion criteria were concomitant diseases or medication use that may influence the natural course of ADPKD [e.g. diabetes mellitus or chronic use of non-steroidal anti-inflammatory drugs (NSAIDs), somatostatin analogues or vasopressin V2 receptor antagonists].

Of the 664 patients included, 582 had fasting blood samples available for glucagon measurement. After exclusion of patients that started disease-modifying drugs during follow-up (e.g. somatostatin analogues or vasopressin V2 receptor antagonists), 527 patients remained. Additionally we excluded patients with diabetes mellitus at baseline [haemoglobin A1c (HbA1c) >53 mmol/mol (n = 8) or glucose >7 mmol/L (n = 18)], because in diabetes mellitus, glucagon concentrations are generally elevated and could interfere with the analyses [10], leaving 501 patients available for analyses.

The DIPAK observational study was approved by the Institutional Review Board of the University Medical Centre Groningen (METc. 2013/040) and was conducted in adherence with the International Conference on Harmonization Good Clinical Practice guidelines. Written informed consent was obtained from all participants.

Data collection

Patients visited the outpatient clinic every year, fasting from midnight onward. Blood was drawn for PKD mutation analysis and to measure glucagon, creatinine, glucose, HbA1C, high-density lipoprotein (HDL) cholesterol, total cholesterol, non-HDL cholesterol and copeptin as a surrogate for vasopressin. Height, weight, blood pressure and use of antihypertensives were assessed at all visits. Body mass index (BMI) was calculated as weight in kilograms divided by height in square meters. Standardized abdominal magnetic resonance imaging (MRI) was performed every 3 years to assess total kidney volume (TKV).

Measurements

Creatinine was measured using an isotope dilution mass spectrometry traceable enzymatic method. eGFR was calculated with the creatinine-based Chronic Kidney Disease Epidemiology Collaboration formula [11]. TKV was measured using automated segmentation, by trained reviewers blinded to patient identity, using a validated deep learning-based approach [12, 13]. The height-adjusted TKV (htTKV) was calculated by dividing the TKV by height in meters. The Mayo risk class was assessed using htTKV and age, as previously described [14]. The creatinine excretion rate (CER), a validated marker for muscle mass [15], was calculated by urinary creatinine concentration times the 24-h urine volume. Copeptin was measured using a sandwich immunoassay (B.R.A.H.M.S, Thermo Fisher Scientific, Berlin, Germany).

Glucagon measurement

Glucagon is derived from its precursor proglucagon [16]. Proglucagon is formed in intestinal and pancreatic tissue, where it is cleaved not only to produce glucagon, but also various other peptide hormones [16]. These peptide hormones can cross-react with glucagon, because they share in part the same molecular structure [16, 17]. Therefore we selected a validated enzyme-linked immunosorbent assay (ELISA; Merckodia A/S, Uppsala, Sweden) with low cross-reactivity and an ability to measure glucagon at known low physiological levels [17]. The reported sensitivity is 1.7 pmol/L, with a lower detection limit of 1.4 pmol/L and cross-reactivity with oxyntomodulin of 3.1–3.6% [18]. The average recovery of dilutional linearity is 103% (99–108%) and of parallelism 106% (98–120%) [18]. The samples were measured at Haemoscan (Groningen, The Netherlands).
Evaluation of glucagon measurement

The assay performance was validated by measuring the intra- and interassay coefficient of variation and a spike-and-recovery test. Two samples of low, medium and high glucagon concentration were measured on every plate. The spike-and-recovery rate was measured to determine if the concentration–response relationship was similar in the calibration curve and the samples [19]. The ideal recovery rate was set at 80–120% [19].

Statistical analysis

For statistical analyses, we used SPSS version 23 (IBM, Armonk, NY, USA) and Stata SE 14 (StataCorp, College Station, TX, USA) in case of linear mixed model analyses. A two-sided P-value <0.05 was considered statistically significant. Normally distributed data were described using means ± SD, non-normally distributed data using the median and interquartile range (IQR) and categorical data using frequencies with percentages. Logarithmic transformation was applied to all variables that did not meet the requirements for normal distribution to meet the assumptions of linear regression and mixed modelling for these variables.

The baseline characteristics were stratified according to sex-adjusted quartiles of glucagon, because glucagon levels in males were significantly higher than in females, in line with previous observations [20]. The differences between the quartiles were tested with one-way analysis of variance (ANOVA) for normally distributed data, Kruskal–Wallis test for non-normally distributed data and the chi-squared test for categorical data. In normally distributed data the P for trend was determined by one-way ANOVA and in non-normally distributed data by the Jonckheere–Terpstra test.

