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Glucagon dynamics and metabolic phenotypes

Patterns of plasma glucagon dynamics do not match metabolic phenotypes in young women

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Abbreviations

IFG: impaired fasting glucose

IGT: impaired glucose tolerance

oGTT: oral glucose tolerance test

ivGTT: intravenous glucose tolerance test

NG: normoglycemic

PGM: pathological glucose metabolism

GDM: gestational diabetes mellitus

hsCRP: high-sensitivity c-reactive protein

RIA: radioimmunoassay

ELISA: Enzyme-linked Immunosorbent Assay

Context: The role of hyperglucagonemia in type 2 diabetes is still debated.
Objective: We analyzed glucagon dynamics during oral glucose tolerance testing (oGTT) in young women with 1 out of 3 metabolic phenotypes: healthy control (normoglycemic after a normoglycemic pregnancy); normoglycemic high-risk (normoglycemic after a pregnancy complicated by gestational diabetes); and prediabetes/screening-diagnosed T2D. We asked if glucagon patterns were homogeneous within the metabolic phenotypes.
Design and Setting: 5-point oGTT; sandwich ELISA for glucagon; functional data analysis with unsupervised clustering.
Participants: Cross-sectional analysis of 285 women from the mono-center observational study PPSDiab ("Prediction, Prevention and Subclassification of gestational and type 2 Diabetes"), recruited between November 2011 and May 2016.
Results: We found 4 patterns of glucagon dynamics that did not match the metabolic phenotypes. Elevated fasting glucagon and delayed glucagon suppression was overrepresented with prediabetes/diabetes, but this was only detected in 21% of this group. It also occurred in 8% of the control group.
Conclusions: We conclude that hyperglucagonemia may contribute to type 2 diabetes in a subgroup of affected individuals but that it is not a sine qua non for the disease. This should

be taken into account in future pathophysiological studies and when testing pharmacotherapies addressing glucagon signaling.

Hyperglucagonemia may cause type 2 diabetes, but we saw it only in a subgroup of women with prediabetes/diabetes in our study. Thus it is a possible, but not a universal factor in this disease.

Introduction

Glucagon is the main antagonist of insulin. It raises plasma glucose by reducing glycolysis and increasing gluconeogenesis and glycogenolysis (1, 2). Glucagon secretion from alpha cells is triggered by hypoglycemia and inhibited by insulin from neighboring beta cells. In turn, glucagon inhibits insulin secretion (2).

Unger et al. first postulated that elevated glucagon is a *sine qua non* in the development of diabetes (3, 4). This marked the departure from an *insulinocentric* concept of type 2 diabetes pathogenesis to a *bihormonal* or even *glucagonocentric* model (4). In a *glucagonocentric* model, most metabolic derangements of diabetes are caused by the disinhibition of glucagon secretion (resulting from insulin-resistant alpha cells or impaired insulin release), but not directly by insufficient insulin action in other tissues (4, 5).

The issue of the different pathophysiologic models remains unresolved, at least in part due to technical difficulties: glucagon is unstable, difficult to measure because of many similar peptides in plasma (2, 6), and its concentration is very low (7). Furthermore, alpha cells are harder to isolate than their insulin-producing neighbors (8). This impedes cellular studies.

Current data on plasma glucagon levels in (pre-)diabetic human subjects are also inconsistent. Several studies have found impaired glucagon suppression during an oral glucose tolerance test (oGTT) in prediabetic and diabetic individuals when compared to healthy controls (9-11). Other studies reported on increased fasting glucagon levels (12, 13). In contrast, Ahren et al. saw no differences between IGT and normoglycemic subjects (14), and Wagner et al. observed rising glucagon values during an oGTT in 21 to 34% of healthy, insulin-sensitive individuals (15). These authors even found that this pattern predicted future metabolic health.

New sandwich enzyme-linked immunosorbent assays (ELISAs) with improved specificity for glucagon became available recently, and this prompted us to re-examine the issue in a post-pregnancy cohort of young women. We compared 3 groups of study participants with different metabolic phenotypes: a control group (normoglycemic women, who had recently completed a normoglycemic pregnancy), a normoglycemic high-risk group for type 2 diabetes (normoglycemic women after a recent pregnancy complicated by gestational diabetes (GDM) (16, 17)), and a prediabetes/diabetes group (women with prediabetes or screening-diagnosed type 2 diabetes after GDM).

