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Hyperglucagonaemia and amino acid alterations in individuals with type 2 diabetes and non-alcoholic fatty liver disease

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Abstract

Aims: Hyperglucagonaemia contributes to the pathophysiology in type 2 diabetes (T2D), but the mechanisms behind the inappropriate glucagon secretion are not fully understood. Glucagon and amino acids are regulated in a feedback loop referred to as the liver–α cell axis. Individuals with non-alcoholic fatty liver disease (NAFLD) appear to be glucagon resistant, disrupting the liver–α cell axis resulting in hyperglucagonaemia and hyperaminoacidaemia. We investigated the associations between circulating glucagon, amino acids, and liver fat content in a cohort of individuals with T2D.

Methods: We included 110 individuals with T2D in this cross-sectional study. Liver fat content was quantified using 1H magnetic resonance spectroscopy (MRS). Associations between liver fat content and plasma glucagon and amino acids, respectively, were estimated in multivariate linear regression analyses.

Results: Individuals with NAFLD (n = 52) had higher plasma glucagon concentrations than individuals without NAFLD (n = 58). The positive association between plasma glucagon concentrations and liver fat content was confirmed in the multivariable regression analyses. Plasma concentrations of isoleucine and glutamate were increased, and glycine and serine concentrations were decreased in individuals with NAFLD. Concentrations of other amino acids were similar between individuals with and without NAFLD, and no clear association was seen between liver fat content and amino acids in the regression analyses.
Conclusion: MRS-diagnosed NAFLD in T2D is associated with hyperglucagonaemia and elevated plasma concentrations of isoleucine and glutamate and low plasma concentrations of glycine and serine. Whether NAFLD and glucagon resistance per se induce these changes remains to be elucidated.

Keywords: glucagon; amino acids; type 2 diabetes mellitus; non-alcoholic fatty liver disease; humans

Introduction

Hyperglucagonaemia contributes to the hyperglycaemia characterizing type 2 diabetes (T2D) (1, 2). Nevertheless, high glucagon concentrations are observed in some, but not all individuals with T2D, indicating that hyperglucagonaemia drives hyperglycaemia in only a subgroup of patients with T2D. The mechanisms behind T2D hyperglucagonaemia are not fully established (3, 4). Glucagon secretion is regulated by a complex interplay between endocrine and paracrine mechanisms, substrate availability, and the autonomic nervous system. Potent regulators of glucagon secretion include hypoglycaemia, insulin, and certain amino acids (4, 5).

A feedback loop between the liver and the pancreas, the liver–α-cell axis, controls amino acid concentrations by modulating hepatic uptake and catabolism of amino acids, while prevailing plasma amino acid concentrations, in turn, regulate glucagon secretion from the pancreatic α cells (6, 7, 8, 9). It has been proposed that hepatic fat deposition impairs glucagon-induced turnover of amino acids in the liver, resulting in increased plasma concentrations of amino acids (10, 11). This may increase glucagon secretion, compensating for the hepatic glucagon resistance and thus restoring amino acid turnover. The price for this is, however, that glucose production increases, as glucagon action on glucose production seems to be preserved in non-alcoholic fatty liver disease (NAFLD) (10, 12). The mechanisms underlying this hepatic glucagon resistance at the level of amino acid turnover may involve downregulation of hepatic amino acid transporters and key enzymes in the ureagenesis (10, 13, 14, 15).

The association between glucagon, amino acids, and liver fat has previously been investigated in healthy and individuals with prediabetes (16), in individuals with severe obesity (17), in a small group of individuals with T2D (18), and in diet and/or metformin-treated individuals with T2D (19). To our knowledge, associations between plasma glucagon and amino acid concentrations and liver fat content as assessed by reference standard methodology (1H magnetic resonance spectroscopy (MRS)) have not been studied in larger T2D cohorts, including individuals with and without NAFLD.

Here, we investigated the association between MRS-assessed liver fat content and plasma concentrations of glucagon and amino acids in a cohort of individuals with T2D. We hypothesized that increased liver fat content, i.e. NAFLD, attenuating the effect of glucagon on amino acid turnover in the liver, would associate with increased plasma concentrations of amino acids and hyperglucagonaemia.

