Insulin resistance is accompanied by increased fasting glucagon and delayed glucagon suppression in individuals with normal and impaired glucose regulation

Short title: Glucagon and insulin sensitivity

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ABSTRACT

Hyperinsulinemia is an adaptive mechanism that enables the maintenance of normoglycemia in the presence of insulin resistance. We assessed whether glucagon is also involved in the adaptation to insulin resistance. A total of 1,437 individuals underwent an oral glucose tolerance test with measurements of circulating glucose, insulin, and glucagon concentrations at 0, 30 and 120 min. Early glucagon suppression was defined as suppression in the period from 0 to 30 min and late glucagon suppression as 30 to 120 min after glucose intake. Insulin sensitivity was estimated by the validated insulin sensitivity index. Individuals with screen-detected diabetes had 30% higher fasting glucagon levels, diminished early glucagon suppression, but greater late glucagon suppression when compared to individuals with normal glucose tolerance ($P \leq 0.014$). Higher insulin resistance was associated with higher fasting glucagon levels, less early glucagon suppression, and greater late glucagon suppression ($P < 0.001$). The relationship between insulin sensitivity and fasting glucagon concentrations was non-linear ($P < 0.001$). In conclusion, increased fasting glucagon levels and delayed glucagon suppression, together with increased circulating insulin levels, develop in parallel with insulin resistance. Therefore, glucose maintenance during insulin resistance may depend not only on hyperinsulinemia but also on the ability to suppress glucagon early after glucose intake.

INTRODUCTION

Glucagon acts as a counter-regulatory hormone to insulin and is therefore essential for glucose regulation both in the fasting state and during glucose intake[1]. In type 2 diabetes, fasting blood glucagon levels are elevated, but the reason for this dysregulation is not clearly established[2-5]. Glucagon suppression in response to oral glucose is also diminished in overweight and obese individuals with impaired glucose tolerance[6-8], and the suppression appears related to insulin sensitivity in individuals with normal glucose tolerance[9,10]. However, studies involving isoglycemic oral and intravenous glucose challenges in patients with type 2 diabetes[11] and first-degree relatives[12] have indicated that the failure to suppress glucagon secretion is mainly observed during the first 30-120 min after oral glucose intake, whereas normal suppression is seen after 2-4 hours. These findings could indicate that the timing of glucagon suppression may play an important role for glucose regulation.

The secretion of glucagon is complex, involving a combination of paracrine, autocrine, hormonal as well as autonomic neural mechanisms[13]. Defective suppression of glucagon by glucose and insulin as well as insulin resistance in the alpha cells have been suggested as potential mechanisms for the hyperglucagonemia in type 2 diabetes[1]. However, in addition to glucose and insulin, glucagon secretion is regulated by gut incretin hormones. The most important ones are Glucagon-Like Peptide-1 (GLP-1), which inhibits glucagon secretion[14], and Glucose-dependent Insulinotropic Polypeptide (GIP), which stimulates glucagon secretion[15]. Therefore, the interplay between glucose, insulin, GLP-1 and GIP is important to consider when studying glucagon secretion in vivo.

We hypothesized the existence of a mechanism linking alpha cell hypersecretion/ hyposuppressibility to a reduction in insulin sensitivity as has been established for beta cell

secretion[16]. As a consequence, hyperglucagonemia and a failure to adequately suppress glucagon as insulin resistance increases could contribute to the impaired glucose regulation in pre-diabetes and type 2 diabetes. We examined in a large cohort of individuals at low to high diabetes risk 1) whether plasma glucagon concentrations differed between individuals with normal glucose tolerance (NGT), pre-diabetes and screen-detected type 2 diabetes; 2) whether fasting glucagon concentrations are related to insulin sensitivity; and 3) whether the timing of glucagon suppression after oral glucose intake was linked to insulin sensitivity and glucose tolerance status.

RESEARCH DESIGN AND METHODS

Study population

The study was based on data from the Danish ADDITION-PRO study[17], a risk-stratified cohort study of individuals at low to high risk for developing type 2 diabetes, nested in the ADDITION-Denmark study[18]. Individuals with impaired glucose regulation at the ADDITION-Denmark screening and individuals from a random sub-sample of individuals at lower diabetes risk were invited to a follow-up health examination (2009-2011), and 2,082 participants (50% of those invited) attended[17]. The study was approved by the Ethical Committee of the Central Denmark Region (ref. no. 20080229) and conducted in accordance with the Helsinki Declaration. All participants provided oral and written informed consent before participating in the study.