To assess the validity of the glucagon assay, we explored possible determinants of glucagon at baseline using univariate linear regression and multivariate backward stepwise regression analyses. Based on the literature, the following determinants were taken into account: sex, age, BMI, glucose and copeptin [7, 10, 21]. In the multivariate analyses, all variables with P-values >0.1 were removed stepwise from the model, until only variables remained that contributed to the model with a P-value <0.01. Second, we analysed whether glucagon was associated with its known downstream effects: glucose, HbA1c, total cholesterol, HDL cholesterol and non-HDL cholesterol.

To investigate the cross-sectional association of glucagon with disease severity, multivariate regression analyses were performed with baseline eGFR and htTKV as dependent variables and baseline glucagon as an independent variable (crude model). Next, these associations were adjusted for potential confounders: sex and age (Model 1), additionally for BMI (Model 2), additionally for copeptin (Model 3) and additionally for PKD mutations (Model 4).

To investigate the longitudinal association of glucagon with disease progression a mixed model repeated measures analysis was performed. All patients who had at least two eGFR values (at baseline and Year 1) or who had at least two htTKV values (at baseline and Year 3) were included in the mixed model analysis. The antilog of the derived estimated effect from the mixed model analysis shows the annual percentage growth of htTKV. All measurements during the study were included for slope analysis. Linear mixed models used an unstructured covariance structure. Intercept and slope were allowed to vary randomly. Baseline concentration glucagon, time and the interaction with time were added to further models. The models used are exactly the same as for the cross-sectional analyses. Covariates were added to the models as measured at baseline. In each model, participants with missing data were excluded listwise.

Various sensitivity analyses were performed. Glucagon was replaced by glucose, HbA1c and glucagon:glucose ratio in the multivariate linear regression and mixed model analyses to assess if these variables, which are regulated by glucagon, are associated with disease severity and progression. We used the glucagon:glucose ratio as it is potentially a more accurate marker of ketogenic status. When energy supply is limited, during low availability of glucose, the glucagon concentration is elevated and induces ketogenesis [7]. CER, a marker of muscle mass, was added to the cross-sectional analysis of eGFR to assess if it could be a confounder for BMI. Additionally, the interaction between CER and BMI was determined using linear regression.

RESULTS

Participant characteristics

Glucagon had a median value of 5.0 pmol/L (IQR 3.4–7.2). Males had significantly higher glucagon concentrations than females [6.6 pmol/L (IQR 4.5–9.5) versus 4.3 (2.9–6.0); P < 0.001, respectively]. Baseline characteristics were stratified according to sex-adjusted quartiles of baseline glucagon concentration (Table 1). Patients within the highest quartile of glucagon concentration had significantly higher weight, BMI, copeptin concentration, glucose and HbA1c. Furthermore, there were significant differences between the quartiles of glucagon with respect to diastolic blood pressure and Mayo Clinic risk classification, with no specific trend. eGFR at baseline was decreased per increasing quartile of glucagon (P for trend = 0.01), whereas htTKV was higher per increasing quartile of glucagon (P for trend = 0.01) (Figure 1).

Validity of glucagon measurement

Assay specifics. The glucagon measurement was validated by calculating the intra- and interassay coefficient of variation and spike-and-recovery rate, which showed acceptable to good results with values of 7.0%, 11.7% (ideally <15%) and 85.3% (ideally 80–120%), respectively.

Stability over time. In 30 patients, glucagon was measured at baseline and after 12 weeks. These patients were selected to represent a broad eGFR range: 60–90 (n = 10), 45–60 (n = 10) and 15–45 mL/min/1.73 m2 (n = 10). The median glucagon concentration was similar at both time points [2.2 pmol/L (IQR 1.5–4.6) and 2.3 (0.7–3.4), P = 0.38, respectively] and the values at both time points showed good correlation (Pearson’s correlation coefficient 0.893; P < 0.001; Supplementary data, Figure S1).

