



Evidence of a liver–alpha cell axis in humans: hepatic insulin resistance attenuates relationship between fasting plasma glucagon and glucagonotropic amino acids

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Abstract

Aims/hypothesis The secretion of glucagon is controlled by blood glucose and inappropriate secretion of glucagon contributes to hyperglycaemia in diabetes. Besides its role in glucose regulation, glucagon regulates amino acid metabolism in hepatocytes by increasing ureagenesis. Disruption of this mechanism causes hyperaminoacidaemia, which in turn increases glucagon secretion. We hypothesised that hepatic insulin resistance (secondary to hepatic steatosis) via defective glucagon signalling/glucagon resistance would lead to impaired ureagenesis and, hence, increased plasma concentrations of glucagonotropic amino acids and, subsequently, glucagon.

Methods To examine the association between glucagon and amino acids, and to explore whether this relationship was modified by hepatic insulin resistance, we studied a well-characterised cohort of 1408 individuals with normal and impaired glucose regulation. In this cohort, we have previously reported insulin resistance to be accompanied by increased plasma concentrations of glucagon. We now measure plasma levels of amino acids in the same cohort. HOMA-IR was calculated as a marker of hepatic insulin resistance.

Results Fasting levels of glucagonotropic amino acids and glucagon were significantly and inversely associated in linear regression models (persisting after adjustment for age, sex and BMI). Increasing levels of hepatic, but not peripheral insulin resistance ($p > 0.166$) attenuated the association between glucagon and circulating levels of alanine, glutamine and tyrosine, and was significantly associated with hyperaminoacidaemia and hyperglucagonaemia. A doubling of the calculated glucagon–alanine index was significantly associated with a 30% increase in hepatic insulin resistance, a 7% increase in plasma alanine aminotransferase levels, and a 14% increase in plasma γ -glutamyltransferase levels.

Conclusions/interpretation This cross-sectional study supports the existence of a liver–alpha cell axis in humans: glucagon regulates plasma levels of amino acids, which in turn feedback to regulate the secretion of glucagon. With hepatic insulin resistance, reflecting hepatic steatosis, the feedback cycle is disrupted, leading to hyperaminoacidaemia and hyperglucagonaemia. The glucagon–alanine index is suggested as a relevant marker for hepatic glucagon signalling.

Keywords Amino acids · Glucagon · Insulin resistance · Liver damage

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Research in context

What is already known about this subject?

- Glucagon increases amino acid turnover in mice
- Amino acids regulate secretion of glucagon, and prolonged exposure results in alpha cell hyperplasia
- A liver–alpha cell axis seems to exist in rodents, which, when disrupted, impairs the effect of glucagon on amino acid turnover, leading to increased amino acid levels and hyperglucagonaemia

What is the key question?

- Does a similar liver–alpha cell axis exist in humans?

What are the new findings?

- In 1408 individuals with a wide range of blood glucose levels, glucagon and glucagonotropic amino acids were significantly associated
- Increasing liver fat (reflected by HOMA-IR) modified the association between glucagon and amino acids
- A glucagon–alanine index was developed and may serve as a marker of hepatic glucagon sensitivity

How might this impact on clinical practice in the foreseeable future?

- An increased glucagon–alanine index may indicate impaired hepatic glucagon sensitivity suggestive of impaired liver function

Abbreviations

ALT	Alanine aminotransferase
BCCA	Branched-chain amino acid
GGT	γ -Glutamyltransferase
ISI _{0–120min}	Peripheral insulin sensitivity as calculated based on the first 120 min of OGTT
NAFLD	Non-alcoholic fatty liver disease

Introduction

Glucagon is an important glucose-regulatory hormone, which via a feedback cycle regulates hepatic glucose production, while the resulting glucose levels regulate the secretion of glucagon from the pancreatic alpha cells [1]. With the use of glucagon receptor antagonists in rodents and in individuals with type 2 diabetes, it has finally been agreed that inappropriate glucagon secretion makes an important contribution to hyperglycaemia in diabetes [2–4]. However, in addition to its glucose-regulatory effects, glucagon also regulates amino acid metabolism (ureagenesis) in the liver [5] and, conversely, amino acids stimulate the secretion of glucagon [6], forming a feedback loop between the liver and the pancreatic alpha cells [7, 8]—a liver–alpha cell axis. This glucagon–amino acid feedback loop may be as important for metabolism as the glucagon–glucose loop [9]. In line with this, individuals with complete disruption of glucagon signalling develop not hypoglycaemia but increasing amino acid levels, leading to severe alpha cell hypersecretion and hyperplasia [7, 10]. In

addition, hyperglucagonaemia (as in individuals with glucagon-producing tumours) does not always cause diabetes, but it severely decreases amino acid levels, resulting in muscular wasting and decreased cellular proliferation in the skin [11, 12].