Examination and measurements

At the examination in 2009-2011, participants without known diabetes received a standard 75 g oral glucose tolerance test (OGTT) after an overnight fast of ≥8 hours. Blood samples were drawn at 0, 30 and 120 min for assessment of serum concentrations of insulin and plasma concentrations of glucose, glucagon, GLP-1 and GIP. Body weight was measured with participants wearing light

indoor clothing without shoes to the nearest 0.1 kg with a Tanita Body Composition Analyzer (Tokyo, Japan), and height was measured to the nearest millimeter using a fixed rigid stadiometer (Seca, Medical Scales and Measuring Systems, Hamburg, Germany). The ADDITION-PRO study is described in detail elsewhere[17].

Plasma glucose concentration was determined using the Hitachi 912 system (Roche Diagnostics, Mannheim, Germany) or the Vitros 5600 system (Ortho Clinical Diagnostics, Illkirch Cedex, France). Values measured by the Vitros 5600 system were converted to correspond to values from the Hitachi 912 system using a validated regression equation[17,19]. Serum insulin concentrations were measured by immunoassay (AutoDELFIA, Perkin Elmer, Waltham, MA, USA). Blood samples for measurement of glucagon, GLP-1 and GIP were taken in tubes containing EDTA and put on ice immediately, centrifuged for plasma content and stored at –80°C. Radioimmunological determinations of glucagon were performed as previously described[2,4,5] using a C-terminal which reliably measures intact glucagon as validated by sandwich ELISA and mass spectrometry[20,21]. The analytical detection limit was 1 pmol/l, and intra- and inter-assay coefficients of variation were <6% and <15%, respectively. Radioimmunological determinations of total plasma GLP-1 concentration (intact GLP-1 + the metabolite GLP-1 9-36amide) were performed as described previously[22-24]. The analytical detection limit was 1 pmol/l and intra- and inter-assay coefficients of variation were 6.0% and 1.5%, respectively at GLP-1 plasma concentrations of 20 pmol/l. Total plasma GIP concentrations (the sum of intact GIP plus the metabolite GIP 3–42) were measured by radioimmunoassay (RIA) as previously described[24,25]. The assays for glucagon, GLP-1 and GIP measure the sum of the intact, active hormones and the metabolites generated by dipeptidylpeptidase-4 (GIP3–42 and GLP-1 9–36-amide). The results therefore reflect the secretion of the hormones. The samples for determination of glucagon, GLP-1 and GIP were analyzed

consecutively during two months using identical quality controls and identical batches for all reagents.

Calculations and classification of participants

Study participants were classified according to the WHO 2006 criteria as having NGT, pre-diabetes (impaired fasting glucose and/or impaired glucose tolerance), or screen-detected type 2 diabetes. Participants with known diabetes (n=336), those fasting <8 hours prior to the health examination (n=20), those who could not be classified due to missing information on fasting or 2 hour plasma glucose concentrations (n=12), and those with no glucagon measurements (n=289) were excluded, leaving 1,437 individuals for analysis.

The relative glucagon suppression during the first 30 min after oral glucose administration was calculated as $(1 - (\text{glucagon}_{30\ \text{min}} / \text{glucagon}_{0\ \text{min}})) \times 100\%$. Similarly, suppression of glucagon from 30 to 120 min was calculated as $(1 - (\text{glucagon}_{120\ \text{min}} / \text{glucagon}_{30\ \text{min}})) \times 100\%$, and glucagon suppression during the entire OGTT was calculated as $(1 - (\text{glucagon}_{120\ \text{min}} / \text{glucagon}_{0\ \text{min}})) \times 100\%$. Accordingly, this resulted in positive values for those who did suppress glucagon and negative values for those who did not suppress glucagon during the OGTT.

As a measure of insulin sensitivity we calculated the insulin sensitivity index (ISI_{0-120}), reflecting whole-body insulin sensitivity: $75000\ \text{mg} + (\text{glucose}_{0\ \text{min}} - \text{glucose}_{120\ \text{min}}) \times 0.19 \times \text{weight}) / (\text{glucose}_{0\text{min}} + \text{glucose}_{120\text{min}}) / 2) / \log (\text{insulin}_{0\text{min}} + \text{insulin}_{120\text{min}}) / 2)$[26]. .

Statistical analyses

Characteristics of the study population are shown by glucose tolerance status. Data are presented as means with standard deviations (SD) for normally distributed variables and as geometric means

with corresponding 95% confidence intervals (CI) for variables with a skewed distribution. For proportions, exact 95% CIs were calculated. An overall ANOVA was used to assess differences in characteristics between groups, and if significant, post hoc t-tests were used to study pairwise differences.