Determinants and downstream effects of glucagon. The biological plausibility of the measured glucagon values was evaluated by testing determinants of glucagon. Baseline glucagon was univariately significantly associated with the known determinants sex, BMI and copeptin (all P < 0.01) (Table 2). In the multivariate stepwise backward analysis, sex [standardized β (st.β) = −0.241, P < 0.001], BMI [st.β = 0.182, P < 0.001] and copeptin [st.β = 0.168, P = 0.001] remained significantly associated with glucagon. The association between glucagon and copeptin remained significant after correcting for the potential confounders thiazide use,
Table 1. Baseline characteristics of the overall study cohort as well as according to sex-stratified quartiles of glucagon

| Variables | Total | Sex-stratified quartiles of baseline glucagon (pmol/L) | M < 4.38 | M 4.38–6.57 | M 6.57–9.45 | M > 9.45 | P-value |
|-----------|-------|-----------------------------------------------------|---------|-------------|-------------|---------|---------|
| Total, n  | 501   | 125                                                 | 126     | 127         | 123         |         | <0.001 |
| Glucagon (pmol/L), median (IQR) | 5.0 (3.4–7.2) | 2.5 (2.0–3.0) | 4.1 (3.6–5.1) | 5.8 (5.0–7.2) | 10.0 (7.3–12.4) |       |         |
| Female, n (%) | 307 (61.3) | 77 (61.6) | 77 (61.3) | 78 (61.4) | 75 (61.0) | 1.00 |
| Age (years) | 47.1 ± 11.9 | 46.5 ± 11.0 | 46.0 ± 10.9 | 47.5 ± 13.0 | 46.3 ± 12.5 | 0.61 |
| Height (cm) | 175.7 ± 9.6 | 175.6 ± 8.2 | 175.6 ± 9.2 | 176.0 ± 9.4 | 175.8 ± 10.8 | 0.9 |
| Weight (kg) | 81.3 ± 16.6 | 77.1 ± 15.0 | 80.0 ± 14.8 | 82.8 ± 16.5 | 85.3 ± 18.8 | 0.001 |
| BMI (kg/m²) | 26.3 ± 4.6 | 24.9 ± 4.0 | 25.9 ± 4.1 | 26.6 ± 4.6 | 27.6 ± 5.3 | <0.001 |
| Genetic mutation, n (%) | - | - | - | - | - | - |
| PKD1 T | 197 (39.3) | 41 (32.8) | 48 (38.1) | 53 (41.7) | 55 (44.7) | - |
| PKD1 NT | 136 (27.3) | 42 (33.6) | 27 (21.4) | 34 (26.8) | 33 (26.8) | - |
| PKD2 | 127 (25.3) | 33 (26.4) | 37 (29.4) | 28 (22.0) | 29 (23.6) | - |
| Missing/other | 41 (8.2) | 9 (7.2) | 14 (11.1) | 12 (9.4) | 6 (4.9) | - |
| Blood pressure (mmHg) | - | - | - | - | - | - |
| Systolic | 129.6 ± 13.5 | 130.6 ± 13.7 | 129.6 ± 13.2 | 128.5 ± 13.7 | 129.6 ± 13.6 | 0.69 |
| Diastolic | 79.5 ± 8.8 | 81.2 ± 8.6 | 79.6 ± 8.7 | 77.8 ± 8.5 | 79.6 ± 9.2 | 0.03 |
| Antihypertensive use, n (%) | 360 (71.9) | 81 (64.8) | 95 (75.4) | 88 (69.3) | 96 (78.0) | 0.09 |
| Creatinine (µmol/L), median (IQR) | 107 (78–150) | 97 (78–134) | 103 (75–140) | 109 (79–160) | 125 (80–173) | 0.06 |
| eGFR (mL/min/1.73 m²) | 63.5 ± 29.2 | 66.6 ± 26.0 | 65.3 ± 27.1 | 62.0 ± 31.1 | 60 ± 32.1 | 0.26 |
| CKD stage baseline, n (%) | - | - | - | - | - | - |
| Stages 1 + 2 | 246 (49.1) | 73 (58.4) | 66 (52.4) | 57 (44.9) | 50 (40.7) | - |
| Stage 3A + B | 193 (38.5) | 41 (32.8) | 49 (38.9) | 52 (40.9) | 51 (41.5) | - |
| Stages 4 + 5 | 62 (12.4) | 11 (8.8) | 11 (8.7) | 18 (14.2) | 22 (17.9) | - |
| Copeptin (pmol/L), median (IQR) | 8.0 (4.4–15.7) | 6.0 (3.5–12.3) | 6.7 (4.3–13.4) | 9.2 (4.4–18.7) | 10.4 (5.5–21.7) | <0.001 |
| htTKV (mL/m), median (IQR) | 834 (481–1327) | 704 (421–1275) | 821 (518–1323) | 914 (477–1354) | 923 (563–1481) | 0.09 |
| Mayo risk classification, n (%) | - | - | - | - | - | - |
| Low | 146 (29.1) | 48 (38.4) | 33 (26.2) | 33 (26.0) | 32 (26.0) | - |
| Medium | 163 (32.5) | 29 (23.2) | 49 (38.9) | 47 (37.0) | 38 (30.9) | - |
| High | 142 (28.3) | 35 (28.0) | 28 (22.2) | 35 (27.6) | 44 (35.8) | - |
| Unknown | 50 (10.0) | 13 (10.4) | 16 (12.7) | 12 (9.4) | 9 (7.3) | - |
| Glucose (mmol/L) | 5.2 ± 0.5 | 5.2 ± 0.5 | 5.1 ± 0.5 | 5.3 ± 0.5 | 5.3 ± 0.6 | 0.01 |
| HbA1c (mmol/mol) | 36.5 ± 4.6 | 35.3 ± 3.7 | 35.7 ± 3.3 | 36.5 ± 3.4 | 36.5 ± 4.6 | 0.03 |
| Total cholesterol (mmol/L) | 4.8 ± 1.0 | 4.8 ± 0.9 | 4.9 ± 0.8 | 4.9 ± 1.0 | 4.8 ± 1.0 | 0.03 |
| HDL cholesterol (mmol/L) | 1.4 ± 0.4 | 1.5 ± 0.4 | 1.5 ± 0.4 | 1.5 ± 0.5 | 1.4 ± 0.4 | 0.43 |
| Non-HDL cholesterol (mmol/L) | 3.4 ± 1.0 | 3.4 ± 1.0 | 3.4 ± 0.8 | 3.4 ± 1.0 | 3.5 ± 1.0 | 0.80 |