We first confirmed that average fasting plasma glucagon was higher and glucagon suppression during an oGTT was impaired in the normoglycemic high-risk and the prediabetes/diabetes group, similarly to what was seen in the majority of previous studies. However, our main research goal was to determine whether glucagon dynamics within each metabolic group were homogeneous or followed heterogeneous patterns. We used functional data analysis and unsupervised clustering to address this question.

Research Design and Methods

Study cohort

Study participants were women enrolled in the prospective, mono-center observational study PPSDiab (“Prediction, Prevention and Subclassification of gestational and type 2 Diabetes”) between November 2011 and May 2016 (18). The cohort includes women with GDM during

their last pregnancy and women following a normoglycemic pregnancy in a 2:1 ratio, recruited consecutively from the diabetes center and the obstetrics department of the University Hospital (Klinikum der Universität München) in Munich, Germany.

Premenopausal women, 3 to 16 months after a singleton (n=295) or twin (n=9) pregnancy with live birth(s) were eligible to participate. The GDM-diagnosis was based on a 75 g oGTT with cut-off values for GDM according to the International Association of the Diabetes and Pregnancy Study Groups (IADPSG) recommendations (plasma glucose: fasting 92 mg/dl, 1 hour 180 mg/dl, and 2 hours 153 mg/dl). Women without a history of GDM and either a normal 75 g oGTT (n=294) or a normal 50 g screening oGTT (<135 mg/dl plasma glucose after 1 hour, n=10) after the 23rd week of gestation were included in the normoglycemic group.

Exclusion criteria for this study were alcohol or substance abuse, pre-pregnancy diabetes, and chronic diseases requiring continuous medication, except for hypothyroidism (n=52), bronchial asthma (n=8), mild hypertension (n=4), gastroesophageal reflux (n=2), and history of pulmonary embolism resulting in rivaroxaban prophylaxis (n=1).

Written informed consent was obtained from all study participants, and the protocol was approved by the ethical review committee of the Ludwig-Maximilians-Universität (study ID 300-11).

Data used in this analysis were collected at the baseline visit of the PPSDiab study, 3 to 16 months after the index pregnancy. In addition to the baseline visit, post-GDM women also attend yearly follow-up visits with a 75 g oGTT.

Groups

We compared 3 groups of women: a control group (women normoglycemic at the baseline visit and after a normoglycemic pregnancy), a normoglycemic high-risk group (women normoglycemic at the baseline visit but with GDM during the preceding pregnancy), and a prediabetes/diabetes group (women with impaired fasting glucose (IFG), impaired glucose tolerance (IGT), combined IFG plus IGT, or screening-diagnosed type 2 diabetes at the baseline visit and with GDM during the preceding pregnancy). IFG (fasting plasma glucose ≥100 mg/dl (5.6 mmol/l)), IGT (2h plasma glucose ≥140 mg/dl (7.8 mmol/l)), and diabetes (fasting plasma glucose ≥126 mg/dl (7.0 mmol/l) or 2h plasma glucose ≥200 mg/dl (11.0 mmol/l)) were defined according to the criteria of the American Diabetes Association (19).

Measurements

We conducted a 5-point 75 g oGTT with measurement of plasma glucose (Glucose HK Gen.3, Roche Diagnostics, Mannheim, Germany), serum insulin (CLIA, DiaSorin LIASON systems, Saluggia, Italy), high-sensitivity C-reactive protein (hs-CRP; wide-range CRP, Siemens Healthcare Diagnostics, Erlangen, Germany), and blood lipids (LDL and HDL cholesterol, triglycerides; enzymatic caloric test, Roche Diagnostics, Mannheim, Germany) after an overnight fast.

Plasma glucagon was measured at all five time points of oGTT with an ELISA (Glucagon ELISA; Mercodia, Uppsala, Sweden; catalogue no: 10-1271-01) and also a radioimmunoassay (RIA Merck Millipore, Darmstadt, Germany; catalogue no: GL-32K) for 283 subjects. ELISA and RIA measurements gave different results (**Supplemental Table 1** and **Supplemental Fig. 1**). In particular, suppression of plasma glucagon during the oGTT was insufficiently represented in the RIA measurement. Sensitivity and specificity of ELISA for pancreatic glucagon (amino acids 33-61) has been proven to be superior to RIA (20, 21). Thus, for this analysis, we exclusively used glucagon data measured by ELISA (n=299). Plasma for glucagon measurements was collected in BD p800 tubes, which contain specific proteinase inhibitors to stabilize glucagon and other metabolically important hormones. Plasma was immediately separated by centrifugation and directly frozen in aliquots on dry

ice, before being transferred to a -80°C freezer within one hour from completion of the oGTT. Glucagon measurements were done in one batch and only from aliquots that had not been thawed previously.