Absolute glucagon levels as well as relative glucagon suppression during the OGTT were studied by glucose tolerance status and by tertiles of insulin sensitivity. As an initial step, data were investigated graphically. This indicated a non-linear association between fasting glucagon and insulin sensitivity. Therefore, we fitted a hyperbolic relationship between fasting glucagon levels and insulin sensitivity and tested for a modifying effect of glucose tolerance status on the association.

In multivariate linear regression analyses, we modelled the association of early- and late glucagon suppression with insulin sensitivity in separate models, using a stepwise approach. First, the data were adjusted for age, sex and initial glucagon level, i.e. fasting glucagon level for early suppression and 30 min glucagon level for late suppression (Model 1). Secondly, data were further adjusted for fasting- and 2-hour plasma glucose concentration (Model 2). Lastly, further adjustment for the relative change in GIP and GLP-1 was performed (from 0 to 30 min for early glucagon suppression as outcome, and from 30 to 120 min for late glucagon suppression as outcome) (Model 3).

Statistical analyses were performed in R, version 3.2.1 (The R Foundation for Statistical Computing) and SAS version 9.2 (SAS Institute, Cary, NC, USA). A two-sided 5% level of significance was used for all analyses.

RESULTS

Glucagon levels in relation to glucose tolerance status

Table 1 shows characteristics of the study population stratified by NGT, pre-diabetes and screen-detected type 2 diabetes. Absolute glucagon levels and the percentage glucagon suppression during the OGTT in these groups are shown in Figure 1A and Figure 1B. Individuals with screen-detected diabetes had on average 12 to 59% higher glucagon levels at all measured time points (0, 30 and 120 min) than the other groups ($P<0.05$). Furthermore, individuals with screen-detected diabetes did not suppress glucagon levels from 0 min to 30 min during the OGTT ($P=0.776$). Persons with pre-diabetes had on average 12 to 22% higher glucagon levels at 0 and 30 min compared with the NGT group, and their level of early glucagon suppression was lower than the NGT group ($P<0.001$). Noteworthy, individuals with screen-detected diabetes suppressed glucagon more than individuals with NGT from 30 to 120 min after oral glucose intake ($P<0.001$).

When exploring glucagon levels in subgroups of pre-diabetes and type 2 diabetes, we found differences between individuals diagnosed by the fasting glucose levels versus the 2-hour glucose levels. Individuals with isolated impaired fasting glycaemia (i-IFG) had 24-31% less late and overall glucagon suppression than individuals with impaired glucose tolerance (i-IGT and IFG+IGT), but the groups did not differ in terms of early glucagon suppression (Table 2). Individuals with screen-detected diabetes diagnosed by elevated 2-hour glucose levels had 50-71% higher fasting and 30 min glucagon levels and greater glucagon suppression from 30 to 120 min than those diagnosed by elevated the fasting glucose only (Table 3).

Glucagon levels in relation to insulin sensitivity

Absolute glucagon levels and percentage glucagon suppression during the OGTT stratified by tertiles of insulin sensitivity are shown in Figure 1C and Figure 1D. Fasting glucagon levels increased with decreasing levels of insulin sensitivity, and those with the poorest insulin sensitivity had impaired glucagon suppression at 30 min, but glucagon levels were equal in all three groups at 120 min. Thus, glucagon suppression from 30 to 120 min was most pronounced in those with the poorest insulin sensitivity ($P<0.001$).

The relationship of fasting glucagon concentration with insulin sensitivity stratified by glucose tolerance status is shown on a normal axis in Figure 2A and on a logarithmic axis in Figure 2B. For all glucose tolerance groups, non-linear (inverse function) relationships were present; i.e. fasting glucagon increased exponentially with lower levels of insulin sensitivity. Interestingly, for a given level of glucagon, the insulin sensitivity differed between the glucose tolerance groups with lowest levels for individuals with screen-detected diabetes, intermediate levels for individuals with pre-diabetes, and highest levels for those with NGT ($P<0.001$). Figure 3A-C show fasting plasma glucagon concentrations as functions of insulin sensitivity stratified by the glucose tolerance groups.

Results from linear regression analysis

From the linear regression analysis it was found that a doubling in insulin sensitivity was associated with 28.0 %-points greater early suppression and 12.4 %-points less late suppression (Table 4, $P<0.001$). Adjustment for fasting- and 2-hour plasma glucose attenuated the association by approx. 30% although it remained statistically significant ($P<0.027$). Neither fasting- nor 2-hour plasma glucose was associated with glucagon suppression in Model 2 ($P\geq0.159$). Further adjustment for

changes in GLP-1 and GIP levels during the OGTT attenuated the association of insulin sensitivity with early glucagon suppression, but not with late glucagon suppression (Model 3). In this model, GLP-1 and GIP were not associated with early glucagon suppression ($P \geq 0.147$), but both were associated with late glucagon suppression ($P < 0.001$). A 50% increase in GLP-1 levels from 30 to 120 min was associated with 10%-points greater glucagon suppression, whereas a 50% increase in GIP levels from 30 to 120 min was associated with 1.5%-points less glucagon suppression.

