Markers of glucagon resistance improve with reductions in hepatic steatosis and body weight in type 2 diabetes

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Keywords: Carbohydrate-reduced high-protein diet; metabolic dysfunction-associated steatotic liver, non-alcoholic fatty liver disease; type 2 diabetes; weight loss; weight maintenance

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Abstract

Context: Hyperglucagonaemia 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 versus body weight is unknown.

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

Design and setting: A post-hoc analysis from two 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 ELISA and metabolomics in 94 individuals (n=28 in study 1; n=66 in study 2). Participants were randomized to a 6-week conventional diabetes (CD) or carbohydrate-reduced high-protein (CRHP) diet within both isocaloric and hypocaloric feeding-interventions.

Participants: Individuals with overweight or obesity with type 2 diabetes.

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 impact 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.
Introduction

Hormonal dysregulation in type 2 diabetes—particularly in relation to the concentration and action of glucagon—contributes to fasting hyperglycaemia due to inappropriate increases in hepatic glucose production (HGP). Some, but not all, patients with type 2 diabetes have increased plasma levels of glucagon (hyperglucagonaemia) (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.

Glucagon metabolism in MASLD

Hyperglucagonaemia is evidenced in individuals with metabolic dysfunction-associated steatotic liver disease (MASLD) (previously termed non-alcoholic 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 HGP 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), associates 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 two 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.

Research Design 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 no. NCT03814694 & NCT02764021) and were conducted in accordance with the Declaration of Helsinki.

Study Design

We performed additional biochemical analyses on a subset of samples from two previously published studies (26-29) (study design shown in Figure 1). Inclusion criteria for both studies included men and women with type 2 diabetes with HbA1c of 48-97 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 two trials have previously been published (26-29). The following sections include a short description of the essential information from these two 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 only included the first six weeks of the study. Therefore, previously published data (e.g., hepatic steatosis) on the isocaloric study may not be identical to what is reported here due to this selection. Fourteen individuals consumed an iso-energetic CD diet, and 14 individuals consumed an iso-energetic CRHP 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 hypo-energetic 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 hypo-energetic CD diet, and 34 individuals consumed a hypo-energetic CRHP diet.

We only included time points 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 randomized 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 (TEE) (30), and in the hypocaloric study, the provided daily energy was calculated based on TEE adjusted for the intended weight loss.

MRI analysis

Hepatic steatosis was evaluated by MRI using a 3.0 T Ingenia MRI system (Philips Healthcare, Best, the Netherlands) 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 previously been published (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 ELISA (Mercodia catalogue 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 HbA1c 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) x fasting alanine (mmol/L) [(12)]. The homeostatic model assessment for insulin resistance (HOMA-IR) was calculated as fasting glucose (mmol/L) x 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 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 delta 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 two observations were deleted due to missing values, leaving 91 subjects for the multiple linear regression analyses (Model 1 and Model 2). Data from the six-week time point was selected for the regression analyses. A p-value of <0.05 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 (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: HbA1c, 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<0.05) 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) (Figure 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<0.0001, Figure 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<0.0001, Figure 2B). The glucagon-alanine index decreased following the hypocaloric intervention (3.0 ± 1.9 vs 2.2 ± 1.3 pmol/L*mmol/L, p<0.0001) but did not change following the isocaloric intervention (3.2 ± 2.8 vs 2.8 ± 2.4 pmol/L*mmol/L, p=0.32, Figure 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<0.001), but was unaltered following the isocaloric intervention (5.5 ± 4.2 vs 4.9 ± 3.2 mmol/L*µU/ml, p=0.8, Figure 2D). Following the
hypocaloric feeding intervention, plasma concentrations of total amino acids (1761 ± 273 vs 1650 ±
243 µmol/L, p<0.001) and glucagon (8.3 ± 4 vs 6.8 ± 3.6 pmol/L, p<0.001) decreased (Figure 2E-
F). 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 (BCAA: leucine, isoleucine, and valine), in addition to glutamic acid,
tyrosine, phenylalanine, proline, tryptophan, and methionine (Figure 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 (Figure 2E-F, Figure 3A-B). Both the isocaloric
and hypocaloric interventions caused a decline in HbA1c (Figure 2G). Serum concentrations of
insulin decreased following hypocaloric feeding but were unaltered after isocaloric feeding (Figure
2H).

