Nonsuppressed Glucagon After Glucose Challenge as a Potential Predictor for Glucose Tolerance

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Glucagon levels are classically suppressed after glucose challenge. It is still not clear as to whether a lack of suppression contributes to hyperglycemia and thus to the development of diabetes. We investigated the association of postchallenge change in glucagon during oral glucose tolerance tests (OGTTs), hypothesizing that higher postchallenge glucagon levels are observed in subjects with impaired glucose tolerance (IGT). Glucagon levels were measured during OGTT in a total of 4,194 individuals without diabetes in three large European cohorts. Longitudinal changes in glucagon suppression were investigated in 50 participants undergoing a lifestyle intervention. Only 66–79% of participants showed suppression of glucagon at 120 min (fold change glucagon$_{120/0}$ <1) during OGTT, whereas 21–34% presented with increasing glucagon levels (fold change glucagon$_{120/0}$ ≥1). Participants with nonsuppressed glucagon$_{120}$ had a lower risk of IGT in all cohorts (odds ratio 0.44–0.53, $P < 0.01$). They were also leaner and more insulin sensitive and had lower liver fat contents. In the longitudinal study, an increase of fold change glucagon$_{120/0}$ was associated with an improvement in insulin sensitivity ($P = 0.003$). We characterize nonsuppressed glucagon$_{120}$ during the OGTT. Lower glucagon suppression after oral glucose administration is associated with a metabolically healthier phenotype, suggesting that it is not an adverse phenomenon.

Under normal circumstances, glucagon levels are expected to decline after oral glucose load. Rodent diabetes models suggest that glucagon action is an important cause of hyperglycemia (1). In humans, elevated fasting glucagon is clearly associated with insulin resistance (2) and diabetes (3). Important studies involving 20–31 participants showed that a suppression of glucagon after an oral glucose load is reduced in diabetes and impaired glucose tolerance (IGT) (4–6). Thus, if glucagon made a causal contribution to diabetes pathogenesis, people with IGT would present with only a slight suppression (or even an increase of glucagon) during the oral glucose tolerance test (OGTT).

We therefore proposed that a reduced suppression of glucagon during OGTT is associated with IGT. To test this hypothesis, we analyzed cross-sectional data from three

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large European cohorts. All participants underwent standardized OGTTs. Two of the cohorts are enriched for prediabetes. On the basis of previous data, in which a sustained suppression of glucagon for up to 300 min after oral glucose challenge (4–6) was observed, we compared fasting glucagon with 2-h postchallenge glucagon levels to determine glucagon suppression during OGTT. We also studied the longitudinal changes of glucagon suppression in a subgroup of individuals undertaking a 9-month lifestyle intervention.

RESEARCH DESIGN AND METHODS

Subjects

Tübingen Family Study and Tübingen Lifestyle Intervention Program

Data were analyzed from subjects who had participated in the Tübingen Family Study (TUEF) and the Tübingen Lifestyle Intervention Program (TULIP). In TUEF, people without diabetes with a family history of type 2 diabetes, BMI >27 kg/m^2, or a previous diagnosis of IGT underwent metabolic phenotyping. Glucagon was originally measured in 625 subjects of the early TUEF cohort, 23 of whom had incident diabetes. The latter were excluded from the study. Clinical characteristics are given in Supplementary Table 1.

TULIP was a standardized lifestyle intervention study designed to identify predictors of metabolic changes over 9 months of moderate exercise and diet. In this project, glucagon was measured during OGTTs in 50 subjects.

The study protocols were approved by the ethics committee of the University of Tübingen. All participants gave written informed consent.

Prevalence, Prediction and Prevention of Diabetes-Botnia Study

The Prevalence, Prediction and Prevention of Diabetes (PPP)-Botnia Study is a population-based study in the Botnia region of Western Finland (7). It was designed to obtain accurate estimates of prevalence and risk factors for diabetes and prediabetes. The baseline study was conducted from 2004 to 2008 in five Botnia centers comprising a total population of 135,000 people. In total, 5,208 women and men participated (54.7% of those invited). The participants gave their written informed consent, and the study protocol was approved by the ethics committee of Helsinki University Hospital.

