Journal of the Endocrine Society, 2023, **7**, 1–11
https://doi.org/10.1210/jendso/bvad122
Advance access publication 21 September 2023
Clinical Research Article

Markers of Glucagon Resistance Improve With Reductions in Hepatic Steatosis and Body Weight in Type 2 Diabetes

Sasha A. S. Kjeldsen,[1,2] Mads N. Thomsen,[3] Mads J. Skytte,[3] Amirsalar Samkani,[3] Michael M. Richter,[1,2] Jan Frystyk,[4] Faidon Magkos,[5] Elizaveta Hansen,[6] Henrik S. Thomsen,[6] Jens J. Holst,[7,8] Sten Madsbad,[9] Steen B. Haugaard,[3,10] Thure Krarup,[3,5] and Nicolai J. Wewer Albrechtsen[1,2]

[1]Department of Clinical Biochemistry, Copenhagen University Hospital–Bispebjerg and Frederiksberg, Copenhagen, 2400, Denmark
[2]Novo Nordisk Foundation Center for Protein Research, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, 2200, Denmark
[3]Department of Endocrinology, Copenhagen University Hospital–Bispebjerg and Frederiksberg, Copenhagen, 2400, Denmark
[4]Department of Endocrinology, Odense University Hospital, Odense, 5000, Denmark
[5]Department of Nutrition, Exercise and Sports, University of Copenhagen, Frederiksberg, 1958, Denmark
[6]Department of Radiology, Copenhagen University Hospital–Herlev, Herlev, 2730, Denmark
[7]Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, 2200, Denmark
[8]Novo Nordisk Foundation Center for Basic Metabolic Research, University of Copenhagen, Copenhagen, 2200, Denmark
[9]Department of Endocrinology, Copenhagen University Hospital–Hvidovre, Hvidovre, 2650, Denmark
[10]Institute of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, 2200, Denmark

Correspondence: Nicolai J. Wewer Albrechtsen, MD, PhD, Department of Clinical Biochemistry, Copenhagen University Hospital–Bispebjerg and Frederiksberg, Nielsine Nielsens Vej 4B, Bispebjerg, 2400, Denmark. Email: nicolai.albrechtsen@regionh.dk.

Abstract

Context: Hyperglucagonemia may develop in type 2 diabetes due to obesity-prone hepatic steatosis (glucagon resistance). Markers of glucagon resistance (including the glucagon-alanine index) improve following diet-induced weight loss, but the partial contribution of lowering hepatic steatosis vs body weight is unknown.

Objective: This work aimed to investigate the dependency of body weight loss following a reduction in hepatic steatosis on markers of glucagon resistance in type 2 diabetes.

Methods: A post hoc analysis was conducted from 2 previously published randomized controlled trials. We investigated the effect of weight maintenance (study 1: isocaloric feeding) or weight loss (study 2: hypocaloric feeding), both of which induced reductions in hepatic steatosis, on markers of glucagon sensitivity, including the glucagon-alanine index measured using a validated enzyme-linked immunosorbent assay and metabolomics in 94 individuals (n = 28 in study 1; n = 66 in study 2). Individuals with overweight or obesity with type 2 diabetes were randomly assigned to a 6-week conventional diabetes (CD) or carbohydrate-reduced high-protein (CRHP) diet within both isocaloric and hypocaloric feeding-interventions.

Results: By design, weight loss was greater after hypocaloric compared to isocaloric feeding, but both diets caused similar reductions in hepatic steatosis, allowing us to investigate the effect of reducing hepatic steatosis with or without a clinically relevant weight loss on markers of glucagon resistance. The glucagon-alanine index improved following hypocaloric, but not isocaloric, feeding, independently of macronutrient composition.

Conclusion: Improvements in glucagon resistance may depend on body weight loss in patients with type 2 diabetes.

Key Words: carbohydrate-reduced high-protein diet, metabolic dysfunction-associated steatotic liver, nonalcoholic fatty liver disease, type 2 diabetes, weight loss, weight maintenance

Abbreviations: BMI, body mass index; CD diet, conventional diabetes diet; CRHP diet, carbohydrate-reduced high-protein diet; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; MASLD, metabolic dysfunction–associated steatotic liver disease.

Hormonal dysregulation in type 2 diabetes—particularly in relation to the concentration and action of glucagon—contributes to fasting hyperglycemia due to inappropriate increases in hepatic glucose production. Some, but not all, patients with type 2 diabetes have increased plasma levels of glucagon (hyperglucagonemia) [1-3]. Several studies demonstrate that glucagon partially regulates systemic amino acid homeostasis via actions on the liver following amino acid–stimulated secretion of pancreatic glucagon [4-7], recognized as the liver-alpha cell axis [8, 9]. Thus, glucagon acts on hepatocytes to augment the uptake [10, 11] and metabolism [12] of amino acids.

Received: 24 July 2023. **Editorial Decision:** 19 September 2023. **Corrected and Typeset:** 9 October 2023

Hyperglucagonemia is evidenced in individuals with metabolic dysfunction–associated steatotic liver disease (MASLD) (previously termed *nonalcoholic fatty liver disease*) and may reflect a state of hepatic glucagon resistance with respect to amino acid catabolism [13-15]. In contrast, glucagon's effect on hepatic glucose production is not reduced in MASLD, indicating that hepatic steatosis per se does not cause resistance to glucagon-mediated glucose production. The glucagon-alanine index, a validated [14, 16-18] plasma marker for glucagon resistance [12], is associated with hepatic steatosis [12, 17].Weight loss reduces hepatic steatosis [19-21] and may be accompanied by reductions in the glucagon-alanine index [14]. However, the independent contribution to the evidenced improvements in glucagon resistance following reduction in hepatic steatosis from that of weight loss is not clear. Additionally, changes in systemic amino acid availability by altered macronutrient composition may also affect glucagon resistance by altering pancreatic glucagon secretion [7, 22, 23] since increased protein intake affects hepatic amino acid metabolism via effects on the alpha cells [24, 25].

To improve our understanding of how differences in energy-restriction and, secondarily, macronutrient composition may affect markers of glucagon sensitivity, we performed a post hoc analysis on 2 recently published clinical trials [26-29] including new measurements of plasma glucagon and the metabolome. In these studies, it was demonstrated that a 6-week carbohydrate-reduced high-protein (CRHP) diet improved glucose and lipid metabolism more compared to a conventional diabetes (CD) diet as observed in a parallel group trial with calorie restriction causing body weight loss [26, 27], and in a crossover trial aiming at weight maintenance [28, 29]. In this study, we investigated the dependency of body weight loss following a reduction in hepatic steatosis on markers of glucagon resistance in type 2 diabetes. We hypothesized that a reduction in hepatic steatosis would improve glucagon sensitivity independently of body weight loss.

Materials and Methods

Ethical Approvals

Participants provided written, informed consent to the study protocols, which were approved by the Health Ethics Committee of Copenhagen and the Danish Data Protection Agency. Both studies are registered with ClinicalTrials.gov (registration Nos. NCT03814694 and NCT02764021) and were conducted in accordance with the Declaration of Helsinki.

Study Design

We performed additional biochemical analyses on a subset of samples from 2 previously published studies [26-29] (study design shown in Fig. 1). Inclusion criteria for both studies included men and women with type 2 diabetes with glycated hemoglobin A_{1c} (HbA_{1c}) of 48 to 97 mmol/mol (6.5%-11.0%), which was assessed at screening according to best clinical practice by following international guidelines on type 2 diabetes diagnostics. These studies (study 1: isocaloric feeding; study 2: hypocaloric feeding) investigated the effect of a CRHP compared to a CD diet on glucose and lipid metabolism, and hepatic steatosis. The aim of the present study was to compare the effects of isocaloric and hypocaloric feeding, both with similar effects on hepatic steatosis, on markers of glucagon resistance. The primary outcome measure was changes from baseline to 6 weeks in a validated [14, 16-18] biochemical marker of glucagon resistance, termed *glucagon-alanine index* [12]. Detailed descriptions of the 2 trials have been published previously [26-29]. The following sections include a short description of the essential information from these 2 studies.