Materials and methods

Cohort of participants with T2D

The present analyses were conducted on data and material obtained from the baseline visit in the randomized clinical trial ‘Effect of the mineralocorticoid receptor antagonist eplerenone on liver fat and metabolism in patients with type 2 diabetes (MIRAD trial)’ (20). In the primary study, 140 participants were recruited from the diabetes outpatient clinic at Herlev and Gentofte Hospital – University of Copenhagen, Herlev, Denmark, based on criteria that included diagnosis of T2D according to WHO criteria of >3 months duration, stable treatment with glucose-lowering and antihypertensive therapy according to guidelines, a history of cardiovascular disease (defined as previous myocardial infarction, significant coronary stenosis, peripheral artery disease, and/or previous stroke) or high risk of cardiovascular disease (identified by N-terminal pro-brain natriuretic peptide (NT-proBNP) ≥70 ng/L or albuminuria) (20). The study was approved by the Ethical Committee of the Capital Region of Denmark and the Data Protection Agency and registered at ClinicalTrials.gov (NCT02809963) and conducted in accordance with the Declaration of Helsinki. For the present secondary explorative analyses, we excluded 20 MIRAD trial participants without MRS measurements of liver fat content and 10 MIRAD trial participants for whom frozen plasma samples for glucagon analyses were not available. In total, 110 MIRAD trial participants with complete data on liver fat content, glucagon, and amino acids were included in this study.

1H MRS

Liver fat content was measured in a region of interest by 1H MRS 3T Achieva MRI system (Philips Medical Systems) as described previously (20, 21). NAFLD was defined according to guidelines as liver fat content ≥5.6% (22).
Biochemical analyses
Blood samples were collected after an overnight fast of a minimum of 8 h and after 30 min of rest. Plasma was stored at −80°C until batch analyses. Plasma glucagon was measured from EDTA plasma using a sandwich ELISA (Mercodia, Uppsala, Sweden) (23) with an intra-assay and inter-assay coefficient of variation (CV) of <10% (24). Individual amino acids were measured from lithium-heparinized plasma using liquid chromatography–tandem mass spectrometry (LS-MS/MS) (TSQ Quantiva, Thermo Fisher Scientific) (25). The remaining biochemical analyses presented here were described previously (20).

Calculations
The homeostatic model assessment of insulin resistance (HOMA-IR) was used as an estimate of hepatic insulin resistance and calculated as fasting plasma glucose (mmol/L) × fasting serum insulin (μIU/mL)/22.5 (26). The Matsuda index was used to estimate whole-body insulin sensitivity and calculated from four-point measurements of plasma glucose and serum insulin from a 2-h 75 g oral glucose tolerance test (27). Estimated glomerular filtration rate (eGFR) was calculated from the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (derived from serum creatinine, gender, and age) (28). To assess liver fibrosis, the Fibrosis-4 (FIB-4) score (derived from age, alanine transaminase, aspartate transaminase, and platelets) (29) and NAFLD fibrosis score (NFS) (derived from age, BMI, impaired fasting glucose, and presence of diabetes) (30) were calculated. The total amino acid concentration was calculated as the sum of individual amino acids.

Statistical analyses
All variables were inspected graphically to evaluate the pattern of distribution. Normally distributed variables are reported as mean ± s.d., non-normally distributed variables as median with interquartile range (IQR), and categorical variables as n and %. The cohort was grouped according to liver fat content < or ≥5.6%. Characteristics between groups were compared using unpaired t-test, Wilcoxon test, or chi–squared test, as appropriate. Multivariate linear regression analyses were used to evaluate the association between glucagon (dependent variable) and liver fat content (independent variable). In a stepwise approach, we adjusted for preselected confounders with the potential of affecting the dependent variable. First, data were adjusted for gender, age, BMI, and eGFR (model glucagon 1). Second, data were adjusted for fasting plasma glucose (model glucagon 2). Last, data were adjusted for diabetes medications (model glucagon 3). Similarly, the associations between amino acids (dependent variables) and liver fat content (independent variable) were evaluated by multivariate linear regression analyses adjusted for gender, age, BMI, and eGFR (model amino acids 1). Data were further adjusted for fasting plasma glucagon concentrations (model amino acids 2). Estimates from all regression analyses are displayed as a crude β coefficient with 95% CI. Liver fat content and glucagon were log-transformed to meet the assumption of normality of the residuals. P < 0.05 was considered statistically significant in all tests. Statistical software R version 4.1.0 was used for statistical evaluation and graphical presentations.