Plasma glucagon was measured in participants recruited after 2006. We excluded 98 subjects with incident diabetes, and 1,369 had complete data sets for all intermediary traits. Clinical details are given in Supplementary Table 2.

Malmö Diet and Cancer Study

The study population consisted of those men and women who had participated in the 2007–2012 (reexamination) investigation of the cardiovascular cohort of the Malmö Diet and Cancer Study (MDCS) (8,9). All participants in the baseline study (n = 6,103) who were alive and who had not emigrated from Sweden (n = 4,924) were invited to the reexamination. A total of 3,734 subjects attended the follow-up investigation (76% of the eligible population). Of these, 2,371 had complete information for all analyzed traits. A further 148 participants had since developed diabetes and were therefore excluded from the analysis. All participants provided written informed consent, and the study was approved by the Lund University Ethics Review Committee.

Measurements

In all studies, subjects participated in a 75-g OGTT after an overnight fast.

In TUEF and TULIP, blood samples were drawn after 0, 30, 60, 90, and 120 min. They were immediately put on ice, centrifuged, and stored at −80°C. The glucose-oxidase method was used to determine plasma glucose (Yellow Springs Instruments). Plasma insulin was determined by chemiluminescence assays for ADVIA Centaur (Siemens Medical Solutions).

In all studies, glucagon was collected in EDTA tubes containing aprotinin and directly stored at −80°C (TUEF and TULIP) or −20°C (MDCS and PPP-Botnia). In TUEF/TULIP and PPP-Botnia, the samples were thawed only for the glucagon assay after a mean lag of 6 months and 1.3 years. respectively, well before the actual analysis of the data. For practical reasons, the analyses were performed in MDCS and PPP-Botnia after all samples had been collected. Glucagon was measured at fasting and 120 min in the data sets of all study centers. In TUEF, glucagon was also measured after 30, 60, and 90 min (n = 598, 447, and 446, respectively). We used the commercially available radioimmunoassay (Linco Research/Millipore, St. Charles, MO) (10). The analytical detection limit was 5.3 pmol/L, and intra- and interassay coefficients of variation were <6.8% and <13.5%, respectively. Liver fat was measured in 148 participants by MRS as previously described (10).

In PPP-Botnia and MDCS, blood samples were obtained after 0, 30, and 120 min. Plasma glucose was measured using the glucose dehydrogenase method (Hemocue). Serum insulin was measured by a fluoroimmunometric assay (AutoDELFIA; PerkinElmer) in PPP-Botnia and by ELISA (Dako, Glostrup, Denmark) in MDCS.

Calculations and Statistics

Classification of prediabetes was performed according to American Diabetes Association criteria (normal glucose tolerance [NGT], impaired fasting glycemia [IFG], and IGT). The areas under the curve (AUCs) were calculated by the trapezoid method (for glucagon using time points 0, 30, and 120; for other measures all five time points in TUEF). The OGTT-derived insulin sensitivity index (ISI-OGTT) was estimated as ISI-OGTT = $10{,}000\sqrt{[(Glu_0 \cdot Ins_0 \cdot Glu_{mean} \cdot Ins_{mean})]}$. Insulin secretion was assessed by calculating the insulinogenic index [IGI = (insulin 30 − insulin 0)/(glucose 30 − glucose 0)].

Table 1—TUEF study: difference in metabolic traits during OGTT between the phenotypes with suppressed and nonsuppressed glucagon