Study 1—isocaloric study

The study was designed as a 6 + 6-week open-label, randomized, crossover-controlled trial with 28 participants. For the data presented here, we included only the first 6 weeks of the study. Therefore, previously published data (eg, hepatic steatosis) on the isocaloric study may not be identical to what is reported here due to this selection. Fourteen individuals consumed an isoenergetic CD diet, and 14 individuals consumed an isoenergetic CHRP diet.

Study 2—hypocaloric study

The study was designed as an open-label, parallel, randomized controlled trial with 72 included participants allocated in a 1:1 ratio to a hypoenergetic CD or CRHP diet for 6 weeks. Five participants withdrew their consent before study completion and one participant had missing values for most parameters investigated here, leaving 66 participants for data analysis. Thirty-two individuals consumed a hypoenergetic CD diet, and 34 individuals consumed a hypoenergetic CHRP diet.

We included time points only from baseline to the end of the first 6-week diet intervention for study 1 and study 2. This is due to the potential bias on plasma amino acid levels for those individuals initially randomly assigned to the CRHP diet (due to high protein intake).

Diet Interventions

The CD diet provided 50 E% carbohydrate, 17 E% protein and 33 E% fat, and the CRHP diet provided 30 E% carbohydrate, 30 E% protein, and 40 E% fat. In the isocaloric study, the provided daily energy corresponded to the participants' total energy expenditure [30], and in the hypocaloric study, the provided daily energy was calculated based on total energy expenditure adjusted for the intended weight loss.

Magnetic Resonance Imaging Analysis

Hepatic steatosis was evaluated by magnetic resonance imaging (MRI) using a 3.0 T Ingenia MRI system (Philips Healthcare) with a dStream torso coil and evaluated at baseline and following 6 weeks' diet intervention. Total hepatic fat fractions were measured by single-voxel MR spectroscopy (Point RESolved Spectroscopy [PRESS]) [31, 32]. These data have been published previously [26-29].

Biochemical Analysis

Blood was sampled after a 10-hour overnight fast in precooled EDTA tubes and centrifuged. Samples obtained at baseline and following 6 weeks' diet intervention were evaluated. Plasma levels of total amino acids were measured using a commercially available L-Amino Acid Assay kit (Abcam, ab65347). Plasma concentrations of glucagon were measured according to the manufacturer's protocol with a validated [33] sandwich enzyme-linked immunosorbent assay (Mercodia catalog

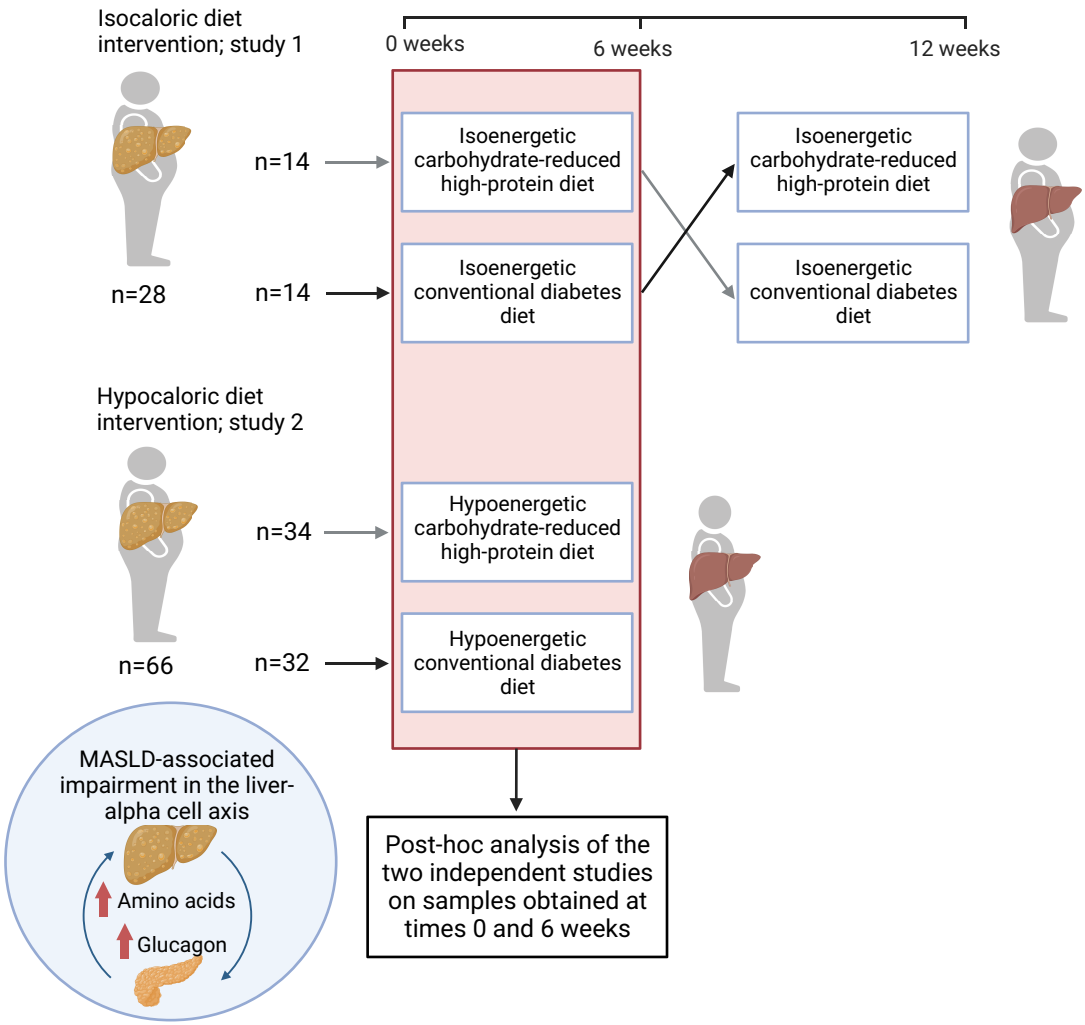

Figure 1. Study designs of the previously published studies. The isocaloric study intervention (study 1) was designed as a randomized crossover study with 2 × 6 weeks' diet intervention. The hypocaloric study intervention (study 2) was designed as a randomized parallel study with 6 weeks' diet intervention. The present study reports on data from both studies at weeks 0 and 6 (as illustrated in the box). Illustration using Biorender.

No. 10-1271-01; RRID: AB_2737304). Samples for measuring individual amino acids (metabolomics) were derivatized with methyl chloroformate and measured using a slightly modified version of a previously described method [34], and processed as previously described [35]. The remaining measurements (glucose, insulin, and HbA_{1c} levels) were measured as previously described and have been published previously [26-29].

Calculations

The glucagon-alanine index was calculated as fasting glucagon (pmol/L) × fasting alanine (mmol/L) [12]. The Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) was calculated as fasting glucose (mmol/L) × fasting insulin (μU/mL)/22.5.