Height and waist circumference were measured to the nearest 1 cm. Body mass and body fat mass was determined by a bioelectrical impedance analysis scale (Tanita BC-418; Tanita Corporation, Tokyo, Japan) (22, 23). Blood pressure was calculated as the mean out of 2 measurements in a resting seated position.

In addition to these basic tests, all study subjects were asked to participate in a magnetic resonance imaging (MRI) measurement and an intravenous glucose tolerance test (ivGTT) on a voluntary basis.

MRI (3 Tesla system, Ingenia or Achieva; Philips Healthcare, Hamburg, Germany) included determination of abdominal visceral adipose tissue volumes and liver fat content, using an mDixon low fat fraction map. In the ivGTT a glucose bolus of 0.3 g/kg body weight was injected over 1 minute with subsequent frequent blood sampling at 0, 2, 4, 6, 8, 10, 20, 30, 45 and 60 minutes. The measurements were used for the calculation of first phase insulin response (FPIR).

A detailed description of the study design, anthropometric, clinical, and MRI measurements, and methodologies of blood sampling and analysis can be found elsewhere (24).

Calculations

$$\text{Mean blood pressure} = \left(\text{diastolic value} * 2 + \text{systolic value}\right)/3$$

The insulin sensitivity index (ISI) according to Matsuda and De Fronzo was calculated from the oGTT (25):

$$ISI = 10000 \Big/ \sqrt{\left[\left(glucose\,0' * insulin\,0'\right) * \left(glucose\,0' + 2 * \left(glucose\,30' + 60' + 90'\right) + glucose1\right.\right.}$$

The disposition index (DI) was calculated as (26):

$$DI = ISI * IR30$$

with

$$IR30 = insulin\,30' - insulin\ 0'$$

ISI and IR30 were previously validated with data from ivGTT-euglycemic clamp tests in this cohort (24).

Glucagon suppression indices were calculated as (27):

$$Early\ suppression = \left(1 - \left[\,glucagon\,30' / glucagon\,0'\,\right]\right) * 100\%$$

$$Late\ suppression = \left(1 - \left[\,glucagon\,120' / glucagon\,30'\,\right]\right) * 100\%$$

$$Overall\ suppression = \left(1 - \left[\,glucagon\,120' / glucagon\,0'\,\right]\right) * 100\%$$

Area under the glucagon curve (AUC glucagon) was calculated using the trapezoidal rule.

First phase insulin response in the ivGTT test was calculated as the incremental area under the insulin curve from 0 to 10 minutes.

Statistical analysis

All metric and normally distributed variables are reported as mean±standard deviation; non-normally distributed variables are presented as median [first quartile–third quartile].

Categorical variables are presented as frequency and percentage. The Kruskal-Wallis test was used to compare metric variables, and the Chi-squared or Fisher's exact test was used to compare categorical variables. For post hoc analysis, Dunn's test was used. P-values <0.05 were considered statistically significant.

Functional data analysis methods were used for the analysis of the oGTT measurements (28). In the first step, the 5-point oGTT measurements were converted into continuous, smooth curves based on B-spline basis functions (29). Afterward, a functional principal component analysis (FPCA) was performed based on the fitted curves to analyze the temporal variation (28). In the next step, a cluster analysis was conducted to identify patients with similar plasma glucagon dynamics. Hierarchical Clustering was performed on the first 3 principal components of the FPCA via the Hierarchical Clustering on Principal Components function of Husson et al. (30). Hierarchical clustering was performed using the Ward's criterion on the selected principal components. The number of clusters was chosen based on the growth of between-inertia. For the final partitioning the k-means algorithm were performed with the partition obtained from the hierarchical tree as the initial partition. All statistical calculations were performed using SAS statistical software package version 9.3 (SAS Institute, Inc., Cary, NC, USA) or R version 3.1.3 (http://www.R-project.org).

Results

Mean glucagon curves differ between metabolic groups

We recruited 304 women into the PPSDiab study cohort but excluded 19 from this analysis. Two women were excluded because of type 1 diabetes diagnosed during follow-up, 2 because of overt hyperthyroidism, 1 because of an acute upper respiratory infection at baseline, 8 women were excluded from the control group due to pathological glucose tolerance at the baseline visit, and 6 women were excluded due to missing glucagon values.