Apart from these extremes, however, it is not clear how the feedback loop between amino acids and glucagon operates in healthy individuals, and in particular in individuals with impaired liver function, for example due to non-alcoholic fatty liver disease (NAFLD) [13]. Hypothetically, even slightly impaired liver function (appearing as hepatic insulin resistance) might attenuate glucagon-induced amino acid turnover (glucagon resistance), resulting in increased plasma levels of amino acids and hence increased glucagon secretion. In order to address this hypothesis, we studied whether fasting plasma glucagon concentrations were associated with fasting plasma concentrations of branched-chain amino acids (BCAAs) as well as four non-BCAAs (alanine, histidine, tyrosine and glutamine) and phenylalanine in a cross-sectional study including 1408 individuals with progressive stages of risk of type 2 diabetes. Furthermore, we examined whether hepatic insulin resistance (reflected by an increased HOMA-IR [14]) would modify glucagon-induced amino acid turnover.

Methods

Study population The study was based on data from the Danish ADDITION-PRO study [15], a risk-stratified cohort

of individuals with low to high risk of developing type 2 diabetes that is nested within the ADDITION-Denmark study [16]. Individuals with impaired glucose regulation as well as a random subsample of individuals found at the ADDITION-Denmark screening (2001–2006) to have a lower risk of diabetes were invited to a follow-up health examination (2009–2011), and 2082 participants (50% of those invited) attended [15]. The study was approved by the Ethics Committee of the Central Denmark Region (reference no. 20080229) and was conducted in accordance with the Declaration of Helsinki. All participants provided oral and written informed consent before participating in the study.

Examination and measurements Full details on the health examination and measurements as part of the ADDITION-PRO study have previously been described [15]. In brief, at the examination in 2009–2011, participants without known diabetes underwent a standard 75 g OGTT after an overnight fast of 8 h or longer. Blood samples were drawn at 0, 30 and 120 min to assess serum concentrations of insulin, and plasma concentrations of glucose, glucagon and amino acids. Using a Tanita Body Composition Analyser (Tokyo, Japan), body weight was measured to the nearest 0.1 kg with participants wearing light indoor clothing without shoes, and height was measured to the nearest millimetre using a fixed rigid stadiometer (Seca, Hamburg, Germany). As this was a secondary analysis, we included data on metabolites that had already been measured, but also included new measurements of liver enzymes and urea.

Plasma alanine aminotransferase (ALT) and plasma glucose concentrations were determined using the Hitachi 912 system (Roche Diagnostics, Mannheim, Germany) or the Vitros 5600 system (Ortho Clinical Diagnostics, Illkirch, France). Values measured by the Vitros 5600 system were converted to correspond to values from the Hitachi 912 system using a validated regression equation [15, 17].

Serum insulin concentrations were measured by immunoassay (AutoDELFIA; PerkinElmer, Waltham, MA, USA). Blood samples for the measurement of glucagon, urea and γ -glutamyltransferase (GGT) were obtained in tubes containing EDTA, immediately put on ice and centrifuged, and the plasma was stored at -80°C .

Radio-immunological determinations of glucagon were performed as previously described [18] using a C-terminus-specific antibody (codename 4305), which reliably measures pancreatic glucagon as validated by sandwich ELISA and mass spectrometry [19]. The analytical detection limit was 1 pmol/l, and the intra-assay and inter-assay CVs were $<6\%$ and $<15\%$, respectively. All samples for determination of glucagon were analysed consecutively over 2 months using identical quality controls and identical batches of all reagents.

A targeted NMR spectroscopy-based approach was used to measure plasma amino acid levels. This method, including

CVs, has previously been described in detail [20]. A total of four non-BCAAs (alanine, histidine, tyrosine and glutamine), phenylalanine and three BCAAs (isoleucine, leucine and valine) were measured. We calculated ‘total non-BCAA’ as the sum of the concentrations of alanine, histidine, tyrosine and glutamine, and ‘total BCAA’ as the sum of isoleucine, leucine and valine (not including phenylalanine).

GGT and urea were measured in plasma on a Cobas 8000 instrument, c802 module (Roche, Mannheim, Germany) using Cobas calibrators and reagents according to the manufacturer’s instructions.

Calculations and statistical analyses Participants with known diabetes ($n = 336$) and those who had fasted for less than 8 h prior to the health examination ($n = 20$) were excluded from the analysis. We further excluded participants who had not had blood samples taken for measurement of plasma glucagon ($n = 281$), those in whom no amino acids were measured ($n = 26$) and those without data on fasting serum insulin ($n = 11$), leaving 1408 (68%) individuals for analysis.

Insulin resistance was calculated according to the HOMA-IR [21]. As the model is based on glucose and insulin concentrations measured in the fasting state, HOMA-IR predominantly reflects hepatic insulin resistance [14, 22]. The HOMA-IR values for the study population are presented as tertiles of HOMA-IR: lower tertile, 0.10–1.06; middle tertile, 1.07–1.84; upper tertile, 1.85–24.05.