Statistical Analysis

The primary outcome measure was the changes from baseline to 6 weeks in the glucagon alanine index. Data distribution and homoscedasticity were evaluated by histograms, residual plots, and Q-Q plots. A mixed-effects analysis with repeated measurements was used to compare study interventions (isocaloric vs hypocaloric) over time (baseline vs 6 weeks). Similarly, the effect of diet (CD vs CRHP) within studies were evaluated by mixed-effects analysis with repeated measurements over time. If the mixed-effects analysis revealed statistical significance (main effects or interactions), Sidak's post hoc test was applied to adjust for multiple comparisons and evaluate significant comparisons. Unpaired *t* tests were used to evaluate differences between changes expressed as Δ values (baseline-subtracted) between trials (isocaloric vs hypocaloric), or between diets (CD vs CRHP). Multiple linear regression was employed to evaluate possible predictors of the glucagon-alanine index for values obtained at week 6 (at study completion for the first randomized diet). Categorical variables were coded as binary variables. One outlier was removed (based on visual assessment of residual and Q-Q plots), and 2 observations were deleted due to missing values, leaving 91 individuals for the multiple linear regression analyses (model 1 and model 2). Data from the 6-week time point was selected for the regression analyses. A *P* value of less than .05 was considered statistically significant. Statistical calculations (unpaired *t* tests and mixed-effects analyses) were performed in GraphPad Prism (version 9.4.1 for Windows; GraphPad Software). Simple and multiple linear regressions were performed using the built-in lm function (base package) in R

Table 1. Baseline anthropometric, biometric, and blood biochemistry data

Variable	Isocaloric study			Hypocaloric study		
	CD/CRHP diets pooled (n = 28)	CD diet (n = 14)	CRHP diet (n = 14)	CD/CRHP diets pooled (n = 66)	CD diet (n = 32)	CRHP diet (n = 34)
Age, y	64 ± 8	63 ± 8	65 ± 6	67 ± 8	67 ± 9	66 ± 7
Sex, % F/% M	29/71	36/64	21/79	47/53	53/47	41/59
Liver fat, %	10 ± 8	10 ± 9	10 ± 7	10 ± 9	9 ± 8	10 ± 10
Body weight	90 ± 20	88 ± 22	90 ± 15	98 ± 20	98 ± 25	98 ± 14
BMI	30 ± 5	30 ± 5	30 ± 4	33 ± 5[b]	33 ± 5	34 ± 5
Metformin, % Y/% N	89/11	86/14	93/7	68/32	63/37	74/26
HbA_{1c}, mmol/mol	60 ± 8	61 ± 9	59 ± 8	57 ± 8	57 ± 8	58 ± 8
HOMA-IR	5.5 ± 4	4.5 ± 2	6.5 ± 5	8.7 ± 4[c]	9.2 ± 4.5	8.2 ± 3.4
Plasma glucagon, pmol/L	9.6 ± 9.6	10.5 ± 12	8.7 ± 5	8.1 ± 4	8 ± 4.2	8.5 ± 4.4
Total plasma amino acids, μmol/L	1551 ± 157	1570 ± 142	1533 ± 168	1761 ± 273[c]	1761 ± 285	1761 ± 262
Plasma alanine, μmol/L	0.33 ± 0.07	0.33 ± 0.06	0.34 ± 0.08	0.35 ± 0.08	0.36 ± 0.08	0.36 ± 0.09
Glucagon-alanine index	3.2 ± 2.8	3.3 ± 3	3.1 ± 2.7	3 ± 1.9	3 ± 2.2	3 ± 1.5

Data are presented as mean ± SD.
Abbreviations: CD diet, conventional diabetes diet; CRHP diet, carbohydrate-reduced high-protein diet; F, female; HbA_{1c}, glycated hemoglobin A_{1c}; HOMA-IR, homeostatic model assessment of insulin resistance; M, male; N, no; Y, yes.
Statistically significant differences between isocaloric and hypocaloric interventions are shown by the following: [a]indicates *P* less than .05, [b]indicates *P* less than .01, [c]indicates *P* less than .001, and [d]indicates *P* less than .0001.

(version R 4.2.2). Data are presented as mean ± SD unless otherwise stated.

Results

The baseline characteristics for the patients enrolled in the isocaloric (study 1) and hypocaloric (study 2) study interventions, including subgrouping by diet (CRHP vs CD), are presented in Table 1. Baseline characteristics for both cohorts were overall similar (study 1 vs study 2: HbA_{1c}, 59.7 ± 8.4 vs 57.4 ± 8.0 mmol/mol; age, 64 ± 8 years vs 67 ± 8; glucagon, 9.6 ± 9.8 vs 8.3 ± 4.3 pmol/L; glucagon-alanine index, 3.2 ± 2.8 vs 3.0 ± 1.9 pM × mM), however, individuals in the hypocaloric study had higher body weight (98 ± 20 vs 89 ± 19 kg), HOMA-IR (9 ± 4 vs 6 ± 4), and plasma and serum concentrations of amino acids (1761 ± 273 vs 1551 ± 160 μmol/L) and insulin (134 ± 61 vs 81 ± 58 pmol/L) (*P* < .05) compared to individuals in the isocaloric study. Within each study, there were no baseline differences between participants assigned to the CD or the CRHP diets.

First, we evaluated differences between the isocaloric and hypocaloric study interventions (CD and CRHP data were pooled within each trial) (Fig. 2A-H). Both interventions induced a significant body weight reduction, but participants lost significantly more body weight following the hypocaloric intervention compared to the isocaloric intervention (98 ± 20 to 92 ± 19 vs 90 ± 19 to 88 ± 19 kg, respectively; *P* < .0001; Fig. 2A). The reduction in hepatic steatosis was similar following 6 weeks' hypocaloric or isocaloric feeding interventions (10 ± 9 to 4 ± 4 vs 10 ± 8 to 5 ± 5%, respectively; *P* < .0001; Fig. 2B). The glucagon-alanine index decreased following the hypocaloric intervention (3.0 ± 1.9 vs 2.2 ± 1.3 pmol/L × mmol/L; *P* < .0001) but did not change following the isocaloric intervention (3.2 ± 2.8 vs 2.8 ± 2.4 pmol/L × mmol/L; *P* = .32; Fig. 2C). Hepatic insulin resistance, evaluated by fasting HOMA-IR, decreased following the hypocaloric intervention (8.7 ± 4 vs 5.4 ± 3.6 mmol/L × μU/mL; *P* < .001), but was unaltered following the isocaloric intervention (5.5 ± 4.2 vs 4.9 ± 3.2 mmol/L × μU/mL; *P* = .8; Fig. 2D). Following the hypocaloric feeding intervention, plasma concentrations of total amino acids (1761 ± 273 vs 1650 ± 243 μmol/L; *P* < .001) and glucagon (8.3 ± 4 vs 6.8 ± 3.6 pmol/L; *P* < .001) decreased (Fig. 2E and 2F). Consistent with this, plasma concentrations of several individual amino acids decreased following the hypocaloric intervention, including the glucagonotropic amino acid alanine, and the branched-chain amino acids (leucine, isoleucine, and valine), in addition to glutamic acid, tyrosine, phenylalanine, proline, tryptophan, and methionine (Fig. 3). Plasma concentrations of total amino acids, glucagon, and the individual amino acids did not change following the isocaloric intervention except for cysteine, serine, and valine (Figs. 2E and 2F and 3A and 3B). Both the isocaloric and hypocaloric interventions caused a decline in HbA_{1c} (Fig. 2G). Serum concentrations of insulin decreased following hypocaloric feeding but were unaltered after isocaloric feeding (Fig. 2H).