DISCUSSION

Regulation of glucagon secretion is complex and involves auto- and paracrine mechanisms as well as intrinsic mechanisms in the alpha cell related to glucose sensing[1]. By exploring the relationship between insulin sensitivity and glucagon response in a large Danish cohort of individuals at low to high risk of diabetes, we found the following: 1) Individuals with screen-detected type 2 diabetes had higher circulating glucagon levels in the fasting state, and 30 and 120 min after oral glucose intake as compared to individuals with NGT or pre-diabetes; 2) Lower insulin sensitivity (i.e. insulin resistance) was associated with higher fasting glucagon levels in a nonlinear manner; and 3) Insulin resistance, pre-diabetes and screen-detected type 2 diabetes were associated with poorer glucagon suppression during the first 30 min after glucose intake, but slightly exaggerated glucagon suppression from 30 to 120 min, which suggests that the timing of glucose-stimulated glucagon suppression is particularly important for maintenance of normoglycemia. . Taken together, these results support our hypothesis that increased circulating glucagon, together with increased insulin, are tightly coupled to a reduction of insulin sensitivity in the early stages of glucose dysregulation[9].

Impaired suppression of glucagon in response to glucose or meal challenges in patients with type 2 diabetes has been reported in several studies[2-5]. We now show in a well powered study that

individuals with newly diagnosed type 2 diabetes only have impairment in the early glucose-stimulated glucagon suppression. Their later relative glucagon suppression is normal or even exaggerated compared with individuals without diabetes, and this is particularly true for individuals diagnosed by the 2-hour glucose concentration after an oral glucose load. Yet, if the high post-OGTT glucose levels observed in this group are considered, it is clear that the late glucagon suppression is not sufficient to secure normal glucose tolerance. Therefore, hyperglycemia seems to be mainly related to impairment of the early glucagon response, which is in analogy to the importance of defective early insulin secretion for hyperglycemia in type 2 diabetes[29]. This combined early defect in both alpha and beta cell secretion during development of type 2 diabetes is of important pathophysiological interest and also supports the indication that the restoration of the early pancreatic responses is an important target for therapy.

The observed novel, non-linear, relationship between fasting glucagon concentration and insulin sensitivity suggests that basal alpha cell secretion depends on insulin sensitivity. It is well established that beta cell secretion depends on insulin sensitivity and is increased in a non-linear manner during development of insulin resistance (i.e. the underlying background for the generation of the disposition index)[16,30]. However, our results also strongly indicate that the resulting glucose tolerance is jeopardized by simultaneous hypersecretion of the alpha cells eventually resulting in hyperglycemia in the presence of hyperinsulinemia – a feature well-known in type 2 diabetes. A shift from the upper right corner towards the lower left corner of the glucagon-insulin sensitivity relationship (Fig. 2A) is associated with a worsening of glucose tolerance status – exactly as seen for the insulin secretion disposition index[16,30]. Unfortunately, from our cross-sectional study it is not possible to conclude whether the changes in basal glucagon secretion precede or parallel changes in insulin secretion in individuals who become insulin resistant.

Another novel finding of our study was that the timing of glucagon suppression after oral glucose intake is particularly important for maintenance of normoglycemia. The postprandial glycemic response is dependent on complex, interdependent relationships between gastric emptying and glucose absorption, secretion and action of incretin hormones, insulin sensitivity as well as insulin and glucagon release. Particularly the rates of gastric emptying and glucose absorption play important roles for the magnitude of both the postprandial glycemic excursion and the incretin hormone responses[34,35]. However, the negative feedback mechanism, which limits the amount of nutrients released into the blood stream during hyperglycemic states[36] makes it difficult to conclude whether delayed gastric emptying results in delayed glucagon suppression or whether a worsening of early glucagon suppression with resulting hyperglycemia leads to delayed gastric emptying[34]. Thus, the precise mechanisms leading to increased basal glucagon levels and failing early glucose-stimulated glucagon suppression in response to insulin resistance are not clearly understood. Fasting hyperglucagonemia has previously been associated with insulin resistance as measured by gold-standard methods in non-diabetic individuals, suggesting that insulin resistance may be present at the level of the pancreatic alpha cells[31]. From studies in patients with Maturity-Onset Diabetes of the Young Type 2 (MODY2) it is clear that it is not the circulating glucose levels per se which drives glucagon secretion but more likely the intracellular metabolism of glucose. In other words, it is likely that glucokinase serves as a metabolic glucose sensor in pancreatic alpha-cells, which then mediate a mechanism for direct regulation of glucagon release by extracellular glucose in the same way as in beta cells [32]. However, alpha cell secretion is also regulated by the gut incretin hormone GLP-1[14], possibly indirectly by somatostatin[33]. In the present study, GLP-1 was not associated with early glucagon suppression, whereas positive changes in GLP-1 levels from 30 to 120 min during the OGTT were associated with greater late glucagon suppression. This supports an inhibitory role of GLP-1 on glucagon secretion during hyperglycemia[14]. Similarly, an increase in GIP levels from