	Suppressed glucagon$_{120}$ during OGTT (n = 474)		Nonsuppressed glucagon$_{120}$ during OGTT (n = 128)			P† adjusted for sex, age, BMI	P† adjusted for sex, age, BMI, glucagon$_0$
	Median/%	IQR/95% CI	Median/%	IQR/95% CI	P*		
Sex (females)	62%	(57–66)‡	81%	(74–87)‡	<0.0001	NA	NA
Age (years)	37.0	(30.0–49.0)	39.0	(31.0–48.0)	0.22	NA	NA
BMI (kg/m^2)	27.3	(23.7–32.7)	24.8	(22.4–28.3)	<0.0001	NA	NA
Fasting glucose (mmol/L)	5.1	(4.8–5.4)	5.1	(4.8–5.3)	0.89	0.67	0.56
Glucose at 30 min (mmol/L)	8.4	(7.3–9.4)	8.1	(7.0–9.1)	0.04	0.24	0.86
Glucose at 60 min (mmol/L)	8.2	(6.7–9.9)	7.6	(5.9–8.9)	0.002	0.05	0.18
Glucose at 90 min (mmol/L)	6.9	(5.6–8.7)	5.9	(4.9–6.9)	<0.0001	<0.0001	0.0003
Glucose at 120 min (mmol/L)	6.3	(5.3–7.6)	5.5	(4.8–6.3)	<0.0001	<0.0001	<0.0001
AUC glucose (mmol/L × 120 min)	14.6	(12.6–16.8)	13.3	(11.8–15.3)	<0.0001	0.001	0.03
Fasting insulin (pmol/L)	50.0	(33.0–81.0)	38.0	(29.0–49.5)	<0.0001	0.002	0.08
Postchallenge insulin$_{120}$ (pmol/L)	323.0	(183.8–607.3)	217.0	(143.0–342.5)	<0.0001	<0.0001	0.002
AUC insulin (pmol/L × 120 min)	380	(245–615)	274	(196–398)	<0.0001	0.003	0.09
Fasting glucagon (pmol/L)	19.2	(15.4–24.3)	14.2	(11.7–17.4)	<0.0001	<0.0001	NA
Postchallenge glucagon$_{120}$ (pmol/L)	14.5	(11.3–17.8)	16.8	(13.9–20.3)	<0.0001	<0.0001	<0.0001
AUC glucagon (pmol/L × 120 min)	16.7	(13.4–20.6)	16.2	(13.4–19.8)	0.27	0.31	<0.0001
ISI (OGTT derived, arbitrary units)	12.2	(7.6–20.8)	17.8	(13.4–25.8)	<0.0001	0.0003	0.03
Insulinogenic index (arbitrary units)	110.7	(69.1–182.9)	96.7	(60.7–164.5)	0.11	0.76	0.31
Hepatic fat content (%) (n = 148)	4.0	(1.8–10.2)	2.3	(1.2–3.7)	0.001	0.009	0.09

*Compared with the Wilcoxon rank sum test for nonnormally distributed variables and with Student t test for variables with normal distribution. Binary outcomes were compared with χ^2 test; †in linear regression models or for binary outcomes, logistic models were adjusted for the given covariates. Nonnormally distributed variables were log transformed prior to analysis; ‡data given are 95% CI. NA, not applicable.

Analytes determined at specific OGTT time points are shown with the minutes of OGTT as subscript (e.g., glucagon$_{120}$ for glucagon at the end of OGTT). Data are shown as mean ± SD or median (interquartile range [IQR]). The Wilcoxon rank sum/Kruskal-Wallis test was used to compare continuous variables with nonnormal distribution. Normally distributed variables were compared using Student t test/ANOVA. Categorical variables were compared by χ^2 tests. Multivariable linear regression models or, in the event of dichotomous outcomes, logistic models were used. Variables

Table 2—MDCS: difference in metabolic traits during OGTT between the phenotypes with suppressed and nonsuppressed glucagon