Values are presented as mean ± SD unless stated otherwise. P-values for differences between groups were tested with one-way ANOVA for normally distributed data, Kruskal-Wallis test for non-normally distributed data and Pearson’s chi-squared test for categorical data. Distribution of Mayo risk classification: low risk includes Classes 1A, 8 and 2; medium risk includes Class 1C, and high risk includes Class 1D and E.

M, men; F, female; T, truncating; NT, non-truncating.

Glucagon versus disease progression in ADPKD

Glucagon concentration and disease severity: cross-sectional analyses

Glucagon was negatively associated with eGFR at baseline (\( \beta = -0.127, P = 0.01 \)) and after adjusting for sex and age (\( \beta = -0.078, P = 0.03 \)). However, after correcting for BMI (\( \beta = -0.055, P = 0.13 \)), glucagon lost significance. Glucagon was positively associated with htTKV (\( \beta = 0.164, P = 0.001 \)) (Table 4), but this association lost significance after adjusting for sex and age (\( \beta = 0.061, P = 0.18 \)).
For the sensitivity analysis, we replaced glucagon with glucose, the glucagon:glucose ratio and HbA1c. No consistent pattern emerged. Glucose and HbA1c were only significantly associated with eGFR and htTKV in crude analyses, whereas after adjustment for covariates, significance was lost. The glucagon:glucose ratio was negatively associated with eGFR at baseline, as well as after adjusting for sex and age (st.β = −0.079, P = 0.02). However, after additionally adjusting for BMI, the glucagon:glucose ratio lost significance (st.β = −0.060, P = 0.08). This ratio was also significantly associated with htTKV at baseline (st.β = 0.137, P = 0.01), but not after adjusting for covariates (st.β = 0.054, P = 0.24). When only including patients with an eGFR > 45 mL/min/1.73 m² (n = 333) or only patients with an eGFR > 60 mL/min/1.73 m² (n = 243), glucagon was not associated with eGFR, and with htTKV only in the crude analysis. When correcting for CER, a measure for muscle mass, as a possible confounder between BMI and eGFR, neither the significance of glucagon nor BMI changed. Additionally there was no significant interaction between CER and BMI.

### Glucagon concentration and disease progression: longitudinal analyses

Follow-up data were available for 465 patients to assess the change in eGFR. The median follow-up time was 3.03 years (IQR 2.0–4.8). The average annual eGFR decline was −2.94 mL/min/1.73 m² [95% confidence interval (CI) −3.29 to −2.59]. Glucagon was significantly and negatively associated with an annual eGFR decline [−1.15 (95% CI −2.07 to −0.23); P = 0.01] (Table 5 and Figure 2). However, after adjusting for sex and age, glucagon lost significance.

A total of 197 patients had follow-up MRIs available. The mean annual growth of htTKV was 4.25% (95% CI 3.54–4.97). Glucagon was significantly associated with the annual growth of htTKV [2.44 (95% CI 0.07–4.89); P = 0.01] (Table 5 and Figure 2).