Plasma concentrations of glucagon and alanine during the OGTT are presented as geometric means. Associations between fasting plasma glucagon (exposure) and fasting plasma levels of amino acids (outcome) were assessed using linear regression analysis. All analyses were adjusted for age and sex (model 1). We further adjusted the analyses for BMI (model 2) and HOMA-IR (model 3). In model 3, we first tested for deviation from linearity by including a quadratic term of glucagon. We next tested for a modifying effect of HOMA-IR on the associations between plasma amino acids and plasma glucagon concentrations. In case of a modifying effect of HOMA-IR, the associations are shown for the median, lower quartile and upper quartile of HOMA-IR. In a sensitivity analysis, we substituted, in model 3, HOMA-IR with peripheral insulin sensitivity (calculated based on the first 120 min of OGTT [$\text{ISI}_{0-120\text{min}}$]), which is an estimate of whole-body/peripheral insulin sensitivity [23].

To facilitate direct comparisons of the strength of association between the eight amino acids and fasting glucagon, plasma levels of amino acids were standardised prior to analysis. The 1408 participants had complete data on all eight amino acids. Fasting glucagon was \log_2 -transformed prior to analysis because the requirement for a normal distribution of model residuals was not met.

Because of the possible bidirectional relationship between amino acids and glucagon, we also performed analyses with

fasting plasma glucagon as outcome and the different amino acids as explanatory factors in linear regression models adjusted for age, sex and HOMA-IR. Additionally, we combined the eight measured amino acids in order to assess the proportion of residual variance in fasting plasma glucagon explained by these amino acids after adjustment for age and sex. For comparison, we also calculated the proportion explained by HOMA-IR and by the amino acids in combination with HOMA-IR.

Finally, to generate a potential surrogate marker for the hepatic actions of glucagon on ureagenesis, we calculated a glucagon–alanine index using the following formula:

$$\text{glucagon – alanine index} = \text{fasting plasma glucagon (pmol/l)} \\ \times \text{fasting plasma alanine (mmol/l)}$$

Scatter plots of fasting concentrations of plasma glucagon and alanine were plotted, together with estimated levels of the new index for tertiles of HOMA-IR, plasma ALT and GGT. In addition, linear regression analysis adjusting for age and sex was used to assess the associations between the new index and HOMA-IR, ALT and GGT in separate analyses. Again, because the data did not meet the requirement for a normal distribution of model residuals, HOMA-IR, ALT and GGT were \log_e -transformed prior to analysis, and, similarly, the glucagon–alanine index was \log_2 -transformed.

Statistical analyses were performed in R version 3.2.3 (R Foundation for Statistical Computing, www.R-project.org) and SAS version 9.4 (SAS Institute, Cary, NC, USA).

Results

Characteristics of the study participants The characteristics of the 1408 study participants according to tertiles of insulin resistance (HOMA-IR) are presented in Table 1. The mean fasting plasma glucagon concentration was significantly higher in individuals in the upper tertile compared with those in the middle and lower tertiles. Higher mean plasma levels of all BCAAs and alanine, tyrosine and glutamine, but not histidine, were also observed in individuals with higher levels of HOMA-IR.

HOMA-IR modifies circulating glucagon and alanine responses during an OGTT Plasma concentrations of glucagon and alanine decreased during the OGTT (Fig. 1a, b); however, the absolute concentrations of alanine differed by tertile of HOMA-IR. The 75 g glucose intake did not significantly affect the plasma concentration of alanine for individuals in the lower tertile of HOMA-IR (fasting: 0.26 ± 0.06 mmol/l; change in alanine 120 min after the OGTT: -0.003 mmol/l [95%CI $-0.007, 0.000$ mmol/l], $p = 0.076$). However, for the

middle and upper tertiles of HOMA-IR, fasting plasma concentrations of alanine were higher (0.27 ± 0.06 and 0.28 ± 0.06 mmol/l, respectively), and in these two tertiles alanine concentration decreased in response to glucose intake (change in alanine 120 min after the OGTT: -0.010 [95% CI $-0.014, -0.007$] and -0.018 mmol/l [95% CI $-0.022, -0.015$ mmol/l], respectively; $p < 0.001$ for both) (Fig. 1b).

A proposed index for estimating the hepatic actions of glucagon Tertiles of HOMA-IR were significantly associated with higher levels of the glucagon–alanine index (Fig. 1c; $p < 0.001$), with the highest levels of the index in the upper tertiles. The glucagon–alanine index also differed significantly between the tertiles of ALT and GGT ($p < 0.001$), although the differences were small. In linear regression analyses, we observed that a doubling of the glucagon–alanine index was associated with a 30% (95% CI 25%, 35%; $R^2 = 0.14$) increase in HOMA-IR ($p < 0.001$), a 7% (95% CI 5%, 10%; $R^2 = 0.09$) increase in plasma ALT ($p < 0.001$), and a 14% (95% CI 10%, 19%; $R^2 = 0.09$) increase in GGT concentration after adjustment for age and sex.