	Suppressed glucagon during OGTT (n = 1,461)		Nonsuppressed glucagon during OGTT (n = 762)			P† adjusted for sex, age, BMI	P† adjusted for sex, age, BMI, glucagon$_0$
	Median/%	IQR	Median/%	IQR	P†		
Sex (females)	53%		75%		<0.0001	NA	NA
Age (years)	72	(67–76)	73	(68–78)	0.006	NA	NA
BMI (kg/m^2)	26.5	(24.2–29.1)	25.3	(23.2–27.8)	<0.0001	NA	NA
Fasting glucose (mmol/L)	5.8	(5.4–6.2)	5.7	(5.4–6.2)	0.002	0.40	0.6
Glucose at 120 min (mmol/L)	6.8	(5.6–8.2)	6.1	(5.0–7.3)	<0.0001	<0.0001	<0.0001
Fasting insulin (pmol/L)	56.9	(40.3–77.4)	42.4	(32.6–59.0)	<0.0001	<0.0001	<0.0001
Insulin at 30 min (pmol/L)	305.6	(211–435.5)	282.0	(201.4–404.3)	0.019	0.43	0.24
Insulin at 120 min (pmol/L)	291.0	(189.6–463.9)	219.8	(147.1–348.3)	<0.0001	<0.0001	<0.0001
Fasting glucagon (pmol/L)	22.7	(19.5–26.93)	18.9	(15.8–22.4)	<0.0001	<0.0001	NA
Glucagon at 120 min (pmol/L)	19.2	(16.4–22.4)	21.8	(18.3–25.3)	<0.0001	<0.0001	NA
ISI (OGTT derived, arbitrary units)	4.8	(3.1–7.2)	6.6	(4.4–10.1)	<0.0001	<0.0001	<0.0001

†In linear regression models or for binary outcomes, logistic models adjusted for the given covariates. Nonnormally distributed variables were log transformed. NA, not applicable.

with skewed distributions were $\log_e$ transformed. The relative change of glucagon from 0 to 120 min ($glucagon_{120}$/$glucagon_0$) is referred to as fold change $glucagon_{120/0}$ throughout the article. Calculations were performed with JMP10 for TUEF and SPSS22 for PPP-Botnia and MDCS.

RESULTS

Cross-sectional Analysis

Fasting glucagon ($glucagon_0$) was consistently higher in subjects with IFG+IGT than in subjects with NGT ($P < 0.02$). As anticipated, $glucagon_{120}$ was lower than $glucagon_0$ in all cohorts ($P < 0.0001$), indicating that a gross suppression of glucagon occurs during OGTT. We calculated the fold change of glucagon between fasting and 120 min (fold change $glucagon_{120/0}$), which had high variation in all cohorts (TUEF: 0.84 ± 0.25; PPP-Botnia: 0.87 ± 0.24; MDCS: 0.94 ± 0.19). A substantial proportion of participants (21–34%) in all three cohorts had higher glucagon 120 min after glucose load (nonsuppressed $glucagon_{120}$).

Contrary to our primary hypothesis, nonsuppressed $glucagon_{120}$ (fold change $glucagon_{120/0} \geq 1$) was associated with a lower IGT risk (odds ratio [OR] 0.44–0.53 in all cohorts, $P \leq 0.009$); this remained essentially unchanged after adjustment for sex, age, and BMI (OR 0.44–0.47, $P < 0.001$). Further phenotypic differences between subjects with suppressed and nonsuppressed glucagon in TUEF and MDCS are shown in Tables 1 and 2. The five-point OGTT course of glucagon, glucose, and insulin in TUEF is shown in Fig. 1*A–C*. To enable us more clearly to distinguish between participants with high and low fold change $glucagon_{120/0}$, we stratified subjects to decreasing, stable, and increasing glucagon (fold change $glucagon_{120/0}$ <0.9, 0.9–1.1, and >1.1, respectively) in the PPP-Botnia cohort (Table 3). Irrespective of stratification, participants with nonsuppressed or increasing $glucagon_{120}$ were leaner and more insulin sensitive in all cohorts, regardless of age, sex, or BMI. To detect potential confounding by substantially lower fasting glucagon levels in the nonsuppressed $glucagon_{120}$ subgroup, we additionally adjusted for fasting glucagon. Nonsuppressed $glucagon_{120}$ was still associated with higher insulin sensitivity after adjustment for fasting glucagon (TUEF: $P = 0.03$; MDCS: $P < 0.0001$).