Next, we performed a subgroup analysis by stratifying the 2 study interventions (isocaloric and hypocaloric studies) on diet (CD vs CRHP) to investigate the effect of macronutrient composition on markers of glucagon resistance. Hepatic steatosis decreased following both CD and CRHP diets in both studies (Fig. 4A and 4B). The CRHP diet caused a larger numerical reduction in hepatic steatosis compared to the CD diet in both isocaloric (*P* = .22) and hypocaloric (*P* = .27) studies. In the isocaloric study, the glucagon-alanine index lowered following the CRHP diet compared to the CD diet (Fig. 4C), but this did not reach statistical significance (Δ, −.7 ± 1.4 vs 0.1 ± 1.0; *P* = .07). Both CRHP and CD diets caused a reduction in the glucagon-alanine index following the hypocaloric intervention (Δ, −0.9 ± 1.2 vs −.7 ± 1.6) (Fig. 4D). Neither CD nor CRHP diets decreased plasma levels of amino acids or glucagon following the isocaloric intervention, whereas both CD and CRHP diets reduced plasma

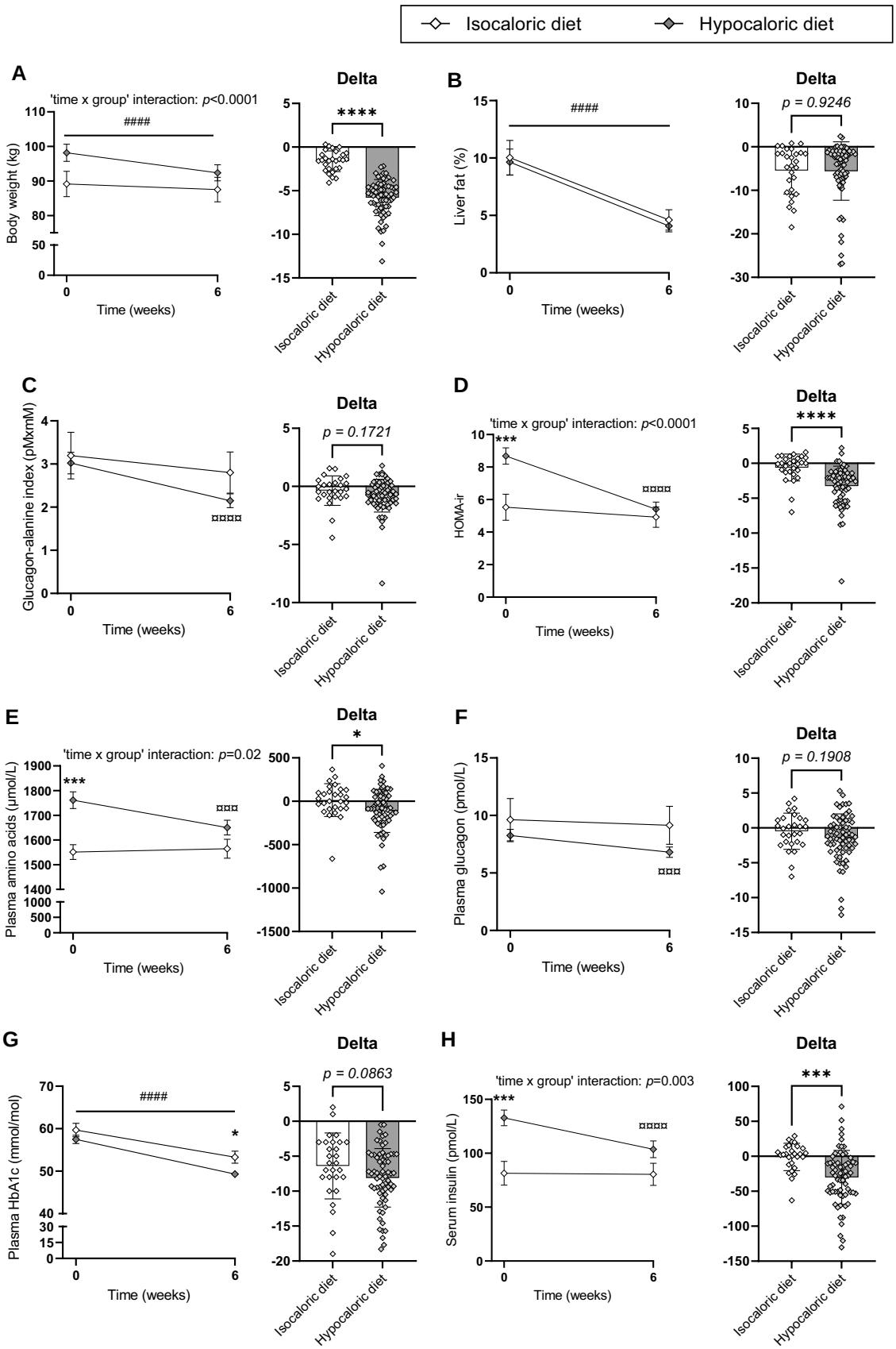

Figure 2. Improvement in glucagon sensitivity following a reduction in hepatic steatosis depends on a concurrent weight loss. Effect of 6-week isocaloric or hypocaloric diets in patients with type 2 diabetes on A, body weight; B, hepatic steatosis; C, glucagon-alanine-index; D, fasting Homeostatic Model Assessment of Insulin Resistance (HOMA-IR); E, total amino acids; F, plasma glucagon; G, glycated hemoglobin A_{1c} (HbA_{1c}); and H, plasma insulin. Both absolute and Δ values are shown. Mixed-effects analyses followed by Sidak's multiple comparisons test for A to H, and unpaired *t* tests between Δ, were performed using GraphPad Prism (version 9.4.1). Data are presented as mean ± SEM. Statistical significance is marked by * for comparisons between isocaloric and hypocaloric trials; ¤ for effect of time for the hypocaloric study intervention; and # above a horizontal line for main effect of time. One symbol indicates *P* less than .05; 2 symbols indicate *P* less than .01; 3 symbols indicate *P* less than .001, and 4 indicate *P* less than .0001.

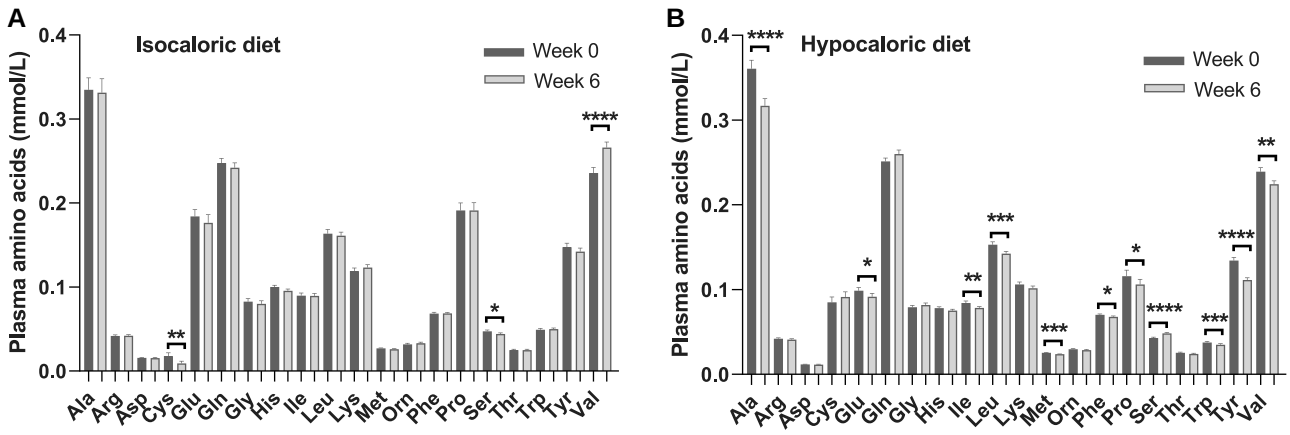

Figure 3. Changes in fasting plasma concentrations of individual amino acids depend on body weight loss. Individual amino acids were compared by paired *t* tests before and 6 weeks after an A, isocaloric or B, hypocaloric diet-intervention. Data are presented as mean ± SEM. One symbol (*) indicates *P* less than .05; 2 symbols indicate *P* less than .01; 3 symbols indicate *P* less than .001, and 4 indicate *P* less than .0001. Ala, alanine; Arg, arginine; Asp, aspartic acid; Cys, cysteine; Glu, glutamic acid; Gln, glutamine; Gly, glycine; His, histine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Orn, ornithine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine.

concentrations of amino acids and glucagon following the hypocaloric intervention (Fig. 4E and 4H). The effect of diets and caloric restriction on the individual amino acid levels are shown in Table 2. Both the composition of the macronutrient and the caloric load had a differential effect on the individual amino acid concentration but were also dependent across the measured amino acids. Interestingly, the glucagonotropic amino acid alanine reduced with the CRHP diet compared to the CD diet within both isocaloric and hypocaloric study interventions (see Table 2). In contrast, the branched-chain amino acids appeared more regulated by the caloric load than by that of the diet. HbA_{1c} declined more following the CRHP diet compared to the CD diet in both the isocaloric and the hypocaloric study (Fig. 4I and 4J), as previously reported [26, 28].