Our final sample consisted of 285 study participants: 93 normoglycemic women after a normoglycemic pregnancy (control group), 121 normoglycemic women who had GDM (normoglycemic high-risk group), and 71 women with impaired fasting glucose, impaired glucose tolerance, or newly-diagnosed type 2 diabetes (prediabetes/diabetes group).

Baseline characteristics of the study cohort are shown in **Table 1**. Mean age and low-density lipoprotein (LDL) cholesterol were comparable, but mean blood pressure, waist circumference, triglycerides, c-reactive protein, liver fat content, intra-abdominal fat, fasting and 2-h plasma glucose increased, and high-density lipoprotein (HDL) cholesterol and insulin sensitivity decreased from the control over the normoglycemic high-risk to the prediabetes/diabetes group (all significant over the 3 groups, results of pairwise post hoc tests shown in **Table 1**).

We next compared plasma glucagon levels during the oGTT in the 3 groups (**Table 1**). Fasting plasma glucagon was significantly elevated, and early glucagon suppression was diminished in the prediabetes/diabetes group compared to the control group (median (Q1-Q3) for fasting plasma glucagon: 6.0 (4.6-8.2) [pmol/l] vs. 7.7 (5.6-11.2) [pmol/l]; early glucagon suppression; 47.6(32.8-57.9) [pmol/l] vs. 32.0 (14.5-51.3) [pmol/l], respectively). The normoglycemic high-risk group lay in between for these variables, but closer to the control group and not statistically different from it (median (Q1-Q3) for fasting plasma glucagon: 6.6 (4.5-8.4) [pmol/l]; early glucagon suppression: 41.3 (22.9-58.3) [pmol/l]) (**Fig. 1** and **Table 1**). Total glucagon suppression was similar in all 3 groups.

Similar to a recent publication by Faerch et al. (27), we further examined fasting glucagon values and glucagon suppression indices in women with isolated IFG compared to those with isolated IGT and combined IFG+IGT (**Supplemental Fig. 2** and **Supplemental Table 2**). Late and overall glucagon suppression was smaller in women with isolated IFG compared to both other groups (median (Q1-Q3) late suppression: 41.8 (16.5-50.4) [%] vs. 58.1 (43.1-

71.3) [%] vs. 58.9 (46.1-69.6) [%] and overall suppression: 58.9 (39.8-70.2) [%] vs. 71.2 (68.4-81.0) [%] vs. 73.7 (63.8-81.0) [%] in IFG vs. IGT vs. IFG+IGT, respectively). We observed no significant differences in early glucagon suppression and fasting glucagon.

Plasma glucagon patterns are heterogeneous within each metabolic group

The 5-point glucagon curves in response to oral glucose were heterogeneous between individuals (**Fig. 2a**). To examine this further, we calculated continuous, smooth curves from the 5 measurements during the oGTT based on B-spline basis functions (**Fig. 2a**). Stratified by group, these curves confirmed within-group heterogeneity of plasma glucagon dynamics (**Supplemental Fig. 3**). To permit pattern identification, we added a principal component analysis of the curves. The first 3 principal component factors explained 79%, 17%, and 3% of curve variance, respectively (**Fig. 2b**). We used these 3 principal components as input for an unsupervised cluster analysis (**Fig. 2c**). This identified 4 clusters corresponding to 4 distinct patterns of plasma glucagon dynamics (**Fig. 2d**).

Cluster 3 was the largest (n=188; **Table 2**) and showed low mean fasting glucagon and rapid suppression during the oGTT (**Fig. 2d and 3a**). Cluster 2, the second largest (n=62), had higher mean fasting glucagon but equally rapid suppression. Cluster 1 (n=21) had high mean fasting glucagon and delayed suppression and cluster 4 (n=7) had low mean fasting glucagon and a rising curve after glucose ingestion (**Fig. 3a** and **Table 2**).

Cluster 1 contained the highest proportion of women from the prediabetes/diabetes group (53%), followed by cluster 2, cluster 3, and cluster 4. Women in cluster 1 had significantly higher body mass index (BMI), waist circumference, triglycerides, liver fat content, intra-abdominal fat, and lower HDL cholesterol and ISI than those in the other 3 clusters. The disposition index (DI) of cluster 1 was significantly lower than those of cluster 2 and 3 (**Table 2**). Cluster 4 included lean, insulin-sensitive women with a tendency toward low glucose values (**Fig. 3b, c**, and **Table 2**).