30 to 120 min was associated with a poorer late suppression of glucagon. Although the effect size was small, the finding is in line with a stimulating role of GIP on glucagon secretion as previously reported[15]. The lack of association of GLP-1 and GIP with early glucagon suppression underscores that control of glucagon secretion is complex and multifactorial, involving a combination of paracrine, autocrine, hormonal, and autonomic neural mechanisms[13]. In addition, recent evidence from pancreatectomized patients suggests that glucagon may be secreted not only from the pancreas, but also from extrapancreatic tissue in humans, probably the gut[21]. Whether gut-derived glucagon secretion is partly responsible for fasting hyperglucagonemia in insulin resistant individuals warrants further studies.

One of the major strengths of this study is the large sample size and the 3-point OGTT, which enabled us to study subgroups of individuals with different patterns of glucagon suppression. Another strength is that we used a well-documented assay method with high specificity and sensitivity for glucagon, which was directed against the C-terminus of the glucagon molecule and therefore mainly measures glucagon of pancreatic origin[37]. Insulin sensitivity was not assessed by the gold-standard clamp technique because of the large size of the cohort, which could appear as a limitation of our study. However, $ISI_{0\text{-}120}$ is a valid measure of insulin sensitivity across different categories of glucose tolerance status and obesity when compared with the gold-standard clamp technique[26,35], supporting the validity of our results. Unfortunately, we were not able to assess the optimal time point for distinguishing between early versus late glucagon suppression, since this would have required more measurements during the OGTT. The 30 min measurement in the ADDITION-PRO study was chosen to be able to assess early insulin secretion, but we cannot exclude the possibility that samples for measurement of glucagon taken 45 or 60 min after oral glucose intake would have given slightly different results.

In conclusion, we found a non-linear inverse relationship between fasting glucagon levels and insulin sensitivity, which means that fasting glucagon concentrations increase exponentially with deterioration of insulin sensitivity. Interestingly, individuals with screen-detected diabetes exhibited a lack of glucagon suppression from 0 to 30 min, but their glucagon suppression from 30 to 120 min was normal or even exaggerated. . Our findings suggest that the fasting hyperglucagonemia observed in individuals with type 2 diabetes is likely to develop in parallel with insulin resistance. A novel finding in our study is that the timing of glucagon suppression is crucial for maintenance of normal glucose homeostasis. While early glucagon suppression is associated with adequate insulin sensitivity and normal glucose tolerance, delayed glucagon suppression is associated with insulin resistance and higher risk of type 2 diabetes. Future studies should examine whether an improvement in insulin sensitivity contributes to a restoration of the early pancreatic response. The specific mechanisms linking insulin resistance to increased fasting glucagonemia and delayed glucagon suppression also needs further study.

AUTHOR CONTRIBUTIONS

BA, GP and JJH conceived the idea. KF, DV, GP, SST, BA, and JJH developed the hypotheses. DV performed the statistical analyses. KF and DV drafted first version of the manuscript. NBJ, DRW, and TL designed the ADDITION-PRO study. AJ, OP and TH designed the incretin and glucagon part of the ADDITION-PRO study, and JJH and SST generated glucagon data. MEJ contributed intellectually to the discussion of the results. All authors reviewed and edited the manuscript and approved the final version.

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CONFLICTS OF INTEREST STATEMENT

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Table 1: Characteristics of the study population by glucose tolerance status