### Table 2. Associations of possible determinants of glucagon with glucagon concentration

| Variables       | Univariate | Multivariate stepwise backward |
|-----------------|------------|--------------------------------|
|                 | st.β       | P-value | st.β | P-value |
| Sex             | −0.317     | <0.001  | −0.241 | <0.001 |
| Age (years)     | 0.043      | 0.34    |       |        |
| BMI (kg/m²)     | 0.214      | <0.001  | 0.182 | <0.001 |
| Copeptin log (mmol/L) | 0.286 | <0.001  | 0.168 | <0.001 |

st.β and P-values were calculated using linear regression analysis. The dependent variable is glucagon (pmol/L). The independent variables are sex, age, glucose and copeptin log.

### Table 3. Associations of glucagon concentration with possible downstream effects of glucagon

| Variables           | st.β | P-value |
|---------------------|------|---------|
| Glucose (mmol/L)    | 0.132| 0.01    |
| HbA1c (mmol/mol)    | 0.142| 0.01    |
| Total cholesterol (mmol/L) | 0.000 | 0.9    |
| HDL cholesterol (mmol/L) | −0.225 | <0.001 |
| Non-HDL cholesterol (mmol/L) | 0.102 | 0.02   |

st.β and P-values were calculated using linear regression analysis. The dependent variables are glucose, HbA1c, total cholesterol, HDL cholesterol and non-HDL cholesterol. The independent variable is glucagon (pmol/L).

### Table 4. Cross-sectional associations of fasting glucagon concentration with eGFR (n = 489) and htTKV (n = 442) at baseline using linear regression analyses

| Variables       | Crude | Model 1 | Model 2 | Model 3 | Model 4 |
|-----------------|-------|---------|---------|---------|---------|
|                 | st.β | P value | R²      | st.β | P value | R²      | st.β | P value | R²      | st.β | P value | R²      |
| eGFR            | −0.127 | 0.01    | 0.02    | −0.078 | 0.03    | 0.48    | −0.055 | 0.13    | 0.49    | 0.019  | 0.51    | 0.65    | 0.027  | 0.38    | 0.68    |
| Glucagon log    |       |         |         |       |         |         |       |         |         |       |         |         |       |         |         |
| Sex             | −0.672 | <0.001  | −0.064  | 0.06   | 0.06    | −0.669  | <0.001  | −0.576  | <0.001  | −0.639  | <0.001  | −0.447  | <0.001  | −0.426  | <0.001  |
| Age (years)     |       |         |         |       |         |         |       |         |         |       |         |         |       |         |         |
| BMI (kg/m²)     |       |         |         |       |         |         |       |         |         |       |         |         |       |         |         |
| Copeptin log    | −0.101 | 0.01    | −0.067  | 0.02   | −0.67   | 0.01    | −0.447  | <0.001  | −0.426  | <0.001  | −0.106  | 0.001   |       |         |         |
| PKD2 (ref)      |       |         |         |       |         |         |       |         |         |       |         |         |       |         |         |
| PKD1 NT         |       |         |         |       |         |         |       |         |         |       |         |         |       |         |         |
| PKD1 T          |       |         |         |       |         |         |       |         |         |       |         |         |       |         |         |
| Other/missing   |       |         |         |       |         |         |       |         |         |       |         |         |       |         |         |
| htTKV           |       |         |         |       |         |         |       |         |         |       |         |         |       |         |         |
| Glucagon log    | 0.164  | 0.001   | 0.061   | 0.18   | 0.012   | 0.79    | −0.046  | 0.29    | −0.058  | 0.18    | −0.058  | 0.18    | −0.046  | 0.29    | −0.058  | 0.18    |
| Sex             | −0.287  | <0.001  | −0.286  | <0.001  | −0.180  | <0.001  | −0.188  | <0.001  | −0.188  | <0.001  | −0.188  | <0.001  | −0.188  | <0.001  | −0.188  | <0.001  |
| Age (years)     | 0.232  | <0.001  | 0.224   | <0.001  | 0.141   | 0.001   | 0.190   | <0.001  | 0.190   | <0.001  | 0.190   | <0.001  | 0.190   | <0.001  | 0.190   | <0.001  |
| BMI (kg/m²)     | 0.229  | <0.001  | 0.205   | <0.001  | 0.203   | <0.001  | 0.203   | <0.001  | 0.203   | <0.001  | 0.203   | <0.001  | 0.203   | <0.001  | 0.203   | <0.001  |
| Copeptin log    |       |         |         |       |         |         |       |         |         |       |         |         |       |         |         |
| PKD2 (ref)      |       |         |         |       |         |         |       |         |         |       |         |         |       |         |         |
| PKD1 NT         |       |         |         |       |         |         |       |         |         |       |         |         |       |         |         |
| PKD1 T          |       |         |         |       |         |         |       |         |         |       |         |         |       |         |         |
| Other/missing   |       |         |         |       |         |         |       |         |         |       |         |         |       |         |         |