The association between fasting plasma concentrations of glucagon and alanine is modified by HOMA-IR For phenylalanine, isoleucine, leucine and valine, we found statistically significant non-linear associations with fasting glucagon concentration ($p \leq 0.049$). However, we found the deviations from linearity to be small and probably clinically irrelevant (data not shown), and therefore the following results are based on linear associations only.

For alanine, tyrosine, phenylalanine, ‘total non-BCAA’ (alanine, tyrosine, histidine and glutamine), isoleucine, leucine and total BCAA, we found a modifying effect of hepatic insulin resistance on the associations with fasting plasma glucagon ($p \leq 0.040$). Hence, an interaction between fasting plasma glucagon and HOMA-IR was included in the models for these amino acids, and results are shown for different levels of HOMA-IR (the median and interquartile limits). As the estimated effects of glucagon were similar for models 1–3, only results from the fully adjusted model are shown (model 3; Fig. 2).

The standardised effects on fasting plasma amino acids levels from increasing fasting plasma glucagon levels were comparable for alanine, tyrosine, isoleucine, leucine and valine (Fig. 2), whereas histidine and glutamine were not significantly associated with fasting glucagon levels ($p \geq 0.127$).

For the two non-BCAAs alanine and tyrosine a twofold increase in plasma glucagon was significantly associated with lower plasma concentrations of the amino acids. These inverse associations of glucagon with alanine and tyrosine were significantly impaired by increasing levels of HOMA-IR ($p \leq 0.025$) (Fig. 2a).

In contrast to the findings for the non-BCAAs, glucagon concentrations were significantly associated with *higher* plasma concentrations of the three BCAAs (isoleucine, leucine

Table 1 Characteristics of the study participants in the ADDITION-PRO cohort by tertiles of insulin resistance (HOMA-IR)

Variable	Lower tertile (0.10–1.06)	Middle tertile (1.07–1.84)	Upper tertile (1.85–24.05)	<i>p</i> value ^a
<i>n</i>	469	470	469	
Age (years)	66.8 (7.4)	65.9 (7.0)	66.0 (6.7)	0.073
Women (%)	49.7 (45.1, 54.3)	50.6 (46.0, 55.2)	40.7 (36.2, 45.3)* [†]	0.003
BMI (kg/m ²)	24.4 (3.2)	26.8 (3.8)*	29.9 (4.6)* [†]	<0.001
HbA _{1c} (mmol/mol)	43.3 (4.0)	43.9 (4.0)	46.1 (6.0)* [†]	<0.001
HbA _{1c} (%)	5.6 (0.3)	5.6 (0.3)	5.8 (0.5)* [†]	<0.001
Fasting plasma glucose (mmol/l)	5.6 (0.5)	5.9 (0.5)*	6.5 (0.9)* [†]	<0.001
120 min plasma glucose (mmol/l)	5.9 (1.7)	6.5 (1.8)*	8.0 (2.8)*	<0.001
Fasting serum insulin (pmol/l)	21.0 (17.0–25.0)	37.0 (32.0–42.0)*	69.0 (55.0–90.0)* [†]	<0.001
HOMA-IR	0.77 (0.59–0.91)	1.39 (1.22–1.60)*	2.75 (2.25–3.77)* [†]	<0.001
HOMA-β	28.0 (22.1–35.0)	44.4 (35.6–54.7) [†]	73.1 (54.9–94.1)* [†]	<0.001
Fasting plasma glucagon (pmol/l)	8.0 (6.0–11.0)	9.0 (6.0–12.0)	13.0 (9.0–17.0)* [†]	<0.001
Glucagon–alanine index	2.1 (1.5–2.9)	2.3 (1.6–3.2)*	3.5 (2.5–5.2)* [†]	<0.001
GGT (U/l)	22.0 (16.0–33.0)	25.0 (17.0–40.0)*	35.0 (23.0–57.0)* [†]	<0.001
Plasma alanine (mmol/l)	0.26 (0.06)	0.27 (0.06)*	0.28 (0.06)* [†]	<0.001
Plasma histidine (mmol/l)	0.068 (0.011)	0.066 (0.012)	0.067 (0.024)	0.264
Plasma tyrosine (mmol/l)	0.059 (0.010)	0.060 (0.011)	0.065 (0.011)* [†]	<0.001
Plasma glutamine (mmol/l)	0.059 (0.010)	0.060 (0.011)	0.065 (0.011)* [†]	<0.001
Plasma phenyl alanine (mmol/l)	0.038 (0.005)	0.038 (0.006)	0.040 (0.006)* [†]	<0.001
Plasma isoleucine (mmol/l)	0.038 (0.011)	0.041 (0.014)*	0.049 (0.016)* [†]	<0.001
Plasma leucine (mmol/l)	0.050 (0.010)	0.050 (0.012)	0.055 (0.013)* [†]	<0.001
Plasma valine (mmol/l)	0.14 (0.03)	0.15 (0.03)	0.16 (0.03)* [†]	<0.001
Urea (mmol/l)	5.5 (1.5)	5.5 (1.7)	5.7 (2.2)	0.086

Data are shown as means (SD), medians (interquartile range) or percentages (95% CI)

^a Level of significance for the overall test of difference between groups of tertiles of insulin resistance

**p* < 0.05 vs lower tertile; [†] *p* < 0.05 vs middle tertile

and valine) and phenylalanine, and these associations were amplified by increasing levels of HOMA-IR, except for valine (*p* ≤ 0.040) (Fig. 2b).