Next, we performed a subgroup analysis by stratifying the two 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 (Figure 4A-B). The CRHP diet caused a larger numerical reduction in hepatic
steatosis compared to the CD diet in both isocaloric (p=0.22) and hypocaloric (p=0.27) studies. In
the isocaloric study, the glucagon-alanine index lowered following the CRHP diet compared to the
CD diet (Figure 4C), but this did not reach significance (delta, -0.7 ± 1.4 vs 0.1 ± 1.0, p=0.07). Both
CRHP and CD diets caused a reduction in the glucagon-alanine index following the hypocaloric
intervention (delta, -0.9 ± 1.2 vs -0.7 ± 1.6) (Figure 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 concentrations of amino acids and glucagon following the
hypocaloric intervention (Figure 4E-H). 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 differential impact on the individual amino acid concentration but was 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 (Table 2). In contrast, the branched-chain amino acids appeared more regulated by the caloric load than to that of the diet. HbA1c declined more following the CRHP diet compared to the CD diet in both the isocaloric and the hypocaloric study (Figure 4 I-J), 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 (%), BMI (kg/m²), 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 for glucagon-alanine index \( (p<0.0001) \) outcome, while BMI was not \( (p=0.07) \). Energy consumption (isocaloric vs hypocaloric intervention) and macronutrient composition (CD vs CRHP) did not significantly associate with the glucagon-alanine index. Model 1 explained 18% of the variance in the glucagon-alanine index (adjusted R²). Next, we aimed to further adjust our model by additionally including sex, age, and HOMA-IR (Table 3) as possible predictors for 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 \( (p<0.0001) \) (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 hyperglucagonaemia 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 either 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 iso- or hypocaloric feeding interventions. This suggests an improvement in glucagon signalling with carbohydrate-reduced high-protein feeding. However, due to similar changes in plasma levels of glucagon with CRHP and CD diets within both iso- 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 impacted on 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, 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 versus 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 only assessed parameters of glucagon metabolism during fasting, overlooking postprandial excursions, which may contribute substantially to disease, as shown for glucose metabolism (40).
a similar study (23), closely resembling the isocaloric study presented here, individuals with type 2 diabetes followed a 6-week isocaloric high-protein diet which 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 signalling. 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 two 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 hyperglucagonaemia during states of high amino acid availability do not seem to impair glucagon sensitivity but may rather improve on 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 timeframe. 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 ($p=0.7$) 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 glycaemic 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 signalling (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, carbohydrate-reduced high-protein 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 two previously published trials and hence these findings may be viewed as exploratory.
Perspectives and significance

These observations provide important insight into the mechanisms of hyperglucagonaemia and highlight a role for both hepatic steatosis and obesity, and not diabetes alone, as cause for diabetogenic hyperglucagonaemia (44). These data also touch upon 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, carbohydrate-reduced high-protein diets may be more favourable compared to conventional high-carbohydrate diets for improving glucagon sensitivity, however, this warrants further investigation.

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**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.

**Data availability**

The data sets generated are not publicly available but may be shared upon reasonable request and following approval by the Danish Data Protection Agency.
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Table 1. Significant differences between the isocaloric and hypocaloric interventions are shown with the symbol †. One symbol indicates \(p<0.05\), two symbols indicate \(p<0.01\), three symbols indicate \(p<0.001\) and four indicate \(p<0.0001\). Data are presented as mean ± SD. Abbreviations: CD diet, conventional diabetes diet; CRHP diet, carbohydrate-reduced high-protein diet; f, female; m, male; y, yes; n, no.
Table 2. The effect of macronutrient composition on plasma levels of the individual amino acids