	NGT	Pre-diabetes	Type 2 diabetes	P value
N	763	514	160	
Age (y)	65.7 (7.5)	66.8 (6.6)[a]	66.1 (6.5)	0.020
Women (%)	52.2 (48.6;55.8)	43.2 (38.9;47.6)[a]	33.8 (26.5;41.6)[a,b]	<0.001
BMI (kg/m^2)	26.0 (4.0)	27.9 (4.4)[a]	29.8 (5.6)[a,b]	<0.001
Fasting glucose (mmol/L)	5.6 (0.4)	6.3 (0.4)[a]	7.3 (1.0)[a,b]	<0.001
30 min glucose (mmol/L)	8.3 (1.4)	9.7 (1.2)[a]	11.4 (1.9)[a,b]	<0.001
120 min glucose (mmol/L)	5.6 (1.1)	7.4 (1.7)[a]	10.6 (3.1)[a,b]	<0.001
Fasting insulin (pmol/L)	30.4 (29.2;31.7)	43.3 (41.2;45.6)[a]	58.9 (53.0;65.6)[a,b]	<0.001
30 min insulin (pmol/L)	205.6 (196.6;215.1)	223.0 (210.0;236.8)[a]	229.0 (207.5;252.6)[a]	0.038
120 min insulin (pmol/L)	134.8 (128.3;141.5)	247.4 (231.5;264.3)[a]	357.8 (317.1;403.8)[a,b]	<0.001
Glucagon suppression, 0-30 min (%)[*]	16.5 (13.9;19.1)	9.5 (5.7;13.2)[a]	-3.8 (-12.2;3.9)[a,b]	<0.001
Glucagon suppression, 30-120 min (%)[ǂ]	27.9 (25.3;30.5)	39.6 (37.1;42.0)[a]	48.3 (44.4;52.0)[a,b]	<0.001
Glucagon suppression, 0-120 min (%)[§]	39.7 (37.3;42)	45.2 (42.6;47.7)[a]	46.2 (41.7;50.5)[a]	0.002

Data are means (SD), geometric means (95% CI) or percentages (95% CI). [*]Calculated as: (1 - (glucagon$_{30\ min}$ / glucagon$_{0\ min}$)) × 100; [ǂ]Calculated as: (1 - (glucagon$_{120\ min}$ / glucagon$_{30\ min}$)) × 100; [§]Calculated as: (1 - (glucagon$_{120\ min}$ / glucagon$_{0\ min}$)) × 100; [a]: P<0.05 vs. NGT; [b]: P<0.05 vs. pre-diabetes.

Table 2: Glucagon characteristics in individuals with different subtypes of pre-diabetes

	i-IFG	i-IGT	IFG+IGT	P value
N	281	110	123	
Fasting glucagon (pmol/L)	9.7 (9;10.5)	9.8 (8.6;11.1)	10.6 (9.3;11.9)	0.190
30 min glucagon (pmol/L)	8.7 (8.1;9.3)	8.9 (8.2;9.8)	9.7 (8.8;10.7)	0.144
120 min glucagon (pmol/L)	5.8 (5.4;6.3)	4.9 (4.4;5.4)[a]	5.1 (4.5;5.6)[a]	0.002
Glucagon suppression, 0-30 min (%)[*]	10.4 (5.2;15.4)	8.5 (-0.1;16.3)	8.2 (0.1;15.7)	0.864
Glucagon suppression, 30-120 min (%)[ǂ]	33.1 (29.6;36.5)	45.2 (40.8;49.3)[a]	47.8 (43.0;52.3)[a]	<0.001
Glucagon suppression, 0-120 min (%)[§]	39.8 (35.8;43.6)	49.7 (45.2;53.9)[a]	52.1 (47.5;56.3)[a]	<0.001

Data are means (SD), geometric means (95% CI) or percentages (95% CI). i-IFG: isolated impaired fasting glycaemia; i-IGT: isolated impaired glucose tolerance; IFG+IGT: Combined IFG and IGT.

Table 3: Glucagon characteristics in individuals with different subtypes of screen-detected diabetes

	F-DM	2h-DM	F-2h-DM	P value
N	80	42	37	
Fasting glucagon (pmol/L)	8.8 (7.3;10.6)	14.3 (11.8;17.3)[a]	14.8 (12.9;17)[a]	<0.001
30 min glucagon (pmol/L)	9.3 (8.0;10.8)	13.9 (12.1;16.1)[a]	15.9 (13.8;18.3)[a]	<0.001
120 min glucagon (pmol/L)	5.6 (4.8;6.5)	6.5 (5.4;8.0)	6.9 (5.7;8.3)	0.355
Glucagon suppression, 30 min (%)[*]	-6.2 (-21.6;7.3)	2.3 (-10.7;13.8)	-7.4 (-17.7;2.1)	0.613
Glucagon suppression, 30-120 min (%)[ǂ]	41.0 (33.4;47.7)	53.1 (47.1;58.3)[a]	56.6 (51.5;61.2)[a]	0.001
Glucagon suppression, 120 min (%)[§]	37.1 (28.7;44.4)	54.2 (46.3;60.8)[a]	53.4 (47.7;58.5)[a]	<0.001

Data are means (SD), geometric means (95% CI) or percentages (95% CI). F-DM: Diabetes diagnosed by fasting glucose only; 2h-DM: Diabetes diagnosed by 2-hour glucose only; F-2h-DM: Diabetes diagnosed by both fasting and 2-hour glucose.