Discussion

In our first analysis, we found that women with prediabetes/screening-diagnosed type 2 diabetes had higher fasting glucagon and delayed glucagon suppression during an oGTT compared to healthy control subjects (normoglycemic women after a normoglycemic pregnancy). Normoglycemic women after GDM, a high-risk group for type 2 diabetes (16, 17), lay in between, with values closer to and not statistically different from the control group.

These results are in line with most previous studies that saw the highest fasting glucagon and most impaired glucagon suppression in subjects with diabetes, followed by those with prediabetes, and, at the low end, normoglycemic individuals (10-13, 27). In several non-diabetic cohorts, fasting glucagon was higher in insulin-resistant than in insulin-sensitive subjects (31-33). A majority of studies also found a positive association of plasma glucagon with obesity in groups with similar glucose tolerance (11, 13, 31). Some earlier studies had different findings. Ahren et al. reported that fasting and postprandial glucagon did not differ between IGT and normoglycemic subjects in 84 postmenopausal women (14). Wagner et al. analyzed cohorts of non-diabetic individuals and found that, in 21 to 34% of subjects, glucagon was not suppressed until 120 minutes into the oGTT (15). These individuals were lean and insulin-sensitive, and also had a favorable prognosis of insulin sensitivity over time.

In their recent study, Faerch et al. described that glucagon curves differed between individuals with IFG and those with IGT (27). They found a smaller overall decrease in glucagon during an oGTT in the group with isolated IFG compared to isolated IGT and combined IFG+IGT. Our analysis confirms this result, with the difference in overall glucagon

suppression mainly caused by the late phase of the oGTT (**Supplemental Fig. 2** and **Supplemental Table 2**).

In our second analysis, we saw that plasma glucagon dynamics in the study cohort followed 4 different patterns, based on an unsupervised cluster analysis. The clusters detected did not fully or even closely match the predefined metabolic groups. We consider this the main finding of this paper. Subjects from the prediabetes/diabetes group were over-represented in cluster 1 (with high fasting glucagon and diminished suppression), but still only made up 50% of this cluster, which also contained 25% control subjects. Conversely, the majority of women from the prediabetes/diabetes group (n=39; 55%) fell into cluster 3, the "most normal" cluster (with low fasting glucagon and rapid suppression). Therefore, hyperglucagonemia was not a universal prerequisite for impaired glucose metabolism or early type 2 diabetes. It only affected a subgroup of individuals.

Delayed glucagon suppression was clearly associated with obesity and metabolic syndrome markers in our study. This is evident from the clinical characteristics, e.g. waist circumference, blood lipids, plasma glucose, intra-abdominal and liver fat of the subjects in cluster 1 compared to the other clusters (**Table 2**).

Hepatic steatosis may even be a cause of hyperglucagonemia, as it disrupts hepatic glucagon sensitivity and probably leads to reactive hypersecretion of the hormone (34). The association of liver fat and hyperglucagonemia was found independent of the presence of disrupted glucose metabolism (34, 35).

Impaired early insulin secretion could be another cause of delayed postprandial glucagon suppression, but we do not find evidence for this relationship. Early insulin and c-peptide levels during the oGTT and first-phase insulin secretion in the ivGTT were not reduced in the women in cluster 1. The reduced disposition index results from lower insulin sensitivity (ISI) in this cluster, but not from reduced early insulin secretion (IR30) (**Table 2**). Alpha-cell resistance to inhibition by insulin or a reactive glucagon hypersecretion due to a resistance of the liver are therefore the most likely explanations for our findings.

Another noteworthy observation was the small cluster 4 (n=7; 2.5% of participants), with low fasting glucagon, but rising glucagon levels during the oGTT. The women in this cluster were lean, insulin-sensitive, and had low glucose levels. In this group, the rising glucagon probably is a physiologic response to avoid post-challenge hypoglycemia as a result of an overactive insulin response, which is not uncommon in lean, young women (36). Wagner et al. (15) associated rising glucagon during an oGTT with a favorable metabolic prognosis. Our small and probably not representative sample does not confirm this finding. Five of the 7 women in cluster 4 had had GDM (**Table 2**) and all of these 5 women developed prediabetes or diabetes during the follow-up of this study (mean duration of follow-up was 38.2 months; data not shown). In our cohort, this phenotype is also much less common than reported in the previous publication. However, given the small number of subjects in cluster 4, we find these observations interesting and worth following up on, but we do not claim that they constitute scientific evidence by themselves.