Results
Cohort characteristics
Baseline characteristics are displayed in Table 1. Overall, the cohort was overweight with a mean BMI of 30 ± 3.8 kg/m² and a mean HbA₁c of 60 ± 14 mmol/mol. The group was divided into T2D without NAFLD and T2D with NAFLD according to the NAFLD criteria (Table 1). The median liver fat content was 12% (IQR 9–17) in T2D with NAFLD and 1% (IQR 1–3) in T2D without NAFLD (P < 0.001). Individuals with T2D with NAFLD were younger compared to T2D − NAFLD (60 ± 8 years vs 66 ± 9 years, P < 0.001) and had a shorter duration of diabetes (10 ± 6 years vs 13 ± 8 years, respectively, P=0.017). Markers of glucose homeostasis and insulin resistance were more impaired in the T2D with NAFLD group with higher C-peptide and insulin concentrations, higher HOMA-IR, and lower Matsuda index (all P ≤ 0.005). Liver enzymes (alanine transaminase and aspartate transaminase) were higher in the T2D with NAFLD group compared to T2D without NAFLD (all P ≤ 0.001). There were no statistically significant differences in fibrosis scores (NFS and FIB-4) or diabetes medications between groups. One participant in the T2D without NAFLD group qualified as an extreme outlier with glucagon concentration of 81 pmol/L, which was extraordinarily high compared with other participants who ranged from 3 to 44 pmol/L. All analyses were performed with and without this outlier, with no differences in significance values or overall conclusions. Therefore, for all data presented, the outlier is included.

Association between glucagon and liver fat content
The T2D with NAFLD group had higher plasma glucagon concentrations compared to the T2D without NAFLD group (15 pmol/L (IQR 10–21) vs 11 pmol/L (IQR 7–15), 95% CI −6 to −1, P=0.003) (Table 1 and Fig. 1A). When adjusting for age, gender, BMI, and eGFR in the multivariate linear regression analysis (model glucagon 1), there was still a positive association between liver fat content and plasma glucagon concentration (Fig. 1B, C and Supplementary Table 1,
Table 1  Participant characteristics presented as the entire cohort and divided into groups based on liver fat content.