Finally, to evaluate possible predictors of glucagon sensitivity, we performed multiple linear regression analyses. In the first model (model 1), we investigated whether anthropometric variables or interventional factors influenced glucagon sensitivity by using hepatic steatosis (%), body mass index (BMI), study (categorical: isocaloric vs hypocaloric intervention), and diet (categorical: CD vs CRHP) as possible predictors for glucagon-alanine index outcome. Hepatic steatosis was a significant predictor of glucagon-alanine index ($P < .0001$) outcome, whereas BMI was not ($P = .07$). Energy consumption (isocaloric vs hypocaloric intervention) and macronutrient composition (CD vs CRHP) was not significantly associated with the glucagon-alanine index. Model 1 explained 18% of the variance in the glucagon-alanine index (adjusted R^2). Next, we aimed to further adjust our model by additionally including sex, age, and HOMA-IR (Table 3) as possible predictors of glucagon-alanine index outcome (model 2). Hepatic steatosis continued to be a significant predictor for glucagon-alanine index outcome, but also HOMA-IR and sex emerged as significant predictors, the former being consistent with the literature. This model (model 2) explained 30% of the variance for the glucagon-alanine index ($P < .0001$) (see Table 3).

Discussion

In this post hoc analysis, we demonstrate that improvements in glucagon sensitivity, as evaluated by the validated [12, 14, 17, 18] glucagon-alanine index, may depend on body weight loss and not only reduction in hepatic steatosis in individuals with overweight or obesity and type 2 diabetes. These data uncover that glucagon resistance may depend on additional features than hepatic steatosis, which has not previously been observed. Our findings highlight obesity as a cause of glucagon resistance by mechanisms not explained by hepatic steatosis alone and implicate body weight loss as a pertinent approach to improve glucagon resistance. Interpreting these findings in the context of hyperglucagonemia as a risk factor for type 2 diabetes development, our study indicates altered glucagon sensitivity as a potential underlying mechanism for the increased risk of diabetes in obesity.

Similarly, other studies find that glucagon sensitivity improves (corresponding to a decline in the glucagon-alanine index) following body weight loss induced by diet [14], surgery [18], or pharmacotherapy [16]. Remission of hepatic steatosis is often accompanied by body weight loss, and the effect of reducing hepatic steatosis without a concurrent body weight reduction has to our knowledge not been investigated previously. Interestingly, plasma levels of the glucagonotropic amino acid alanine reduced with the CRHP diet independently of isocaloric or hypocaloric feeding interventions. This suggests an improvement in glucagon signaling with CRHP feeding. However, due to similar changes in plasma levels of glucagon with CRHP and CD diets within both isocaloric and hypocaloric study interventions, the CRHP diet did not significantly improve the glucagon-alanine index. Similarly to the glucagon-alanine index, HOMA-IR also did not reduce following 6 weeks' isocaloric feeding. Glucagon resistance and insulin resistance are associated as shown here and by others [17]. Therefore, another possibility for the apparent indifference in glucagon sensitivity following isocaloric feeding may also be driven by the lack of change in HOMA-IR. Additionally, the apparent differences at baseline on plasma levels of amino acids and insulin between study 1 and study 2 may also have affected glucagon-alanine index outcome. Finally, other steatotic depots (such as pancreatic steatosis), which have not been investigated here, may also have influenced the results.

Despite a body weight loss dependency for improving the glucagon-alanine index in individuals with type 2 diabetes,

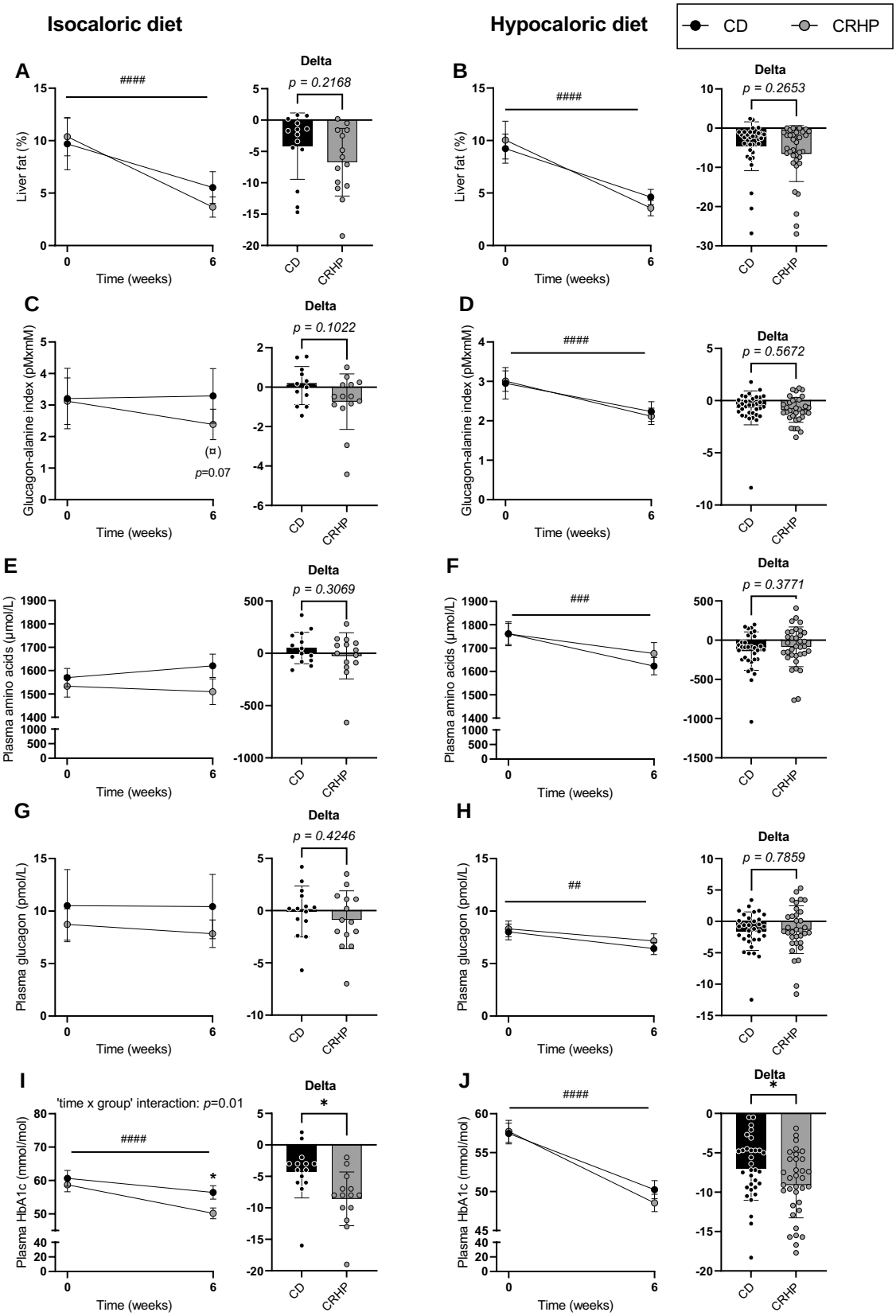

Figure 4. Changes in glucagon sensitivity following an isocaloric or hypocaloric intervention do not depend on dietary macronutrient composition. Effect of a 6-week isocaloric (A, C, E, G, I) or hypocaloric (B, D, F, H, J) diets in patients with type 2 diabetes on A and B, hepatic steatosis; C and D, glucagon-alanine-index; E and F, total amino acids; G and H, plasma glucagon; and I and J, glycated hemoglobin A_{1c} (HbA_{1c}). A mixed-effects analysis followed by Sidak's multiple comparisons test was performed for A to J, and unpaired *t* tests between Δ were performed using GraphPad Prism (version 9.4.1). Data are presented as mean ± SEM. Statistical significance is marked by * for comparisons between conventional diabetes (CD) and carbohydrate-reduced high-protein (CRHP) diets (post hoc analysis following mixed-effects analysis and *t* tests between Δ); ¤ for effect of time for CRHP diet intervention (post hoc analysis following mixed-effects analysis); and # above a horizontal line for main effect of time. One symbol indicates *P* less than .05; 2 symbols indicate *P* less than .01; 3 symbols indicate *P* less than .001; and 4 indicate *P* less than .0001.