Table 4: Estimated %-points change (with 95% CI) in early- and late glucagon suppression by a doubling in insulin sensitivity.

	Early glucagon suppression		Late glucagon suppression	
	Estimate	**P-value**	**Estimate**	**P-value**
Model 1	28.0 (21.0;35.0)	<0.001	-12.4 (-16.4;-8.4)	<0.001
Model 2	20.5 (6.3;34.7)	0.005	-8.9 (-16.8;-1.0)	0.027
Model 3	11.5 (-0.9;23.8)	0.069	-10.6 (-18.3;-2.9)	0.007

Model 1: Adjustment for age, sex and fasting glucagon (early suppression as outcome) or 30 min glucagon level (late suppression as outcome).

Model 2: Further adjustment for fasting- and 2-hour plasma glucose.

Model 3: Further adjustment for relative change in GIP and GLP-1 (from 0 to 30 min for early suppression as outcome, and from 30 to 120 min for early suppression as outcome).

FIGURE LEGENDS

Figure 1: Plasma glucagon (A, C) and glucagon suppression (B, D) during OGTT. Top row (A, B) by glucose tolerance status: NGT (light blue), pre-diabetes (dark blue), and type 2 diabetes (red). Bottom row (C, D) by tertiles of insulin sensitivity ($ISI_{0\text{-}120}$): high insulin sensitivity (light blue), median insulin sensitivity (dark blue), low insulin sensitivity (red). Data are geometric means with 95% CI.

Panel A:

Overall $P_{0\ min} < 0.001$ (Pre-D vs NGT: 0.001; T2D vs NGT: <0.001; T2D vs Pre-D: 0.024);

Overall $P_{30\ min} < 0.001$ (Pre-D vs NGT: <0.001; T2D vs NGT: <0.001; T2D vs Pre-D: <0.001);

Overall $P_{120\ min} = 0.048$ (Pre-D vs NGT: 0.561; T2D vs NGT: 0.014; T2D vs Pre-D: 0.046).

Panel C:

Overall $P_{0\ min} < 0.001$ (median vs high: <0.001; low vs high: <0.001; low vs median: <0.001);

Overall $P_{30\ min} < 0.001$ (median vs high: <0.001; low vs high: <0.001; low vs median: <0.001);

Overall $P_{120\ min} = 0.278$ (median vs high: 0.468; low vs high: 0.383; low vs median: 0.110).

Figure 2: Fasting glucagon concentration as function of insulin sensitivity on the original scale (A) and on the log-scale (B). Curves are plotted within the range of observed insulin sensitivity and shown for NGT (light blue), pre-diabetes (dark blue), and type 2 diabetes (red). The thin grey lines illustrate different levels of insulin sensitivity × fasting glucagon concentrations (hyperbolic function). P<0.001 for all associations.

Figure 3: Fasting glucagon concentration as function of insulin sensitivity stratified by NGT (light blue, A), pre-diabetes (dark blue, B), and type 2 diabetes (red, C). Curves are plotted within the range of observed insulin sensitivity. The thin grey lines illustrate different levels of insulin sensitivity × fasting glucagon concentrations (hyperbolic function).