Since glucagon may be a more important contributor of postchallenge hyperglycemia at earlier time points of the OGTT than $glucagon_{120}$, we also investigated glucagon suppression after 30, 60, and 90 min and its association with IGT. Nonsuppressed $glucagon_{30}$, $glucagon_{60}$, and $glucagon_{90}$ were seen in 40%, 27%, and 23% of subjects of the TUEF cohort, but none were associated with IGT (OR 1.30, $P = 0.31$; OR 1.36, $P = 0.32$; OR 1.12, $P = 0.72$, respectively).

Glycemic data in TUEF and PPP-Botnia stratified by prediabetes categories are shown in Supplementary Tables 1 and 2. Mean levels of glucose, glucagon, and insulin during the five-point OGTT (Supplementary Table 4) with an aggregated plot of relative glucagon courses (Supplementary Fig. 1) suggest that the lack of glucagon suppression was not specific during the last 30 min of the OGTT. When directly comparing glucagon suppression between NGT, IFG, and IGT in the TUEF study at the intervals 0–30, 0–60, 0–90, and 0–120, only fold change $glucagon_{120/0}$ was different (Supplementary Table 5).

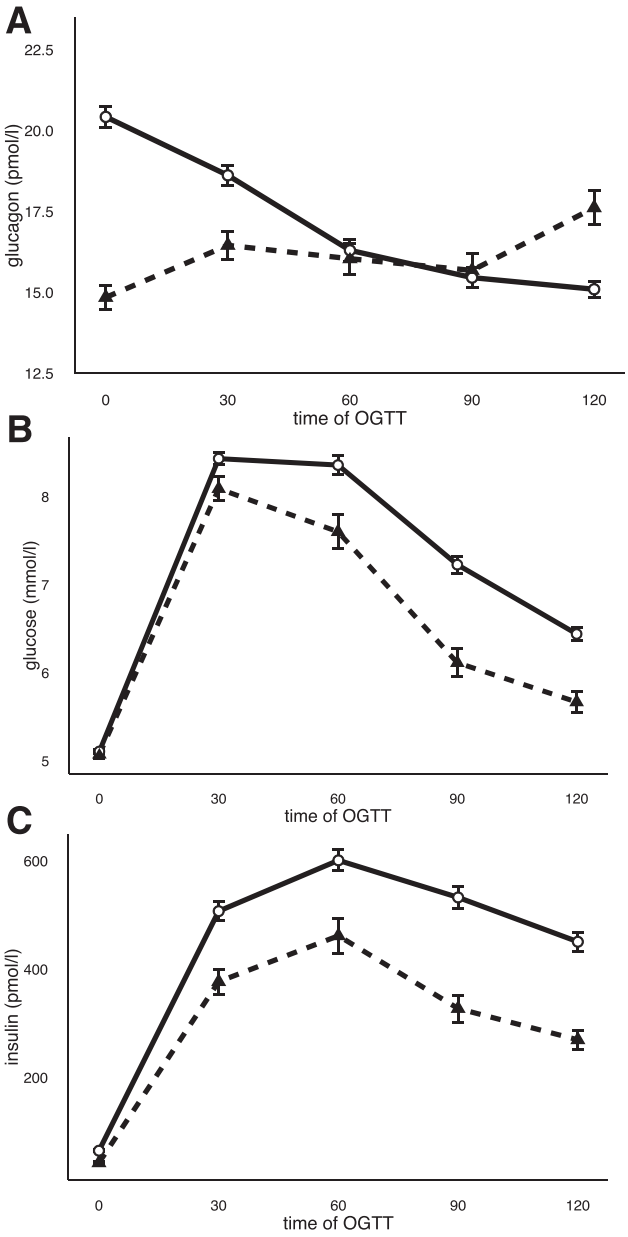

Figure 1—Suppressed and nonsuppressed glucagon during OGTT. Mean glucagon, glucose, and insulin levels per subphenotype (open circles and continuous line represent subjects with suppressed glucagon; closed triangles and dashed line represent subjects with nonsuppressed glucagon) are shown in *A*, *B*, and *C*, respectively. Error bars represent SEM.

In PPP-Botnia, significantly fewer of the 98 individuals with incident diabetes had increasing or stable $glucagon_{120}$ than subjects without diabetes (25 vs. 39%, $P = 0.002$).