st.β and P-values were calculated using multivariate regression analysis. The dependent variables are eGFR (mL/min/1.73 m²) and htTKV (mL/m) at baseline. The independent variables are baseline glucagon log (crude), adjusted for sex, age (Model 1), additionally adjusted for BMI (Model 2), additionally adjusted for copeptin log (Model 3) and additionally adjusted for PKD mutations (Model 4). *PKD mutation was used as a dummy variable with PKD2 as the reference group.
Table 5. Longitudinal associations between fasting glucagon concentration and annual decline of eGFR (n = 465) and annual growth of htTKV (n = 197) using linear mixed model analyses

| Variables          | Crude                          | Model 1                          | Model 2                          | Model 3                          | Model 4                          |
|--------------------|--------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
|                    | Est. (95% CI)                  | P-value                          | Est. (95% CI)                    | P-value                          | Est. (95% CI)                    | P-value                          |
| eGFR/year          |                                 |                                  |                                  |                                  |                                  |                                  |
| Glucagon log       | -1.15 (-2.07 to -0.23)         | 0.01                             | -0.75 (-1.70 to -0.19)           | 0.12                             | -0.55 (-1.53 to -0.43)           | 0.27                             | -0.10 (-1.06 to -0.87)           | 0.85                             | 0.04 (-0.91 to -0.99)           | 0.9 |
| Sex                | 0.82 (0.28–1.36)               | 0.003                            | 0.81 (0.27–1.36)                 | 0.004                            | 0.02 (-0.02–0.02)                | 0.74                             | 0.02 (-0.01–0.04)                | 0.16                             | 0.05 (-0.02–0.02)               | 0.9 |
| Age (years)        | 0.00 (-0.02–0.02)              | 0.82                             | 0.00 (-0.02–0.02)                | 0.74                             | -0.06 (-0.12–0.00)              | 0.05                             | -0.06 (-0.12–0.01)              | 0.07                             | 0.05 (-0.12–0.01)               | 0.08 |
| BMI (kg/m²)        | 0.06 (-0.12–0.00)              | 0.05                             | -1.56 (-2.34 to -0.79)           | <0.001                           | -1.49 (-2.25 to –0.72)          | <0.001                           | -1.19 (-1.88 to -0.51)          | 0.001                           | -1.37 (-2.05 to -0.69)          | <0.001 |
| Copeptin log       |                                 |                                  |                                  |                                  |                                  |                                  |                                  |                                  |                                  |                                  |
| PKD2 (ref)         |                                 |                                  |                                  |                                  |                                  |                                  |                                  |                                  |                                  |                                  |
| PKD1 NT            |                                 |                                  |                                  |                                  |                                  |                                  |                                  |                                  |                                  |                                  |
| PKD1 T             |                                 |                                  |                                  |                                  |                                  |                                  |                                  |                                  |                                  |                                  |
| Other/missing      |                                 |                                  |                                  |                                  |                                  |                                  |                                  |                                  |                                  |                                  |
| htTKV/year         |                                 |                                  |                                  |                                  |                                  |                                  |                                  |                                  |                                  |                                  |
| Glucagon log       | 2.44 (0.07–4.89)               | 0.04                             | 1.59 (-0.75–3.98)                | 0.19                             | 1.53 (-0.88–4.01)                | 0.21                             | 1.39 (-1.01–3.85)                | 0.26                             | 1.21 (-1.12–3.63)               | 0.32 |
| Sex                | -2.49 (-3.86 to -1.09)         | 0.001                            | -2.64 (-4.02 to -1.12)           | <0.001                           | -2.29 (-3.72 to -0.83)           | 0.01                             | -2.23 (-3.70 to -0.85)           | 0.01                             | -2.03 (-3.10–0.02)              | 0.19 |
| Age (years)        | -0.04 (-0.09–0.01)             | 0.14                             | -0.04 (-0.09–0.02)               | 0.20                             | -0.01 (-0.11–0.01)              | 0.08                             | -0.03 (-0.10–0.02)              | 0.19                             | -0.03 (-0.19–0.14)              | 0.76 |
| BMI (kg/m²)        | -0.01 (-0.17–0.16)             | 0.9                               | 1.92 (-0.41–4.30)               | 0.11                             | 1.64 (-0.66–3.99)               | 0.16                             |                                  |                                  |                                  |                                  |
| Copeptin log       |                                 |                                  |                                  |                                  |                                  |                                  |                                  |                                  |                                  |                                  |
| PKD2 (ref)*        |                                 |                                  |                                  |                                  |                                  |                                  |                                  |                                  |                                  |                                  |
| PKD1 NT            |                                 |                                  |                                  |                                  |                                  |                                  |                                  |                                  |                                  |                                  |
| PKD1 T             |                                 |                                  |                                  |                                  |                                  |                                  |                                  |                                  |                                  |                                  |
| Other/missing      |                                 |                                  |                                  |                                  |                                  |                                  |                                  |                                  |                                  |                                  |