In the sensitivity analysis substituting HOMA-IR with ISI_{0–120min}, we found no modifying effect of ISI_{0–120min} on the association between plasma glucagon and plasma

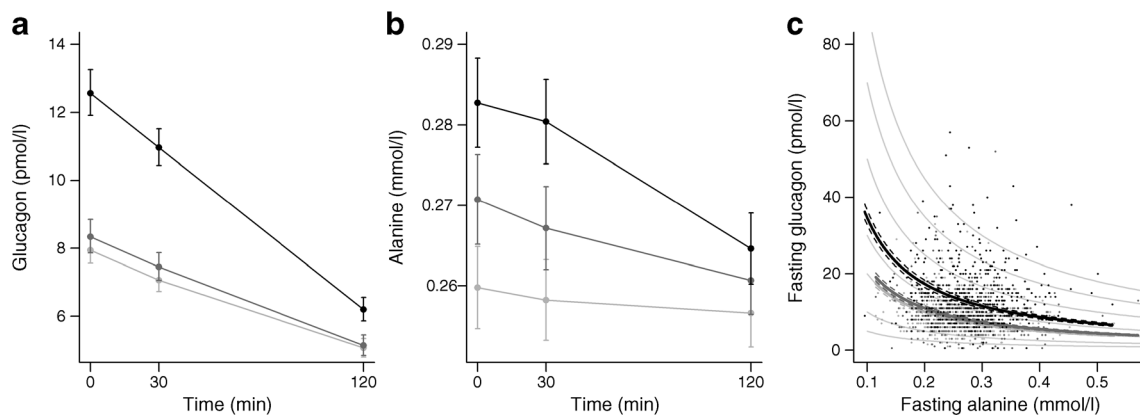


Fig. 1 Plasma concentrations of glucagon and alanine may reflect fasting HOMA-IR. **(a)** Plasma glucagon and **(b)** alanine concentrations during an OGTT by tertiles of HOMA-IR: lower tertile (light grey), middle tertile (dark grey) and upper tertile (black). Data are geometric means with 95% CI for glucagon, and means with 95% CI for alanine. **(c)** Scatter plots of fasting plasma glucagon as a function of fasting plasma alanine,

stratified by tertiles of HOMA-IR. Curves with 95% CI (dotted lines) are plotted within the range of observed fasting plasma alanine and shown for the lower tertile (thick light grey line), middle tertile (thick dark grey line) and upper tertile (thick black line). The thin grey lines illustrate different levels of the (multiplicative) product: fasting plasma glucagon × fasting plasma alanine (hyperbolic function)