Table 2. Effect of macronutrient composition on plasma levels of individual amino acids

	Isocaloric study			Hypocaloric study		
	CD diet	CRHP diet	*P*	CD diet	CRHP diet	*P*
Alanine	28.4 ± 67.8	−34.1 ± 58.5	.02	−21.2 ± 68.1	−65.6± 86.3	.02
Arginine	2.1 ± 7.8	−1.0 ± 9.3	.37	−1.9 ± 8.7	−0.4 ± 11.4	.57
Aspartic acid	0.2 ± 2.4	−0.5 ± 2.1	.44	0.2 ± 4.1	−0.6 ± 2.5	.34
Cysteine	−7.0 ± 12.6	−10.9 ± 17.7	.53	−2.0 ± 40.7	14.3 ± 45.1	.12
Glutamic acid	9.4 ± 33.0	−19.9 ± 25.8	.02	−3.2 ± 27.5	−10.9 ± 18.7	.18
Glutamine	5.2 ± 29.7	−12.6 ± 29.9	.14	17.7 ± 31.2	−0.4 ± 44.2	.06
Glycine	4.9 ± 14.4	−10.5 ± 14.8	.01	3.6 ± 15.8	1.6 ± 14.4	.59
Histidine	−1.0 ± 15.7	−4.8 ± 11.1	.49	−2.6 ± 13.2	−3.1 ± 11.4	.86
Isoleucine	1.5 ± 9.6	−0.3 ± 10.7	.65	−9.0 ± 15.1	−2.8 ± 15.2	.10
Leucine	−2.0 ± 17.4	−2.0 ± 16.9	.99	−16.7 ± 23.2	−4.5 ± 21.5	.03
Lysine	1.9 ± 11.9	7.4 ± 23.3	.47	−7.7 ± 20.5	−1.4 ± 20.0	.21
Methionine	−0.4 ± 2.6	−1.2 ± 4.0	.57	−1.8 ± 4.0	−1.5 ± 3.2	.75
Ornithine	0.03 ± 4.55	2.39 ± 7.98	.38	−2.7 ± 7.6	0.6 ± 7.5	.07
Phenylalanine	0.4 ± 3.7	1.0 ± 6.1	.77	−4.3 ± 7.9	−0.3 ± 6.5	.03
Proline	15.7 ± 34.5	−15.3 ± 34.9	.03	−10.6 ± 36.6	−8.9 ± 27.3	.83
Serine	−2.2 ± 5.8	−3.5 ± 6.3	.61	4.7 ± 8.5	6.0 ± 8.9	.54
Threonine	1.5 ± 4.2	−1.7 ± 3.8	.05	−1.6 ± 5.4	−1.0 ± 6.4	.65
Tryptophan	2.5 ± 4.9	0.8 ± 8.0	.53	−2.6 ± 5.9	−2.9 ± 6.4	.84
Tyrosine	−0.4 ± 18.7	−6.0 ± 24.1	.52	−25.3 ± 31.7	−21.1 ± 19.8	.51
Valine	18.2 ± 25.5	45.6 ± 17.9	.003	−27.3 ± 37.7	−2.3 ± 30.7	.004

Effect of a 6-week CD or CRHP diet within isocaloric and hypocaloric study interventions. Delta values (value at 6 weeks subtracted from baseline) in µmol/L are presented as mean ± SD. *P* values represent *t* testing with correcting for multiple testing between diet for the individual trial (hypocaloric and isocaloric diet).
Abbreviations: CD, conventional diabetes; CRHP, carbohydrate-reduced high-protein.

Table 3. Multiple linear regression analysis, model 2

	Coefficient, β	SE	*T* value	Significance	95% CI, 2.5%	95% CI, 97.5%
Dependent variable						
Glucagon-alanine index	−1.57	1.49	−1.05	0.30	−4.54	1.40
Independent variables						
Hepatic steatosis, %	0.08	0.04	2.31	0.02	0.01	0.15
BMI	0.30	0.03	1.15	0.25	−0.02	0.09
HOMA-IR	0.12	0.05	2.74	0.007	0.03	0.21
Study (hypo)	−0.38	0.30	−1.31	0.19	−0.97	0.20
Diet (CRHP)	−0.11	0.25	−0.45	0.66	−0.61	0.39
Age, y	0.03	0.02	1.63	0.11	−0.01	0.06
Sex (male)	0.64	0.25	2.50	0.01	0.13	1.14
R^2	0.35					
Adjusted R^2	0.30					
n	91					

Multiple linear regression analysis.
Abbreviations: BMI, body mass index; CD diet, conventional diabetes diet; CRHP diet, carbohydrate-reduced high-protein diet; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; hypo, hypocaloric.

BMI was not significantly associated with the glucagon-alanine index. Rather, hepatic steatosis and HOMA-IR were significantly associated with the glucagon-alanine index consistent with previous reports [12, 17]. These data indicate that hepatic steatosis dictates the level of glucagon resistance to a greater extent than excess body weight, but on the other hand, that a reduction in hepatic steatosis alone without weight loss is insufficient for improving markers of glucagon sensitivity. These data implicate differential mechanism(s) of hepatic steatosis vs other dysmetabolic features in obesity as drivers for altered

glucagon secretion. The underlying mechanism for this is unknown and warrants further investigation. Thus, obesity (or other unwanted fat depositions, not measured here) may also cause hypersecretion of glucagon [36] and impaired glucagon-mediated amino acid catabolism [15]. The glucagon-alanine index was also significantly associated with sex, and higher values (indicating more glucagon resistance) were observed in men compared to women, which is comparative to the sexual dimorphic profile regarding insulin resistance as evidenced in the literature [37, 38]. Sexual dimorphism regarding glucagon sensitivity in type 2 diabetes is a possibility that needs further study. However, given that insulin stimulates the secretion of androgens, linked to the development of insulin resistance [39], the sex-dependent differences in the glucagon-alanine index may be secondary to insulin resistance.

We assessed parameters of glucagon metabolism only during fasting, overlooking postprandial excursions, which may contribute substantially to disease, as shown for glucose metabolism [40]. In a similar study [23] closely resembling the isocaloric study presented here, individuals with type 2 diabetes followed a 6-week isocaloric high-protein diet that yielded no changes in fasting levels of glucagon, mirroring our findings. Nevertheless, the authors [23] also assessed the effect of a mixed-meal tolerance test following 6 weeks' high-protein feeding and showed a noteworthy reduction in postprandial alanine levels, while postprandial glucagon levels remained unchanged, indicative of improved glucagon signaling. Indeed, glucagon is also important for the postprandial regulation of glucose and amino acid metabolism [9] and perhaps also lipid metabolism [41]. The stimulating effects of high-protein feeding on postprandial glucagon secretion are well established. However, the CRHP diet did not increase fasting levels of glucagon nor amino acids when compared to the CD diet in either study (isocaloric and hypocaloric). Rather, the 2 most abundant amino acids in plasma, alanine and glutamine, numerically reduced following CRHP feeding in both isocaloric and hypocaloric interventions, indicating improved glucagon sensitivity. Therefore, hourly bouts of hyperglucagonemia during states of high amino acid availability do not seem to impair glucagon sensitivity but may rather improve glucagon resistance as evaluated here in the fasted state.