The estimates and P-values for the interactions of variables with time are depicted. The interaction with time is the effect of the variables on eGFR (mL/min/1.73 m²) over time, which is the effect on the eGFR slope. Crude shows the association of glucagon with the eGFR slope. Model 1 shows the association of glucagon with the eGFR slope adjusted for sex and age. Model 2 shows the association of glucagon with the eGFR slope adjusted for sex, age and BMI. Model 3 shows the association of glucagon with the eGFR slope adjusted for sex, age, BMI and copeptin log. Model 4 shows the association of glucagon with the eGFR slope adjusted for sex, age, BMI, copeptin log and PKD mutations. PKD mutation was used as a dummy variable with PKD2 as the reference group.

Est., estimation.
However, this association lost significance after adjusting for sex and age.

For these longitudinal analyses, sensitivity analyses were performed by replacing glucagon with glucose, the glucagon:glucose ratio and HbA1c in the linear mixed model analyses. Again a non-consistent pattern emerged. Glucose was not associated with the annual growth of htTKV or with eGFR and HbA1c was significantly associated with the annual growth of htTKV in the fully adjusted model [0.26 (95% CI 0.05–0.47); P = 0.02] and with annual decline of eGFR [−0.09 (95% CI −0.16 to −0.02); P = 0.01], but it lost significance after adjusting for copeptin. The glucagon:glucose ratio was significantly associated with the annual decline of eGFR [−1.00 (95% CI −1.93 to −0.06); P = 0.04], but it lost significance after additional adjustment for sex and age and was not associated with the annual growth of htTKV. Furthermore, when only including patients with better kidney function (eGFR >45 mL/min/1.73 m²; n = 315) glucagon was significantly associated with the annual decline of eGFR but lost significance after adjusting for sex, age and BMI. When including patients with an eGFR >60 mL/min/1.72 m² (n = 228), glucagon was only significantly associated in the crude analysis. For the annual growth of htTKV, when including patients with either an eGFR >45 mL/min/1.73 m² (n = 158) or an eGFR >60 mL/min/1.73 m² (n = 131), glucagon was only significantly associated in the crude analyses.

DISCUSSION

In this study we investigated whether glucagon could be a biomarker to predict disease severity and progression in ADPKD. We first assessed the validity of the glucagon measurement and showed acceptable to good intra- and interassay coefficients of variation and stability over time, while we also found associations with the expected determinants and downstream effects of glucagon. However, we did not find the expected associations between glucagon concentration and disease severity and progression in ADPKD.

In the literature, no information is available about glucagon and ADPKD. Previous studies in patients with kidney diseases other than ADPKD describe a negative association between glucagon and kidney function. In these studies, glucagon concentration in patients with kidney failure was 2–4 times higher compared with healthy controls [22, 23] and increased along with CKD severity [20]. These associations may have been caused by cross-reactivity of the assay with proglucagon and non-glucagon derivate of proglucagon, because glucagon was measured by a C-terminal assay. It is known that some of these hormonally inactive derivate accumulate in renal impairment and consequently lead to overestimation of glucagon levels [17]. We tried to avoid this pitfall by using a highly specific sandwich ELISA that is optimized in order to avoid cross-reactivity [16, 17].