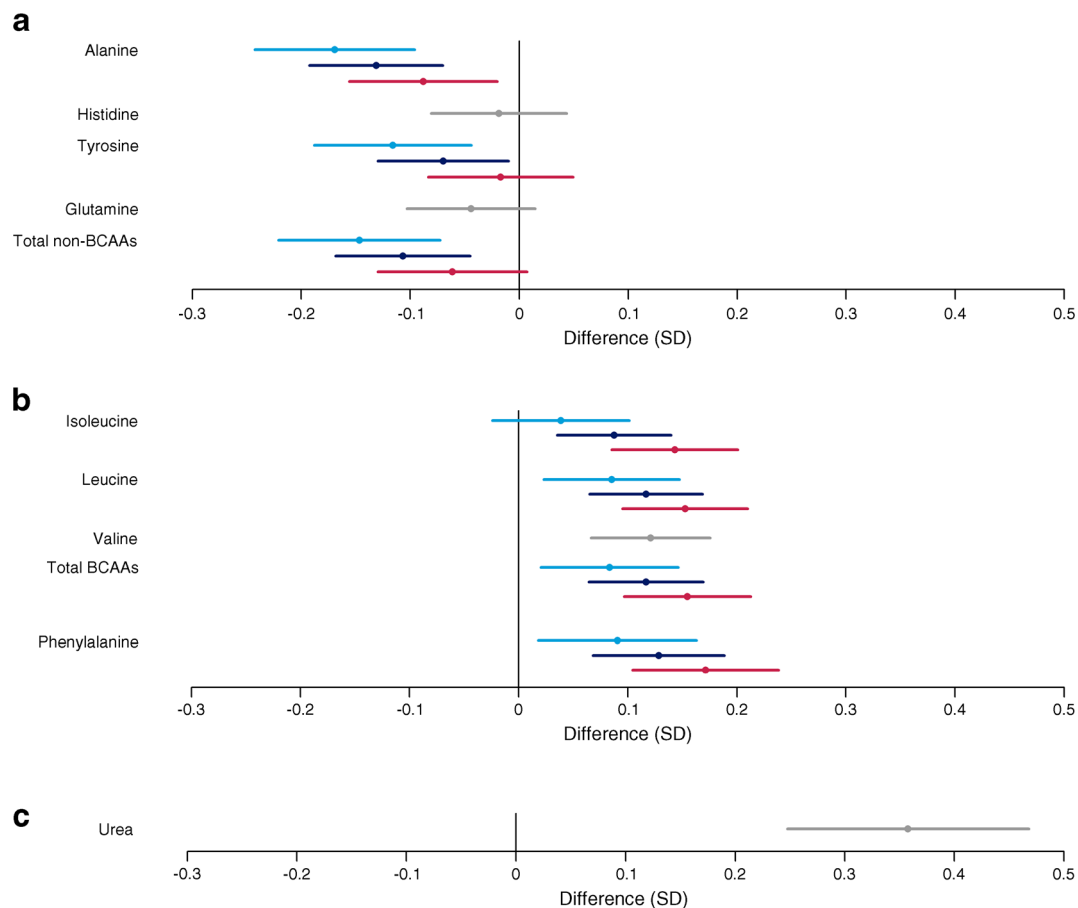


Fig. 2 Differential effect of glucagon on non-BCAAs and BCAAs. Difference (SD units) in plasma concentrations of (a) non-BCAAs, (b) BCAAs and (c) urea by a doubling of fasting plasma glucagon, adjusting for age, sex, BMI and insulin resistance (HOMA-IR). For plasma concentrations of alanine, tyrosine, total non-BCAAs, isoleucine, leucine, total BCAAs and phenylalanine, results are shown for three different

levels of HOMA-IR: median quartile, dark blue (1.39); lower quartile, light blue (0.91); upper quartile, red (2.25). For histidine, glutamine, valine and urea, results are shown as a grey line. Total non-BCAAs is defined as the sum of alanine, histidine, tyrosine and glutamine concentrations. Total BCAAs is defined as the sum of isoleucine, leucine and valine (and does not include phenylalanine)

concentrations of ‘non-BCAAs’ ($p > 0.166$ for all), but $ISI_{0-120min}$ modified the association between glucagon and total BCAAs ($p = 0.036$), with decreasing effect size of glucagon for higher levels of $ISI_{0-120min}$. Assuming that ureagenesis would be the outcome of glucagon action on amino acid metabolism, we also measured plasma urea levels in all samples. The results showed that a twofold increase in plasma glucagon was associated with a significant increase in plasma urea ($p < 0.001$); however, HOMA-IR did not have a significantly modifying effect ($p = 0.621$) (Fig. 2c).

Plasma amino acids and insulin resistance explain the variance in fasting plasma glucagon concentration As shown in Fig. 3, the eight amino acids and HOMA-IR explained an equal proportion (approximately 10%) of the total residual variance in fasting plasma glucagon, but there was also an overlap due to an association between HOMA-IR and plasma amino acid concentrations, supporting the relationship between liver function, amino acids and glucagon secretion.

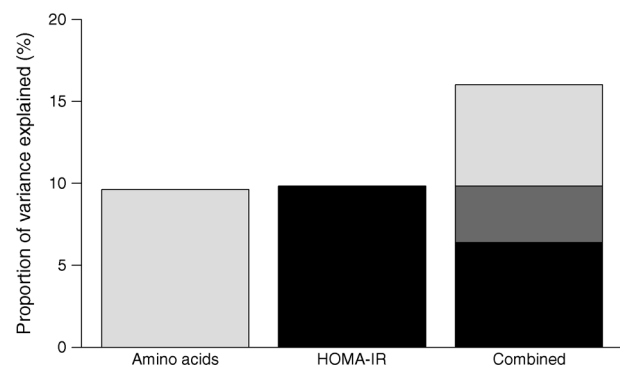


Fig. 3 Plasma concentrations of amino acids may explain variance in plasma glucagon concentrations. Proportion of variance in fasting plasma glucagon explained by all eight plasma amino acids, HOMA-IR, and plasma amino acids and HOMA-IR combined. In the ‘Combined’ bar, the light grey and black areas indicate the proportion of variance explained independently by plasma concentrations of amino acids and HOMA-IR, respectively, whilst the dark grey area indicates the proportion of variance explained by either HOMA-IR or plasma amino acids concentration owing to the correlation between them

Discussion

The data presented here support that a feedback loop exists in humans between glucagon and certain glucagonotropic (alanine, tyrosine and glutamine) amino acids, and when this is disrupted due to hepatic abnormalities (reflected here as elevated HOMA-IR levels), the lowering effect of glucagon on the plasma concentration of amino acids may be gradually impaired. This leads to increased amino acid concentrations, and eventually increased glucagon concentrations, as illustrated by increases in the new, proposed ‘glucagon–alanine index’. Specifically, we found in 1408 individuals with normal and impaired glucose regulation that the fasting plasma levels of four glucagonotropic amino acids were inversely associated with fasting glucagon levels independent of age, sex, BMI and peripheral insulin resistance (ISI_{0-120}), and that the associations were modified by hepatic insulin resistance (HOMA-IR). Plasma concentrations of both glucagon and non-BCCAs (e.g. alanine) during an OGTT were affected by increasing HOMA-IR; the BCAAs were also affected but in the opposite direction, as previously reported [24–26]. These observations support our hypothesis that impaired hepatic glucagon signalling, potentially due to fat accumulation in the liver and resulting hepatic insulin resistance [14], impairs glucagon’s ability to lower plasma levels of non-BCCAs. Taken together, the current findings support the proposed existence and importance of a liver–alpha cell axis in humans [8].