The isocaloric intervention was designed as a crossover study. We did not include data following crossover as the primary objective of the present study was to investigate the change in markers of glucagon sensitivity following a diet-induced reduction in hepatic steatosis with or without a concurrent clinically relevant body weight loss. No additional reduction in hepatic steatosis was evident following crossover (week 6 vs 12). Therefore only 14 participants per diet group were included for these post hoc analyses. This selection enabled a direct comparison of the results on measures of glucagon resistance between the isocaloric study and the hypocaloric study for the same time frame. On the other hand, a lower sample size in the isocaloric study could make some comparisons statistically underpowered. For example, the CRHP diet tended to reduce the glucagon-alanine index more ($P = .7$) compared to the CD diet within the isocaloric feeding intervention. Diets high in protein and low in carbohydrates may offer additional metabolic benefits on glucagon sensitivity in addition to the improvements in glycemic control; however, this hypothesis requires further investigation. As we did not include observations from the isocaloric study following crossover, there are discrepancies between the results from the subset of individuals investigated here and what was reported previously. For the data presented here, a 6-week isocaloric diet induced a body weight loss of 1.8%, and the reduction in hepatic steatosis was similar following a 6-week CD or CRHP diet. When including samples following the crossover, the body weight loss was 1.2% and the CRHP diet induced a larger reduction in hepatic steatosis [28, 29]. Finally, 74% of participants were treated with metformin throughout the studies. In hepatocytes, metformin suppresses glucagon-stimulated gluconeogenesis [42] and hepatic glucagon signaling [43], and the use of metformin may therefore have confounded the interpretation of our results.

In conclusion, a diet-induced reduction in hepatic steatosis and body weight improved glucagon sensitivity as evaluated by the glucagon-alanine index in individuals with type 2 diabetes, whereas a decrease in hepatic steatosis without a concomitant clinically relevant body weight loss (induced by isocaloric feeding) did not, indicating that reductions in hepatic steatosis alone is insufficient for improving glucagon metabolism. However, CRHP feeding may add additional benefits for improving glucagon sensitivity as evidenced by reduced plasma levels of alanine with the CRHP diet independently of caloric intake. Finally, the glucagon-alanine index was associated with MASLD, HOMA-IR, and sex, but not BMI or dietary macronutrient composition as evaluated by linear regression. The study was a post hoc analysis of 2 previously published trials and hence these findings may be viewed as exploratory.

Perspectives and Significance

These observations provide important insight into the mechanisms of hyperglucagonemia and highlight a role for both hepatic steatosis and obesity, and not diabetes alone, as causes of diabetogenic hyperglucagonemia [44]. These data also touch on the potential risks of hepatic steatosis, and the assessment of liver health in diabetes control may potentially improve long-term health outcomes. Hypocaloric feeding, as compared to isocaloric feeding, provides additional metabolic benefits including reductions in plasma levels of glucagon in individuals with overweight or obesity and type 2 diabetes. Thus, for relatively short-term diet interventions, caloric restriction should be advised. Additionally, CRHP diets may be more favorable compared to conventional high-carbohydrate diets for improving glucagon sensitivity; however, this warrants further investigation.

Acknowledgments

We thank Christine Rasmussen for her skillful technical assistance on the biochemical measurements and Nicole J. Jensen for suggesting the collaboration between research groups.

Funding

N.J.W.A. is supported by an Novo Nordisk Foundation Excellence Emerging Investigator Grant–Endocrinology and Metabolism (application No. NNF19OC0055001), an European Foundation for the Study of Diabetes Future Leader Award (No. NNF21SA0072746), and Independent Research Fund Denmark Sapere Aude (No. 1052-00003B). The NNF Center for Protein Research is supported financially by the Novo Nordisk Foundation (grant agreement NNF14CC0001) and S.A.S.K. is supported by the Aase og Ejnar Danielsens Fond (21-10-0287) and A. P. Møller

Fonden (2021-00683). The isocaloric study (NCT02764021) was funded by grants from Arla Food for Health; the Novo Nordisk Foundation Center for Basic Metabolic Research, University of Copenhagen; the Department of Clinical Medicine, Aarhus University; the Department of Nutrition, Exercise and Sports, University of Copenhagen; and Copenhagen University Hospital, Bispebjerg. The hypocaloric study (NCT03814694) was funded by Arla Foods amba, The Danish Dairy Research Foundation, and Copenhagen University Hospital Bispebjerg.

Author Contributions

S.A.S.K. and N.J.W.A. conceived the idea of investigating markers of glucagon resistance in patients with type 2 diabetes after a diet-induced reduction in hepatic steatosis with or without a concurrent body weight reduction. S.A.S.K. wrote the first draft of the manuscript. M.N.T., M.S., A.S., S.B.H., and T.K. contributed with samples, and all authors revised the manuscript and approved the final version for publication.

Disclosures

None of the authors have conflicts of interest to declare.

Data Availability

The data sets generated are not publicly available but may be shared on reasonable request and following approval by the Danish Data Protection Agency.

Clinical Trial Information

ClinicalTrials.gov registration numbers NCT03814694 (registered January 24, 2019) and NCT02764021 (registered April 26, 2016).