Finally, we believe it is important to use highly specific glucagon assays, in particular to study post-prandial glucagon dynamics. We initially used a standard RIA, which strongly underestimated glucagon suppression (**Supplemental Fig. 1**). This was probably due to cross-reactivity with other peptides cleaved from proglucagon, such as oxyntomodulin, glicentin 1-61 (N-terminally elongated glucagon), and miniglucagon. Intestinal secretion of these peptides increases in the post-prandial state, masking glucagon suppression (21, 37-39). Sandwich ELISAs, with antibodies against the N- and the C-terminal end of the glucagon molecule, circumvent this problem.

Strengths of this study are optimal pre-analytic and analytic techniques plus a cohort homogeneous for age and sex and with little medication and concomitant diseases. We used

functional data analysis to interpret glucagon dynamics and also consider this a strength of our work. This method can extract more of the information contained in a function than classic multivariate statistical techniques (40-42). Together with a subsequent cluster analysis, it permits the grouping of datasets according to their curve shapes. Using a recent history of GDM to identify a high-risk cohort early in the process of type 2 diabetes development should have limited secondary metabolic abnormalities to the minimal extent possible in a human study. At the same time, the study cohort can also be interpreted as a weakness, because results may not apply to the general population. Another limitation of this analysis is its cross-sectional design, which precludes the clarification of cause-effect relationships.

We conclude that fasting hyperglucagonemia and delayed postprandial glucagon suppression associate with insulin resistance, prediabetes and diabetes, but are, in reality, only present in subgroups of individuals. Dysglycemia can develop without elevated plasma glucagon and elevated glucagon does not preclude normoglycemia. Fasting hyperglucagonemia and delayed suppression are strongly linked to obesity and metabolic syndrome. Rising glucagon during an oGTT may be a rare phenomenon. It occurs in insulin-sensitive individuals with a tendency toward hypoglycemia, but does not necessarily indicate metabolic health.

Our results have consequences for the pathophysiologic understanding of type 2 diabetes and for the development of precision treatments. At present, glucagon agonists and antagonists are evaluated for diabetes therapy (1, 2, 43, 44). Based on our findings, patients should probably be stratified by glucagon values for such treatments. For those patients with hyperglucagonemia, glucagon antagonists could be an appropriate therapy, whereas for others, agonists may be useful to induce beneficial effects mediated through the glucagon receptor, such as weight loss (2, 44).

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Author Contributions

Conceptualization and Methodology, A.L. and J.S.; Formal Analysis, M.R.; Investigation, C.G., V.S., F.B., S.M., N.H., and D.P.; Resources, C.H.; Data Curation, V.S. and C.G.; Writing – Original Draft, C.G.; Writing – Review & Editing, A.L.; Visualization, M.R., C.G., and A.L.

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Fig. 1: Glucagon during oral glucose tolerance testing stratified by risk-groups (blue = controls, gray = normoglycemic high-risk, red = prediabetes/diabetes).

Fig. 2: Process of functional data analysis. **a)** Based on the 5-point oGTT data curves, continuous, smooth curves were calculated (median indicated by black line). **b)** Then, a principal component analysis of the curves was conducted (median indicated by solid line; extremes indicated by dotted lines). **c)** The 3 principal components were used as input for an unsupervised cluster analysis (* line types used to represent the clusters in Fig. 3). **d)** Fitted glucagon curves during oral glucose tolerance testing stratified by the 4 clusters (colors: original risk groups as used in Table 1 and Figure 1; blue = controls, gray = normoglycemic high-risk, red = prediabetes/diabetes).

Fig. 3: Means of **a)** glucagon, **b)** glucose, **c)** insulin, and **d)** c-peptide curves during oral glucose tolerance testing stratified by the 4 clusters derived from the glucagon curves (Fig. 2).