Alanine and other non-BCCAs, including tyrosine and glutamine, have previously been shown to increase glucagon secretion in humans and dogs, whereas this is not the case for BCAAs [27, 28]. In addition, prolonged administration of non-BCCAs to rodents results in alpha cell hyperplasia and hyperglucagonaemia [7, 29]. These observations have led to the proposal of the existence of a hitherto neglected feedback loop between the pancreatic alpha cells and the liver [8, 30]. Consistent with this proposed feedback loop, individuals with glucagon-producing tumours have decreased levels of plasma amino acids [11, 31], and, conversely, individuals with glucagon receptor mutations [10, 32, 33] and mice with glucagon receptor deficiency [30, 34] exhibit increased levels of plasma amino acids. Hyperglucagonaemia has also been reported in individuals with fatty liver disease independent of their glycaemic status [35], and this has recently been linked to an increased plasma pool of amino acids including alanine, but excluding the BCAAs isoleucine, leucine and valine [36].

In this study, only eight amino acids, including three BCAAs, were measured, which obviously limits the interpretation of our results. Measurement of all circulating amino acids would have been ideal but was unfeasible based on the current NMR-based platform. We may therefore have under- or overestimated the association between glucagon and several of the additional non-BCCAs. Glutamine, although known to spontaneously degrade in solution [37], was included in the

study because in particular this amino acid has been suggested to be of importance for amino acid-induced alpha cell proliferation [7] and glucagon secretion [38]. The importance of the measured amino acids for determining plasma levels of glucagon clearly needs further investigation given that only around 10% of the variance in plasma glucagon could be accounted for. Therefore, our results cannot be extrapolated to general hyperaminoacidaemia, but they do show that a link seems to exist between certain glucagonotropic amino acids and plasma levels of glucagon. Indeed, the non-BCCAs included in our analysis have recently been linked to alpha cell proliferation in animal models [39, 40]. Assuming that ureagenesis would be the outcome of glucagon action on amino acid metabolism, we also measured plasma urea levels in all samples. The results showed that a twofold increase in plasma glucagon was associated with a significant increase in plasma urea, therefore further supporting the hepatic actions of glucagon on amino acid metabolism. Finally, as the data shown here rely on a cross-sectional study design, it is important to note that causality cannot be implied.

In addition, disturbances of glucagon secretion at fasting or during intake of carbohydrates may be related to imbalances of insulin secretion and insulin sensitivity [41, 42], the latter having been associated with increased plasma concentrations of BCAAs and phenylalanine [26, 43]. This may also explain why we observed increasing plasma concentrations of BCAAs with increasing plasma concentrations of glucagon, and may reflect a glucose-related interaction of glucagon and insulin, as recently reported [44].

Interestingly, we recently demonstrated that glucose regulation during development of insulin resistance was associated not also with hypersecretion of insulin, but also with a reduced ability to acutely suppress glucagon after glucose intake [45]. The hypersecretion of glucagon from the pancreatic alpha cells may stem from an impairment of hepatic glucagon signalling (potentially caused by excessive food intake that may result in NAFLD and insulin resistance), which then, due to decreased glucagon-induced amino acid turnover, would result in hyperaminoacidaemia [36]. Finally, the hyperaminoacidaemia, and in particular the increased plasma concentrations of the glucagon-stimulatory amino acids [27, 28] alanine and tyrosine, may, as suggested by Solloway et al [7], result in hyperglucagonaemia, counteracting the NAFLD-induced impairment of glucagon-induced ureagenesis. Indeed, in the current study we found that elevations in plasma glucagon concentrations were associated with increased ureagenesis, as reflected by increased plasma concentrations of urea. However, this association was not significantly modified by HOMA-IR.