<table>
<thead>
<tr>
<th></th>
<th>All</th>
<th>T2D without NAFLD</th>
<th>T2D with NAFLD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number, n (%)</td>
<td>110</td>
<td>58 (53%)</td>
<td>52 (47%)</td>
<td>0.61</td>
</tr>
<tr>
<td>Gender, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>30 (27.3%)</td>
<td>17 (29.3%)</td>
<td>13 (25.0%)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>80 (72.7%)</td>
<td>41 (70.7%)</td>
<td>39 (75.0%)</td>
<td></td>
</tr>
<tr>
<td>Age (years), mean ± s.d.</td>
<td>63.5 ± 9.3</td>
<td>66.3 ± 9.2</td>
<td>60.3 ± 8.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes duration (years), mean ± s.d.</td>
<td>11.7 ± 7.4</td>
<td>13.2 ± 7.9</td>
<td>9.8 ± 6.4</td>
<td>0.017</td>
</tr>
<tr>
<td>BMI (kg/m²), mean ± s.d.</td>
<td>30.1 ± 3.8</td>
<td>29.6 ± 4.2</td>
<td>31.0 ± 3.2</td>
<td>0.060</td>
</tr>
<tr>
<td>HbA1c (mmol/mol), mean ± s.d.</td>
<td>59.5 ± 14.2</td>
<td>59.2 ± 12.6</td>
<td>59.2 ± 12.6</td>
<td>0.82</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/L), median (IQR)</td>
<td>7.8 (6.8–9.4)</td>
<td>7.7 (6.6–9.2)</td>
<td>8.0 (6.9–9.4)</td>
<td>0.33</td>
</tr>
<tr>
<td>Glucagon (pmol/L), median (IQR)</td>
<td>12.0 (9.0–17.0)</td>
<td>11.0 (7.0–15.0)</td>
<td>14.5 (10.0–21.2)</td>
<td>0.003</td>
</tr>
<tr>
<td>C-peptide (pmol/L), mean ± s.d.</td>
<td>867 ± 550</td>
<td>694 ± 449</td>
<td>1062 ± 599</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Insulin (pmol/L), median (IQR)</td>
<td>14.5 (10.6–24.3)</td>
<td>11.9 (8.8–20.0)</td>
<td>17.0 (12.8–28.8)</td>
<td>0.003</td>
</tr>
<tr>
<td>Matsuda index, median (IQR)</td>
<td>0.8 (0.6–1.2)</td>
<td>0.9 (0.7–1.3)</td>
<td>0.7 (0.4–0.9)</td>
<td>0.002</td>
</tr>
<tr>
<td>HOMA-IR index, median (IQR)</td>
<td>5.2 (3.6–8.2)</td>
<td>4.6 (3.2–6.8)</td>
<td>7.0 (4.1–10.1)</td>
<td>0.005</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73 m²), mean ± s.d.</td>
<td>85.4 ± 18.8</td>
<td>82.7 ± 19.3</td>
<td>88.4 ± 17.9</td>
<td>0.12</td>
</tr>
<tr>
<td>Liver fat content (%), median (IQR)</td>
<td>5.2 (1.0–11.6)</td>
<td>1.0 (1.0–3.2)</td>
<td>11.8 (8.8–17.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/L), median (IQR)</td>
<td>31 (25–41)</td>
<td>29.0 (24.3–35.0)</td>
<td>36.5 (27.8–47.2)</td>
<td>0.001</td>
</tr>
<tr>
<td>Aspartate aminotransferase (U/L), median (IQR)</td>
<td>28 (24–35)</td>
<td>26.0 (22.0–30.0)</td>
<td>31.5 (28.0–37.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NAFLD fibrosis score, mean ± s.d.</td>
<td>-0.08 ± 0.9</td>
<td>-0.02 ± 1.0</td>
<td>-0.15 ± 0.8</td>
<td>0.45</td>
</tr>
<tr>
<td>FIB-4 score, median (IQR)</td>
<td>1.4 (1.0–1.7)</td>
<td>1.3 (1.0–1.8)</td>
<td>1.4 (1.1–1.7)</td>
<td>0.68</td>
</tr>
<tr>
<td>Total amino acids (µmol/L), median (IQR)</td>
<td>2796 (2604–3012)</td>
<td>2775 (2608–2991)</td>
<td>2803 (2597–3021)</td>
<td>0.91</td>
</tr>
<tr>
<td>Diabetes medication, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metformin</td>
<td>91 (82.7%)</td>
<td>46 (79.3%)</td>
<td>45 (86.5%)</td>
<td>0.32</td>
</tr>
<tr>
<td>SGLT-2 inhibitor</td>
<td>18 (16.4%)</td>
<td>7 (12.1%)</td>
<td>11 (21.2%)</td>
<td>0.20</td>
</tr>
<tr>
<td>Sulphonylurea</td>
<td>11 (10.0%)</td>
<td>4 (6.9%)</td>
<td>7 (13.5%)</td>
<td>0.25</td>
</tr>
<tr>
<td>DDP-4 inhibitor</td>
<td>24 (21.8%)</td>
<td>9 (15.5%)</td>
<td>15 (28.8%)</td>
<td>0.09</td>
</tr>
<tr>
<td>GLP-1RA</td>
<td>36 (32.7%)</td>
<td>22 (37.9%)</td>
<td>14 (26.9%)</td>
<td>0.22</td>
</tr>
<tr>
<td>Insulin</td>
<td>39 (35.5%)</td>
<td>23 (39.7%)</td>
<td>16 (30.8%)</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Normally distributed data are presented as mean ± s.d., non-normally distributed data as median with IQR in parentheses, and categorical data as n with percent in parentheses. Group comparison with t-test, Kruskal–Wallis test, or chi–squared test, respectively. DDP-4, dipeptidyl peptidase 4; eGFR, estimated glomerular filtration rate; GLP-1RA, glucagon-like peptide 1 receptor agonist; HOMA-IR, homeostatic model assessment for insulin resistance; IQR, interquartile range; SGLT-2, sodium glucose transporter 2.

see section on supplementary materials given at the end of this article. The model estimated that a doubling in liver fat content would associate with an increase in glucagon concentration of 8% (95% CI 1–16, P = 0.039). Further adjustment for the variance in fasting glucose concentrations (model glucagon 2) did not change the estimates. Finally, we adjusted for the variation in diabetes medication (model glucagon 3), resulting in an only minor change in the estimates (Fig. 1B and Supplementary Table 1). According to the models, none of the diabetes medications nor eGFR were significantly associated with plasma glucagon concentration (Fig. 1B and Supplementary Table 1).