Table 1: Baseline characteristics of the PPSDiab study sample

		control	normoglycemic high-risk	prediabetes/diabetes	p-value
n		93	121	71	
Glucose status	NGT	93 (100.0%)	121 (100.0%)	-	
	IFG	-	-	31 (43.7%)	
	IGT	-	-	22 (31.0%)	
	IFG+IGT	-	-	12 (16.9%)	
	type 2 diabetes	-	-	6 (8.5%)	
Age [years]		35.3±4.2	35.2±4.5	35.9±4.5	0.6204
Mean blood pressure [mmHg] (missing=1)		85.8±9.0	89.0±8.6[a]	90.9±10.3[a]	0.0026
BMI [kg/m^2] (missing=4)		23.7±4.0	25.2±5.8	28.2±7.1[a, b]	0.0001
Waist circumference [cm] (missing=5)		78.1±8.9	80.7±11.2	86.6±13.2[a, b]	0.0002
hsCRP [mg/dl]		0.04 (0.01-0.08)	0.06 (0.02-0.25)[a]	0.09 (0.02-0.30)[a]	0.0030
Triglycerides [mg/dl]		61.0 (51.0-77.0)	65.0 (50.0-87.0)	81.0 (62.0-130.0)[a, b]	<0.0001
HDL cholesterol [mg/dl]		64.0 (57.0-73.0)	63.0 (56.0-73.0)	56.0 (46.0-65.0)[a, b]	<0.0001
LDL cholesterol [mg/dl]		104.0 (88.0-118.0)	105.0 (89.0-120.0)	104.0 (85.0-124.0)	0.9035
Plasma glucose 0 min [mg/dl]		89.0 (83.0-92.0)	91.0 (87.0-95.0)	102.0 (97.0-106.0)[a, b]	<0.0001
Plasma glucose 120 min [mg/dl]		93.0 (81.0-108.0)	114.0 (96.0-122.0)[a]	141.0 (113.0-165.0)[a, b]	<0.0001
ISI (missing=1)		6.8 (5.2-8.6)	5.5 (3.7-7.5)[a]	3.3 (2.1-4.6)[a, b]	<0.0001
DI (missing=1)		297.4 (221.4-363.1)	246.6 (179.7-322.0)	160.0 (111.4-207.6)[a, b]	<0.0001
FPIR (missing=152)		2.2 (1.4-3.5)	2.2 (1.6-3.5)	2.3 (1.5-3.9)	0.8218
Liver fat content [%] (missing=132)		0.2 (0.0-0.8)	0.5 (0.0-1.1)	1.7 (0.0-4.1)[a, b]	0.0122
Intra-abdominal fat [l] (missing=124)		1.4 (0.9-2.1)	1.8 (1.1-2.9)[a]	2.3 (1.3-3.2)[a]	0.0046
Glucagon 0 min [pmol/l]		6.0 (4.6-8.2)	6.6 (4.5-8.4)	7.7 (5.6-11.2)[a, b]	0.0069
Glucagon 30 min [pmol/l]		3.0 (2.4-4.7)	3.7 (2.5-4.9)	5.0 (3.0-7.6)[a, b]	<0.0001
Glucagon 60 min [pmol/l]		1.9 (1.4-3.1)	2.6 (1.8-3.7)	2.9 (2.0-4.4)[a]	0.0009
Glucagon 90 min [pmol/l]		2.1 (1.3-3.0)	2.1 (1.6-3.2)	2.5 (1.8-3.9)	0.0527
Glucagon 120 min [pmol/l]		2.3 (1.4-3.5)	2.2 (1.5-3.3)	2.3 (1.6-3.5)	0.5239
AUC glucagon		339.4 (248.5-473.6)	392.1 (283.5-518.2)	511.5 (353.4-615.2)[a, b]	0.0006
Early suppression glucagon (0-30) [%]		47.6 (32.8-57.9)	41.3 (22.9-58.3)	32.0 (14.5-51.3)[a]	0.0055
Late suppression glucagon (30-120) [%]		31.8 (8.9-49.6)	40.9 (14.9-56.7)	47.4 (33.3-63.6)[a, b]	<0.0001
Suppression glucagon (0-120) [%]		61.2 (48.2-76.9)	64.1 (49.5-74.4)	68.5 (57.3-75.0)	0.3130

significant post hoc tests [a]vs. control [b]vs. normoglycemic high-risk

AUC: area under the curve, FPIR: first phase insulin response in intravenous glucose tolerance testing, GDM: previous gestational diabetes, NGT: normal glucose tolerance, IFG: impaired fasting glucose, IGT: impaired glucose tolerance, hsCRP: high-sensitivity c-reactive protein, ISI: insulin sensitivity index.

Table 2: Baseline characteristics of the PPSDiab study sample, stratified by clusters of glucagon dynamics.