A major strength of this study is the large number of study participants encompassing a wide range of glycaemia from normal glucose tolerance to screen-detected and, hence, untreated type 2 diabetes. Importantly the measurement of

glucagon, which is demanding when it comes to analysing the subtle effects of small changes in fasting glycaemia on glucagon concentration, was carried out using a validated, highly sensitive radioimmunoassay, thus diminishing the risk of including erroneous glucagon measures, as may occur with some commercial glucagon assays [46]. Because of the size of the study, hepatic insulin resistance was evaluated by the relatively simple HOMA-IR model, which is often used in epidemiological studies, and not by hyperinsulinaemic–euglycaemic clamp, which is the gold standard in smaller but in-depth physiological studies. Nevertheless, HOMA-IR shows a fairly good correlation with hepatic insulin resistance measured by hyperinsulinaemic–euglycaemic clamp combined with tracer techniques to estimate hepatic glucose production [22], and is therefore a useful surrogate marker of hepatic insulin resistance. Moreover, we did not observe the same modifying effect of ISI_{0-120} as of HOMA-IR on the associations between fasting plasma levels of glucagon and non-BCAAs, which supports the notion of HOMA-IR being mainly related to insulin resistance in the liver. In addition, GGT, a liver-related enzyme that contributes to the transfer of γ -glutamyl groups to other amino acids and which has been suggested to be a potential surrogate marker of hepatic steatosis [47], was here coupled to changes in plasma glucagon and HOMA-IR, thereby supporting the suggestion that the increases in glucagon concentrations might be due to increased hepatic fat. HOMA-IR was recently demonstrated to be closely linked to hepatic steatosis [14].

Taken together, our findings suggest that hyperglucagonaemia may reflect disturbances in the physiological feedback loop between amino acid metabolism and the pancreatic alpha cells, rather than abnormalities of glucose metabolism. Observations of elevated fasting concentrations of plasma glucagon levels in humans should therefore direct attention to the functional status of the liver. Since hyperglucagonaemia is likely to be coupled to impaired amino acid metabolism, a combination of these two entities in the form of a product is likely to be a more sensitive measure of the abnormality than is either variable alone. The proposed glucagon–alanine index may provide an early indication of any abnormality of the feedback cycle. Whereas failures of alpha cell secretion are not well characterised, disturbances of liver function are common. However, based on our novel findings and partly as a consequence of improved assays for glucagon measurement [48], disturbances in alpha cell secretion may now begin to be uncovered. The usefulness of exploring such defects by use of our proposed glucagon–alanine index needs further studies. For instance, it will be interesting to see whether changes in hepatic insulin and glucagon sensitivity (HOMA-IR and the glucagon–alanine index) move in parallel or follow distinct trajectories over time. Moreover, recent research has suggested that disruption of the glucagon–liver cycle (with glucagon receptor antagonists) may also lead to disturbances

in lipid metabolism [49]. This has not been addressed in the present study, but needs to be investigated.

In conclusion, higher fasting plasma glucagon concentrations were associated with lower concentrations of certain non-BCAAs including alanine, tyrosine and glutamine, and with higher concentrations of BCAAs, in a large study population covering a broad glycaemic range from normal glucose tolerance to screen-detected type 2 diabetes. A liver–alpha cell axis may therefore exist in humans by which glucagon controls plasma levels of amino acid through hepatic gluconeogenesis and ureagenesis. Importantly, impaired liver function (increased HOMA-IR) weakened this relationship, resulting in higher amino acid levels and ensuing hyperglucagonaemia. If validated in other studies, a glucagon–alanine index may be a useful surrogate marker of glucagon receptor signalling in humans.

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Data availability Authors can confirm that all relevant data are included in the article.

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Duality of interest At the time of the initiation of the ADDITION-PRO study, the Steno Diabetes Center Copenhagen (SDCC) operated under the

name Steno Diabetes Center A/S, owned by Novo Nordisk A/S, and worked as a research hospital in the Danish National Health Service. The ADDITION-PRO study received internal research and equipment funds from Steno Diabetes Center A/S. As of 1 January 2017, Steno Diabetes Center A/S formally and legally transitioned to the Capital Region of Copenhagen, a public non-profit body, to become the SDCC. The SDCC receives part of its core funding from unrestricted grants from the Novo Nordisk Foundation. NJWA and JJH have received speaker fees from MSD Denmark in relation to glucagon biology. KF, TMJ, MEJ and DV are employed by SDCC. KF, DRW, TMJ, SST, TL, OP, MEJ and DV hold shares in Novo Nordisk A/S. TL has received unrestricted grants between 2000 and 2011 for the ADDITION study (screening and intensive treatment of type 2 diabetes in primary care) from the medical industry: Novo Nordisk AS, Novo Nordisk Scandinavia AB, ASTRA Denmark, Pfizer Denmark, GlaxoSmithKline Pharma Denmark, SERVIER Denmark A/S and HemoCue Denmark A/S. All other authors declare that there is no duality of interest associated with their contribution to this manuscript.

Contribution statement NJWA and KF drafted the manuscript. NJWA, KF, TMJ, JP, DV and JJH researched and interpreted the data. DV performed the statistical analyses. DRW, TL, OP and TH designed the ADDITION-PRO study (the parts related to glucagon and metabolomics). DRW, YM, AEJ, KDG, MWS, SST, TL, OP, FKK, TH and MEJ contributed to interpretation of the data. TMJ, DRW, JP, YM, AEJ, KDG, MWS, SST, TL, OP, FKK, TH, MEJ, DV and JJH revised the manuscript critically. All authors approved the final version of manuscript. JJH and DV are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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