Longitudinal Analysis

We also analyzed longitudinal data from the TULIP intervention study (see Supplementary Table 3). We

Table 3—PPP-Botnia study: difference in metabolic traits during OGTT between the phenotype with decreasing, stable, and increasing glucagon

	Decreasing glucagon n = 833 (61%)		Stable glucagon n = 356 (26%)		Increasing glucagon n = 180 (13%)				
	Median/%	IQR	Median/%	IQR	Median/%	IQR	P§	P† adjusted for sex, age, BMI	P† adjusted for sex, age, BMI, glucagon$_0$
Sex (females)	45.3		64.1		73.7		<0.0001	NA	NA
Age (years)	47.2	37.6 to 61.0	47.5	37.3 to 61.2	48.3	38.5 to 59.9	0.873	NA	NA
BMI (kg/m^2)	26.7	23.9 to 29.5	25.3	23.0 to 28.0	24.1	22.5 to 27.0	<0.0001	NA	NA
Fasting glucose (mmol/L)	5.5	5.2 to 5.9	5.6	5.2 to 5.8	5.6	5.2 to 5.8	0.753	0.25	0.14
Glucose at 30 min (mmol/L)	8.6	7.6 to 9.6	8.3	7.3 to 9.1	7.8	6.8 to 8.8	<0.0001	0.01	0.04
Glucose at 120 min (mmol/L)	5.5	4.5 to 6.7	4.9	4.1 to 5.9	4.7	3.9 to 5.4	<0.0001	<0.0001	<0.0001
ΔGlucose (120 min − 0 min)	0	−0.9 to 1.1	−0.6	−1.4 to 0.3	−0.9	−1.6 to 0.1	<0.0001	<0.0001	<0.0001
Fasting insulin (pmol/L)	44.5	29.9 to 61.9	33.4	24.3 to 46.6	27.8	19.5 to 38.2	<0.0001	<0.0001	<0.0001
Postchallenge insulin (pmol/L)	188.3	117.5 to 315.5	130.7	74.4 to 205.0	107.7	67.4 to 176.5	<0.0001	<0.0001	<0.0001
ΔInsulin (120 min − 0 min)	144.6	81.3 to 259.2	96.6	44.5 to 167.5	84.1	40.3 to 143.9	<0.0001	<0.0001	<0.0001
Fasting glucagon (pmol/L)	19.8	16.4 to 23.8	17.1	14.1 to 20.0	13.8	11.2 to 16.9	<0.0001	<0.0001	NA
Postchallenge glucagon$_{120}$ (pmol/L)	14.1	11.9 to 17.5	16.6	13.8 to 19.5	16.6	14.6 to 21.2	<0.0001	<0.0001	0.98
ISI (OGTT derived, arbitrary units)	6.9	4.7 to 10.2	9.3	6.6 to 12.5	11.2	8.0 to 14.3	<0.0001	<0.0001	<0.0001

Decreasing glucagon, postchallenge glucagon >10% lower than fasting glucagon; increasing glucagon, postchallenge glucagon >10% higher than fasting glucagon; stable glucagon, postchallenge glucagon ± 10% of fasting glucagon. §P value for ANOVA or χ^2 for categorical variables, nonnormally distributed variables were log transformed when appropriate; †logistic regression analysis with decreasing/increasing glucagon as independent variable, stable group excluded. NA, not applicable.

investigated whether individual change of fold change glucagon$_{120/0}$ is capable of predicting the change of ISI-OGTT during lifestyle intervention. Increasing fold change glucagon$_{120/0}$ during the course of the lifestyle intervention was significantly associated with increasing ISI-OGTT after appropriate adjustment for covariates (P = 0.003) (see Supplementary Table 5).

DISCUSSION

Whereas a mixed-meal test elevates glucagon levels (11), postprandial glucagon is expected to decline in subjects without diabetes after oral pure glucose load (4–6). In a smaller study, reduced glucagon suppression was found to be associated with insulin resistance, IGT, and diabetes (4). Thus, we hypothesized that nonsuppressed glucagon after glucose challenge associates with postchallenge hyperglycemia.