		Cluster 1	Cluster 2	Cluster 3	Cluster 4	p-value
n		28	62	188	7	
Risk group	Control	7 (25.0%)	19 (30.7%)	65 (34.6%)	2 (28.6%)	0.0279
	normoglycemic high-risk	6 (21.4%)	27 (43.6%)	84 (44.7%)	4 (57.1%)	
	prediabetes/diabetes	15 (53.6%)	16 (25.8%)	39 (20.7%)	1 (14.3%)	
Glucose status	NGT	13 (46.4%)	46 (74.2%)	149 (79.3%)	6 (85.7%)	0.0099
	IFG	5 (17.9%)	6 (9.7%)	19 (10.1%)	1 (14.3%)	
	IGT	3 (10.7%)	7 (11.3%)	12 (6.4%)	0	
	IFG+IGT	3 (10.7%)	3 (4.8%)	6 (3.2%)	0	
	type 2 diabetes	4 (14.3%)	0	2 (1.1%)	0	
Age [years]		33.5±4.8	35.5±4.4	35.7±4.3[1]	35.0±4.0	0.0315
Mean blood pressure [mmHg] (missing=1)		96.2±8.6	89.4±9.2	87.0±9.0	85.6±7.4	<0.0001
BMI [kg/m^2] (missing=4)		33.3±6.1	26.5±6.4[1]	24.0±4.6[1]	21.6±1.5[1]	<0.0001
Waist circumference [cm] (missing=5)		96.0±11.9	83.8±12.3[1]	78.6±9.3[1]	73.5±4.1[1]	<0.0001

hsCRP [mg/dl]		0.19 (0.07-0.47)	0.05 (0.01-0.17)[1]	0.04 (0.01-0.12)[1]	0.12 (0.05-0.38)	0.0004
triglycerides [mg/dl]		91.5 (58.5-132.0)	62.5 (53.0-83.0)	67.5 (53.0-88.5)	63.0 (58.0-91.0)	0.0898
HDL cholesterol [mg/dl]		49.0 (44.5-61.5)	62.0 (51.0-73.0)	63.0 (56.0-73.0)	65.0 (56.0-70.0)	0.0012
Plasma glucose 0 min [mg/dl]		97.5 (90.5-106.0)	91.0 (88.0-97.0)	91.0 (86.0-97.0)	87.0 (82.0-92.0)	0.0078
Plasma glucose 120 min [mg/dl]		127.0 (115.5-154.5)	113.5 (95.0-130.0)[1]	106.5 (90.0-121.5)[1]	80.0 (74.0-92.0)[1, 2]	<0.0001
ISI (missing=1)		2.5 (1.9-4.3)	5.0 (3.3-6.9)[1]	5.8 (4.2-8.1)[1]	7.9 (5.6-8.3)[1]	<0.0001
DI (missing=1)		152.0 (96.5-247.8)	230.2 (165.3-392.0)[1]	252.8 (176.7-324.4)[1]	232.9 (156.2-276.4)	0.0007
IR30 (missing=1)		55.7 (37.1-82.2)	50.3 (36.4-86.1)	41.6 (30.9-60.1)	28.7 (26.2-41.3)[1, 2]	0.0023
FPIR (missing=152)*		3.9 (2.2-6.2)	3.3 (2.2-4.3)	2.1 (1.4-3.1)	2.1 (1.0-2.7) n=3	0.0140
Liver fat content [%] (missing=131)		2.4 (1.1-6.4)	0.7 (0.0-1.7)[1]	0.3 (0.0-0.8)[1]	0.1 (0.0-0.5)[1]	<0.0001
Intra-abdominal fat [l] (missing=124)		3.4 (2.9-4.4)	2.0 (1.5-3.0)[1]	1.5 (1.0-2.3)[1, 2]	1.1 (0.9-1.6)[1, 2]	<0.0001

Significant post hoc test: [1]significant vs. cluster 1, [2] significant vs. cluster 2
*the post-hoc test for FPIR was conducted both including cluster 4 and after exclusion of cluster 4 (due to the small group size in cluster 4) - in any case, the post-hoc test has not reached significance.
NGT: normal glucose tolerance, IFG: impaired fasting glucose, IGT: impaired glucose tolerance, hsCRP: high-sensitivity c-reactive protein, ISI: insulin sensitivity index, DI: disposition index, IR30: insulin release 0' to 30' in the oral glucose tolerance test, FPIR: first phase insulin response in the intravenous glucose tolerance test.