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<td>Arginine</td>
<td>2.1 ± 7.8</td>
<td>-1.0 ± 9.3</td>
<td>0.37</td>
<td>-1.9 ± 8.7</td>
<td>-0.4 ± 11.4</td>
<td>0.57</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.2 ± 2.4</td>
<td>-0.5 ± 2.1</td>
<td>0.44</td>
<td>0.2 ± 4.1</td>
<td>-0.6 ± 2.5</td>
<td>0.34</td>
</tr>
<tr>
<td>Cysteine</td>
<td>-7.0 ± 12.6</td>
<td>-10.9 ± 17.7</td>
<td>0.53</td>
<td>-2.0 ± 40.7</td>
<td>14.3 ± 45.1</td>
<td>0.12</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>9.4 ± 33.0</td>
<td>-19.9 ± 25.8</td>
<td>0.02</td>
<td>-3.2 ± 27.5</td>
<td>-10.9 ± 18.7</td>
<td>0.18</td>
</tr>
<tr>
<td>Glutamine</td>
<td>5.2 ± 29.7</td>
<td>-12.6 ± 29.9</td>
<td>0.14</td>
<td>17.7 ± 31.2</td>
<td>-0.4 ± 44.2</td>
<td>0.06</td>
</tr>
<tr>
<td>Glycine</td>
<td>4.9 ± 14.4</td>
<td>-10.5 ± 14.8</td>
<td>0.01</td>
<td>3.6 ± 15.8</td>
<td>1.6 ± 14.4</td>
<td>0.59</td>
</tr>
<tr>
<td>Histidine</td>
<td>-1.0 ± 15.7</td>
<td>-4.8 ± 11.1</td>
<td>0.49</td>
<td>-2.6 ± 13.2</td>
<td>-3.1 ± 11.4</td>
<td>0.86</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.5 ± 9.6</td>
<td>-0.3 ± 10.7</td>
<td>0.65</td>
<td>-9.0 ± 15.1</td>
<td>-2.8 ± 15.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Leucine</td>
<td>-2.0 ± 17.4</td>
<td>-2.0 ± 16.9</td>
<td>0.99</td>
<td>-16.7 ± 23.2</td>
<td>-4.5 ± 21.5</td>
<td>0.03</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.9 ± 11.9</td>
<td>7.4 ± 23.3</td>
<td>0.47</td>
<td>-7.7 ± 20.5</td>
<td>-1.4 ± 20.0</td>
<td>0.21</td>
</tr>
<tr>
<td>Methionine</td>
<td>-0.4 ± 2.6</td>
<td>-1.2 ± 4.0</td>
<td>0.57</td>
<td>-1.8 ± 4.0</td>
<td>-1.5 ± 3.2</td>
<td>0.75</td>
</tr>
<tr>
<td>Ornithine</td>
<td>0.03 ± 4.55</td>
<td>2.39 ± 7.98</td>
<td>0.38</td>
<td>-2.7 ± 7.6</td>
<td>0.6 ± 7.5</td>
<td>0.07</td>
</tr>
<tr>
<td>Phenylnalanine</td>
<td>0.4 ± 3.7</td>
<td>1.0 ± 6.1</td>
<td>0.77</td>
<td>-4.3 ± 7.9</td>
<td>-0.3 ± 6.5</td>
<td>0.03</td>
</tr>
<tr>
<td>Proline</td>
<td>15.7 ± 34.5</td>
<td>-15.3 ± 34.9</td>
<td>0.03</td>
<td>-10.6 ± 36.6</td>
<td>-8.9 ± 27.3</td>
<td>0.83</td>
</tr>
<tr>
<td>Serine</td>
<td>-2.2 ± 5.8</td>
<td>-3.5 ± 6.3</td>
<td>0.61</td>
<td>4.7 ± 8.5</td>
<td>6.0 ± 8.9</td>
<td>0.54</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.5 ± 4.2</td>
<td>-1.7 ± 3.8</td>
<td>0.05</td>
<td>-1.6 ± 5.4</td>
<td>-1.0 ± 6.4</td>
<td>0.65</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>2.5 ± 4.9</td>
<td>0.8 ± 8.0</td>
<td>0.53</td>
<td>-2.6 ± 5.9</td>
<td>-2.9 ± 6.4</td>
<td>0.84</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>-0.4 ± 18.7</td>
<td>-6.0 ± 24.1</td>
<td>0.52</td>
<td>-25.3 ± 31.7</td>
<td>-21.1 ± 19.8</td>
<td>0.51</td>
</tr>
<tr>
<td>Valine</td>
<td>18.2 ± 25.5</td>
<td>45.6 ± 17.9</td>
<td>0.003</td>
<td>-27.3 ± 37.7</td>
<td>-2.3 ± 30.7</td>
<td>0.004</td>
</tr>
</tbody>
</table>

1 Table 2. Effect of a 6-week CD or CRHP diet within the iso- and hypocaloric study interventions. Delta values (value at 6 weeks subtracted from baseline) in µmol/L are presented as mean ± SD. P-value represents t-testing with correcting for multiple testing between diet for the individual trial (hypo- and isocaloric diet).