References

1. Wewer Albrechtsen NJ, Junker AE, Christensen M, *et al.* Hyperglucagonemia correlates with plasma levels of non-branched-chain amino acids in patients with liver disease independent of type 2 diabetes. *Am J Physiol Gastrointest Liver Physiol*. 2018;314(1):G91-GG6.
2. Lund A, Bagger JI, Christensen M, Knop FK, Vilsboll T. Glucagon and type 2 diabetes: the return of the alpha cell. *Curr Diab Rep*. 2014;14(12):555.
3. Demant M, Bagger JI, Suppli MP, *et al.* Determinants of fasting hyperglucagonemia in patients with type 2 diabetes and nondiabetic control subjects. *Metab Syndr Relat Disord*. 2018;16(10):530-536.
4. Rocha DM, Faloona GR, Unger RH. Glucagon-stimulating activity of 20 amino acids in dogs. *J Clin Invest*. 1972;51(9):2346-2351.
5. Kuhara T, Ikeda S, Ohneda A, Sasaki Y. Effects of intravenous infusion of 17 amino acids on the secretion of GH, glucagon, and insulin in sheep. *Am J Physiol*. 1991;260(1 Pt 1):E21-E26.
6. Muller WA, Faloona GR, Aguilar-Parada E, Unger RH. Abnormal alpha-cell function in diabetes. Response to carbohydrate and protein ingestion. *N Engl J Med*. 1970;283(3):109-115.
7. Ang T, Bruce CR, Kowalski GM. Postprandial aminogenic insulin and glucagon secretion can stimulate glucose flux in humans. *Diabetes*. 2019;68(5):939-946.
8. Solloway MJ, Madjidi A, Gu C, *et al.* Glucagon couples hepatic amino acid catabolism to mTOR-dependent regulation of alpha-cell mass. *Cell Rep*. 2015;12(3):495-510.
9. Richter MM, Galsgaard KD, Elmelund E, *et al.* The liver-α-cell axis in health and in disease. *Diabetes*. 2022;71(9):1852-1861.
10. Kim J, Dominguez Gutierrez G, Xin Y, *et al.* Increased SLC38A4 amino acid transporter expression in human pancreatic alpha-cells after glucagon receptor inhibition. *Endocrinology*. 2019;160(5):979-988.
11. Kim J, Okamoto H, Huang Z, *et al.* Amino acid transporter Slc38a5 controls glucagon receptor inhibition-induced pancreatic alpha cell hyperplasia in mice. *Cell Metab*. 2017;25(6):1348-1361.e8.
12. Albrechtsen NJ W, Faerch K, Jensen TM, *et al.* Evidence of a liver-alpha cell axis in humans: hepatic insulin resistance attenuates relationship between fasting plasma glucagon and glucagonotropic amino acids. *Diabetologia*. 2018;61(3):671-680.
13. Kjeldsen SAS, Richter MM, Jensen NJ, *et al.* Development of a glucagon sensitivity test in humans: pilot data and the GLUSENTIC study protocol. *Peptides*. 2023;161:170938.
14. Winther-Sørensen M, Galsgaard KD, Santos A, *et al.* Glucagon acutely regulates hepatic amino acid catabolism and the effect may be disturbed by steatosis. *Mol Metab*. 2020;42:101080.
15. Suppli MP, Bagger JI, Lund A, *et al.* Glucagon resistance at the level of amino acid turnover in obese subjects with hepatic steatosis. *Diabetes*. 2020;69(6):1090-1099.
16. Svane MS, Johannesen HH, Hansen AE, *et al.* Four weeks treatment with the GLP-1 receptor analogue liraglutide lowers liver fat and concomitantly circulating glucagon in individuals with overweight. *Int J Obes (Lond)*. 2022;46(11):2058-2062.
17. Gar C, Haschka SJ, Kern-Matschilles S, *et al.* The liver-alpha cell axis associates with liver fat and insulin resistance: a validation study in women with non-steatotic liver fat levels. *Diabetologia*. 2021;64(3):512-520.
18. Pedersen JS, Rygg MO, Kristiansen VB, *et al.* Nonalcoholic fatty liver disease impairs the liver-alpha cell axis independent of hepatic inflammation and fibrosis. *Hepatol Commun*. 2020;4(11):1610-1623.
19. Kirk E, Reeds DN, Finck BN, Mayurranjan SM, Patterson BW, Klein S. Dietary fat and carbohydrates differentially alter insulin sensitivity during caloric restriction. *Gastroenterology*. 2009;136(5):1552-1560.
20. Browning JD, Baker JA, Rogers T, Davis J, Satapati S, Burgess SC. Short-term weight loss and hepatic triglyceride reduction: evidence of a metabolic advantage with dietary carbohydrate restriction. *Am J Clin Nutr*. 2011;93(5):1048-1052.
21. Otten J, Stomby A, Waling M, *et al.* The liver-alpha-cell axis after a mixed meal and during weight loss in type 2 diabetes. *Endocr Connect*. 2021;10(9):1101-1110.
22. Linn T, Santosa B, Grönemeyer D, *et al.* Effect of long-term dietary protein intake on glucose metabolism in humans. *Diabetologia*. 2000;43(10):1257-1265.
23. Zhang J, Pivovarova-Ramich O, Kabisch S, *et al.* High protein diets improve liver fat and insulin sensitivity by prandial but not fasting glucagon secretion in type 2 diabetes. *Front Nutr*. 2022;9:808346.
24. Rose AJ. Role of peptide hormones in the adaptation to altered dietary protein intake. *Nutrients*. 2019;11(9):1990.
25. Elmelund E, Galsgaard KD, Johansen CD, *et al.* Opposing effects of chronic glucagon receptor agonism and antagonism on amino acids, hepatic gene expression, and alpha cells. *iScience*. 2022;25(11):105296.
26. Thomsen MN, Skytte MJ, Samkani A, *et al.* Dietary carbohydrate restriction augments weight loss-induced improvements in glycaemic control and liver fat in individuals with type 2 diabetes: a randomised controlled trial. *Diabetologia*. 2022;65(3):506-517.
27. Thomsen MN, Skytte MJ, Samkani A, *et al.* Weight loss improves β-cell function independently of dietary carbohydrate restriction in people with type 2 diabetes: a 6-week randomized controlled trial. *Front Nutr*. 2022;9:933118.
28. Skytte MJ, Samkani A, Petersen AD, *et al.* A carbohydrate-reduced high-protein diet improves HbA(1c) and liver fat content in weight stable participants with type 2 diabetes: a randomised controlled trial. *Diabetologia*. 2019;62(11):2066-2078.
29. Skytte MJ, Samkani A, Astrup A, *et al.* Effects of carbohydrate restriction on postprandial glucose metabolism, β-cell function, gut hormone secretion, and satiety in patients with type 2 diabetes. *Am J Physiol Endocrinol Metab*. 2021;320(1):E7-E18.

30. Mifflin MD, St Jeor ST, Hill LA, Scott BJ, Daugherty SA, Koh YO. A new predictive equation for resting energy expenditure in healthy individuals. *Am J Clin Nutr*. 1990;51(2):241-247.
31. Chabanova E, Fonvig CE, Bøjsøe C, Holm J-C, Thomsen HS. 1H MRS assessment of hepatic fat content: comparison between normal- and excess-weight children and adolescents. *Acad Radiol*. 2017;24(8):982-987.
32. Chabanova E, Bille DS, Thisted E, Holm J-C, Thomsen HS. MR Spectroscopy of liver in overweight children and adolescents: investigation of 1H T2 relaxation times at 3 T. *Eur J Radiol*. 2012;81(5):811-814.
33. Albrechtsen NJ W, Kuhre RE, Windelov JA, *et al*. Dynamics of glucagon secretion in mice and rats revealed using a validated sandwich ELISA for small sample volumes. *Am J Physiol Endocrinol Metab*. 2016;311(2):E302-E309.
34. Smart KF, Aggio RB, Van Houtte JR, Villas-Bôas SG. Analytical platform for metabolome analysis of microbial cells using methyl chloroformate derivatization followed by gas chromatography-mass spectrometry. *Nat Protoc*. 2010;5(10):1709-1729.
35. Johnsen LG, Skou PB, Khakimov B, Bro R. Gas chromatography—mass spectrometry data processing made easy. *J Chromatogr A*. 2017;1503:57-64.
36. Stern JH, Smith GI, Chen S, Unger RH, Klein S, Scherer PE. Obesity dysregulates fasting-induced changes in glucagon secretion. *J Endocrinol*. 2019;243(2):149-160.
37. Arslanian SA, Heil BV, Becker DJ, Drash AL. Sexual dimorphism in insulin sensitivity in adolescents with insulin-dependent diabetes mellitus. *J Clin Endocrinol Metab*. 1991;72(4):920-926.
38. Velasco M, Ortiz-Huidobro RI, Larqué C, Sánchez-Zamora YI, Romo-Yáñez J, Hiriart M. Sexual dimorphism in insulin resistance in a metabolic syndrome rat model. *Endocr Connect*. 2020;9(9):890-902.
39. Unluhizarci K, Karaca Z, Kelestimur F. Role of insulin and insulin resistance in androgen excess disorders. *World J Diabetes*. 2021;12(5):616-629.
40. Hanssen NMJ, Kraakman MJ, Flynn MC, Nagareddy PR, Schalkwijk CG, Murphy AJ. Postprandial glucose spikes, an important contributor to cardiovascular disease in diabetes? *Front Cardiovasc Med*. 2020;7:570553.
41. Galsgaard KD, Elmelund E, Johansen CD, *et al*. Glucagon receptor antagonism impairs and glucagon receptor agonism enhances triglycerides metabolism in mice. *Mol Metab*. 2022;66:101639.
42. Yu B, Pugazhenthi S, Khandelwal RL. Effects of metformin on glucose and glucagon regulated gluconeogenesis in cultured normal and diabetic hepatocytes. *Biochem Pharmacol*. 1994;48(5):949-954.
43. Miller RA, Chu Q, Xie J, Foretz M, Viollet B, Birnbaum MJ. Biguanides suppress hepatic glucagon signalling by decreasing production of cyclic AMP. *Nature*. 2013;494(7436):256-260.
44. Albrechtsen NJ W, Holst JJ, Cherrington AD, *et al*. 100 Years of glucagon and 100 more. *Diabetologia*. 2023;66(8):1378-1394.