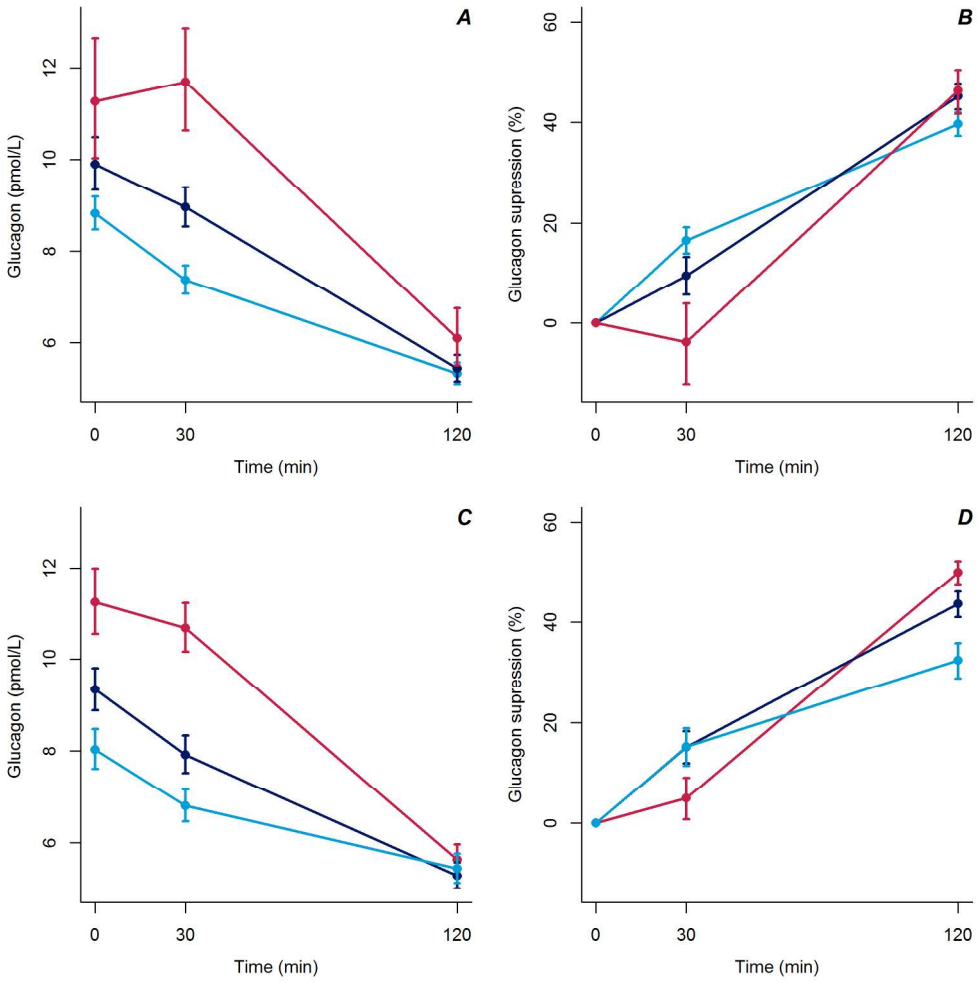

Figure 1

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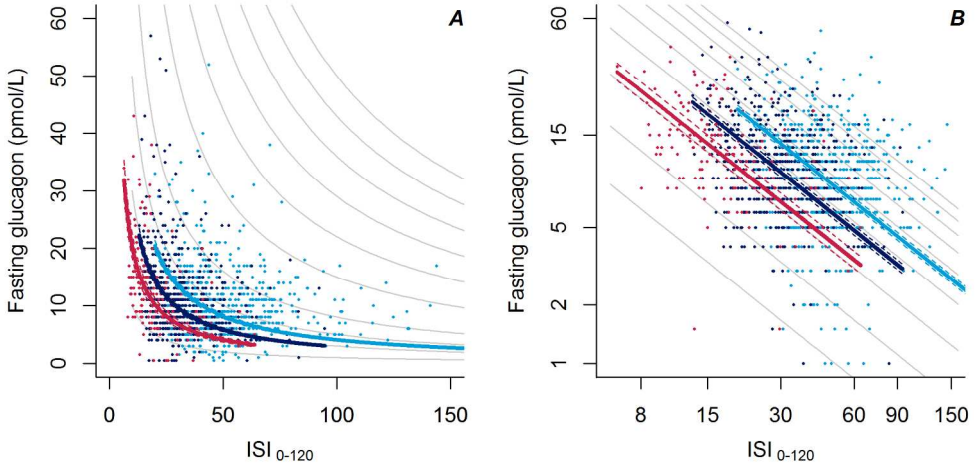

Figure 2

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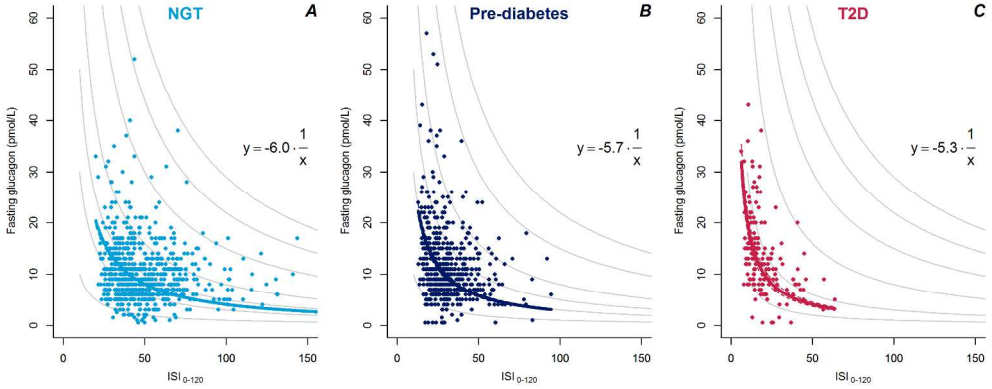

Figure 3

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