Table 3. Multiple linear regression analysis, Model 2

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Coefficient (beta)</th>
<th>Standard Error</th>
<th>T value</th>
<th>Significance</th>
<th>[95% Conf. Interval, 2.5%]</th>
<th>[95% Conf. Interval, 97.5%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucagon-alanine index</td>
<td>-1.57</td>
<td>1.49</td>
<td>-1.05</td>
<td>0.30</td>
<td>-4.54</td>
<td>1.40</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>Coefficient (beta)</th>
<th>Standard Error</th>
<th>T value</th>
<th>Significance</th>
<th>[95% Conf. Interval, 2.5%]</th>
<th>[95% Conf. Interval, 97.5%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatic steatosis (%)</td>
<td>0.08</td>
<td>0.04</td>
<td>2.31</td>
<td>0.02</td>
<td>0.01</td>
<td>0.15</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>0.30</td>
<td>0.03</td>
<td>1.15</td>
<td>0.25</td>
<td>-0.02</td>
<td>0.09</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.12</td>
<td>0.05</td>
<td>2.74</td>
<td>0.007</td>
<td>0.03</td>
<td>0.21</td>
</tr>
<tr>
<td>Study (hypo)</td>
<td>-0.38</td>
<td>0.30</td>
<td>-1.31</td>
<td>0.19</td>
<td>-0.97</td>
<td>0.20</td>
</tr>
<tr>
<td>Diet (CRHP)</td>
<td>-0.11</td>
<td>0.25</td>
<td>-0.45</td>
<td>0.66</td>
<td>-0.61</td>
<td>0.39</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.03</td>
<td>0.02</td>
<td>1.63</td>
<td>0.11</td>
<td>-0.01</td>
<td>0.06</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>0.64</td>
<td>0.25</td>
<td>2.50</td>
<td>0.01</td>
<td>0.13</td>
<td>1.14</td>
</tr>
</tbody>
</table>

R² | 0.35
Table 3. Multiple linear regression analysis. BMI, body mass index; HOMA-IR, homeostatic Model Assessment for insulin resistance; CD diet, conventional diabetes diet; CRHP diet, carbohydrate-reduced high-protein diet.

Figure 1. Study designs of the previously published studies. The isocaloric study intervention (study 1) was designed as a randomized cross-over study with 2x6 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 red box).

Figure 2. Improvement in glucagon sensitivity following a reduction in hepatic steatosis depends on a concurrent weight loss. Effect of six-week isocaloric or hypocaloric diets in patients with type 2 diabetes on body weight (A), hepatic steatosis (B), glucagon-alanine-index (C), fasting HOMA-IR (D), total amino acids (E), plasma glucagon (F), HbA1c (G), and plasma insulin (H). Both absolute and delta values are shown. Mixed-effects analyses followed by Sidak’s multiple comparisons test for A-H, and unpaired t-tests between delta, 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 $p<0.05$, two symbols indicate $p<0.01$, three symbols indicate $p<0.001$ and four indicate $p<0.0001$.

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 $p<0.05$, two symbols indicate $p<0.01$, three symbols indicate $p<0.001$ and four indicate $p<0.0001$. Abbreviations: 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.

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 hepatic steatosis (A and B), glucagon-alanine-index (C and D), total amino acids (E and F), plasma glucagon (G and H), and HbA1c (I and J). A mixed-effects analysis followed by Sidak’s multiple comparisons test was performed for A-J and unpaired t-tests between delta, were performed using GraphPad Prism (version 9.4.1). Data are presented as mean ± SEM. Statistical significance is marked by * for comparisons between CD and CRHP diets (post-hoc analysis following mixed-effects analysis and t-tests between delta); ¤ 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 $p<0.05$, two symbols indicate $p<0.01$, three symbols indicate $p<0.001$ and four indicate $p<0.0001$. 


Figure 1

Post-hoc analysis of the two independent studies on samples obtained at times 0 and 6 weeks

219x208 mm (x DPI)
Figure 2

177x261 mm ( x DPI)
Figure 3
191x277 mm (x DPI)
Figure 4

206x75 mm (x DPI)