In order to confirm that we measured the biologically active hormone glucagon, and not proglucagon 1–61 or other derivate from proglucagon, we analysed the associations between glucagon and its known determinants and downstream effects. Glucagon was positively associated with its determinant BMI [10] and with downstream effects on non-HDL cholesterol, HbA1c and glucose [7]. These associations provide biological plausibility that we accurately measured plasma glucagon. Furthermore, the intra- and interassay variation and spike-and-recovery were both within the ideal range [19, 24]. Moreover, we confirmed that individual glucagon levels remained relatively stable over time. Thus we argue that our glucagon measurement can be considered accurate. However, we noticed individual variability in levels between baseline and Month 3 (Supplementary data, Figure S1). We therefore recommend that in future studies glucagon should be measured on several occasions.

Previous studies showed that ketogenesis and a decrease in mTOR activity are renoprotective in ADPKD [3, 6]. It is known that glucagon can cause ketogenesis as well as mTOR inhibition [8, 10]. Based on our results, it seems that endogenous levels of glucagon do not cause ketogenesis or mTOR inhibition to the extent that it can ameliorate ADPKD progression. Potentially, the level of glucagon only becomes relevant when patients are in ketogenic status due to prolonged fasting or following a ketogenic diet [10]. For the sensitivity analysis we used the glucagon:glucose ratio as a potentially more accurate marker of ketogenic status. When energy supply is limited, during low availability of glucose, the glucagon concentration is elevated and induces ketogenesis [7]. However, this analysis did not reveal any association.

Not finding the expected associations might also be due to the complex interplay of glucagon forms with other hormones associated with glucose metabolism [7, 10]. In healthy individuals, glucagon secretion is inhibited by insulin, glucagon-like peptide-1 and somatostatin, while glucagon increases insulin secretion [7]. It might thus be that not finding a direct association between glucagon and disease severity and progression in ADPKD is the result of the complex interplay among these hormones.

In the multivariate stepwise backward analysis, copeptin remained significantly associated with glucagon, even after adjustment for possible confounder thiazide use, sex and BMI. Copeptin is a surrogate marker for vasopressin and is known to be increased in ADPKD and associated with disease severity and progression [25]. Vasopressin can induce glucagon secretion through V1b receptors and can also stimulate hepatic glycogenolysis and gluconeogenesis through V1a receptors [21, 26]. Our data confirm the relation between vasopressin and glucagon. However, the contribution of vasopressin to glucose homeostasis remains complex and not fully elucidated [21]. Nevertheless, vasopressin might be a major determinant of the glucagon concentration in patients who are not in a ketogenic state.

The strengths of this study are the use of a highly specific and sensitive ELISA to measure glucagon concentration, the
large and well-phenotyped cohort of ADPKD patients, the standardized method of sample collection (fasting at the same time in the morning) and the measurement of disease severity markers eGFR as well as hTKV. Nevertheless, several limitations have to be acknowledged. In the cross-sectional part of our study, we cannot infer causality. However, in the longitudinal analyses, no association was found either. Measuring glucagon is a complex process, mainly due to cross-reactivity with proglucagon 1-61. We aimed to avoid this by using a validated ELISA that has limited cross-reactivity and by validating the glucagon measurements, however, we cannot rule out cross-reactivity in our measurements. Lastly, as reasoned above, there may be confounding due to the complex hormonal interrelationships.

To summarize, in this observational study we did not find an association between higher endogenous glucagon concentrations and less disease severity or slower disease progression in patients with ADPKD. Thus these observational data do not provide evidence for a role of endogenous glucagon as a protective hormone in ADPKD. However, as discussed above, the limitations of our retrospective observational study design and how we measured glucagon do not allow us to completely exclude the possibility that glucagon has a beneficial effect on disease progression in ADPKD. Prospective intervention studies in which glucagon is increased are needed to definitively elucidate the relationship between glucagon and ADPKD disease progression.

SUPPLEMENTARY DATA
Supplementary data are available at ckJ online.

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CONFLICT OF INTEREST STATEMENT
R.T.G. received grants for research from Galapagos, Ipsen, Otsuka and Sanofi-Genzyme. All money is paid to the employing institution. R.T.G. holds the rights for the orphan medical product designation for lanreotide to halt disease progression in ADPKD. The results presented in this article have not been published previously in whole or part, except in abstract format.

DATA AVAILABILITY STATEMENT
Data cannot be shared for legal reasons. Participating subjects signed in their informed consent form that their data will only be used by investigators of the DIPAK Consortium and will not be handed to third parties. However, external investigators can submit a research proposal to the DIPAK Steering Committee. If this body agrees with the proposal, the proposed analyses will be carried out by the epidemiologists working for the consortium. The results will be sent to these investigators and may be used for publication.

APPENDIX
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