Glucagon$_{120}$ was found not to be suppressed in 21–34% of the 4,194 subjects who participated in the three independent cohorts. Individuals with nonsuppressed glucagon$_{120}$ were consistently leaner and had a lower risk of IGT and higher insulin sensitivity. In the subgroup undergoing MRS, these participants also had less liver fat than those with the classic decrease in glucagon. Of note, subjects with nonsuppressed glucagon$_{120}$ had lower fasting glucagon levels that potentially also contribute to the observed phenotype of nonsuppressed glucagon$_{120}$. However, similar AUC glucagon levels and additional controlling for fasting glucagon suggested that fasting glucagon does not solely explain the association between glucagon suppression and insulin sensitivity. Thus, there appears to be an association between nonsuppressed glucagon 2 h after glucose challenge and a metabolically healthier phenotype that is not connected with low fasting glucagon.

A recent investigation by Færch et al. (12) examining glucagon levels in ~1,400 individuals at 0, 30, and 120 min during an OGTT found lower early glucagon suppression between minutes 0 and 30 and higher late glucagon suppression between minutes 30 and 120 in subjects with prediabetes and incident diabetes as compared with subjects with NGT. Whereas our data on late glucagon suppression (fold change$_{120/0}$) are in accordance with this, the five-point OGTT data in the TUEF cohort show a lack of a difference for earlier time intervals in the OGTT between NGT and prediabetes (30/0, 60/0, and 90/0) (Supplementary Table 5). In addition, the article by Færch et al. (12) showed that higher insulin sensitivity was associated with higher early glucagon suppression and lower late glucagon suppression. The latter is, again, corroborated by our data showing higher insulin sensitivity in patients with higher fold change glucagon$_{120/0}$. In sum, these data suggest that the insulin-sensitive and/or NGT phenotype is characterized by a nonsuppression of glucagon$_{120}$.

It has to be noted that glucagon levels were about twofold higher in our study compared with the work of Færch et al. (12). In contrast to the acknowledged proprietary assay in the study by Færch et al., our measurements were performed by a commercial assay in a multicenter setting, and the data were consistent in all our cohorts.

Whether the association of nonsuppressed glucagon$_{120}$ with NGT and higher insulin sensitivity is a physiologic mechanism pertaining to the prevention of late postprandial hypoglycemia cannot be determined from our descriptive studies.

However, it is intriguing to consider the potential physiologic consequences of increasing glucagon levels during the late OGTT. It has been shown that glucagon action in the central nervous system leads to an inhibition of hepatic glucose production, constraining the overshoot of hyperglycemia after a surge of plasma glucagon (13). Glucagon is an important satiety signal and enhances adaptive thermogenesis (14). In our study, individuals with nonsuppressed glucagon$_{120}$ had a significantly lower BMI. Hepatic triglyceride content has a strong inverse association with nonsuppressed glucagon$_{120}$ in our data. The fact that glucagon inhibits hepatic triglyceride synthesis and enhances β-oxidation may raise the possibility that glucagon is protective against hepatic steatosis (15). Indeed, glucagon can treat fatty liver in cows (16). Glucagon/GLP-1 coagonists are under clinical development for the treatment of obesity and diabetes (17). Sodium–glucose cotransporter 2 inhibitors have been implicated in a direct stimulation of glucagon secretion in the α-cell (18), and liraglutide induces a "paradoxical" increase in postchallenge glucagon on chronic use (19). Nonsuppressed glucagon could therefore contribute to the beneficial metabolic effects of these two drug classes.

The associations presented in our data do not allow causal inference. A further limitation is that glucagon measurement can be influenced by degradation and varying assay specificity. However, a systematic bias is unlikely, because samples were treated uniformly within studies and the results are consistent in all investigated cohorts.

In summary, we characterize the increase of glucagon from fasting to 120 min postchallenge as a phenomenon associated with a desirable metabolic phenotype. These data could provide an important context for the evolving appreciation of glucagon's multifaceted role in metabolism.

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