Association between amino acids and liver fat content

There was no difference in total amino acids between groups (Table 1 and Fig. 2C). T2D with NAFLD had a significantly higher average concentration of glutamate and isoleucine and a significantly lower average concentration of glycine and serine compared to T2D without NAFLD (Fig. 2A and Table 2). All other amino acids were not statistically different between groups. No amino acids associated with liver fat content in the linear regression model after adjusting for age, gender, BMI, and eGFR (model amino acids 1). Adjusting for variance in glucagon concentrations (model amino acids 2) did not change the associations between liver fat content and amino acid concentrations (Fig. 2B and Supplementary Table 2).

Association between amino acids and glucagon

From the linear regression models with amino acids as the dependent variables and liver fat content as
the independent variable, we could further estimate the associations between glucagon and amino acids (model amino acids 2), taking the variance of liver fat content, age, gender, BMI, and eGFR into account (Fig. 3A and Supplementary Table 2). Glucagon associated positively with isoleucine, leucine, valine, methionine, and phenylalanine and negatively with glycine and glutamine (Fig. 3B, C, D, E, F, and G and Supplementary Table 2).

Discussion

We hypothesized that hepatic fat deposition attenuates the effect of glucagon on amino acid turnover in the liver, resulting in increased plasma concentrations of amino acids and hyperglucagonaemia. In the present cohort of 110 individuals with T2D with established or at high risk of cardiovascular disease and MRS-determined liver fat content of varying degree (n=52 with NAFLD, n=58 without NAFLD), we found that the T2D with NAFLD group was characterized by elevated fasting plasma glucagon concentration. Furthermore, the T2D with NAFLD group had significantly higher plasma concentrations of glutamate and isoleucine, whereas glycine and serine concentrations were lower compared to T2D without NAFLD. The concentrations of other amino acids were similar between individuals with and without NAFLD.

A positive association between glucagon and liver fat content has previously been reported in a small cohort of individuals with T2D and BMI-matched non-diabetic controls (18) and in individuals with normoglycaemia or prediabetes (16). In contrast, there was no association in a cohort of diet and/or metformin-treated individuals with T2D (19) or in individuals with severe obesity (17). The abovementioned studies used liver biopsy or MRS to assess liver fat content. In the present study, the higher average plasma glucagon concentration in the T2D with NAFLD group was supported by a positive association between liver fat content and fasting plasma glucagon concentration. However, there was a substantial variation in glucagon concentrations within both groups, and liver fat content only contributed little to the overall variation of glucagon, while none of the other included covariates associated significantly with glucagon (Fig. 1B and Supplementary Table 1).

In contrast to our hypothesis, we did not observe hyperaminoacidaemia in individuals with T2D with NAFLD vs individuals with T2D without NAFLD. As mentioned above, the group with T2D with NAFLD...
IRix et al. had higher plasma concentrations of glutamate and isoleucine and lower plasma concentrations of glycine and serine than T2D without NAFLD, whereas other amino acids and total amino acids were similar between the two groups (Fig. 2A). No associations were found between amino acids and liver fat in the regression analyses. Hypothetically, the lack of associations between liver fat content and other amino acids could be explained by compensatory increased glucagon levels in individuals with higher liver fat content, which would lower amino acids despite differences in glucagon sensitivity in the liver. However, in the model with adjustment for variation in glucagon levels, no association between liver fat content and amino acid concentrations was seen (Fig. 2B and Supplementary Table 2). Lack of associations between plasma amino acid concentrations and liver fat content was reported for individuals with T2D (19). Another study showed that individuals with severe obesity had higher concentrations of amino acids compared to lean controls, but the coexistence of NAFLD did not aggravate the hyperaminoacidaemia (17). In contrast, liver fat content and amino acids were positively associated in healthy individuals and individuals with prediabetes (16) and in a small cohort of obese individuals with and without T2D (18).

The lack of associations between liver fat content and most amino acids in our cohort of T2D patients point towards other factors than steatosis alone contributing to hyperaminoacidaemia. Insulin resistance results in increased muscle protein catabolism and release of amino acids from muscle to circulation (31) and may, accordingly, constitute a major determinant of amino acid levels. A large cross-sectional study recently found that individuals with NAFLD (diagnosed by transient elastography) had higher levels of most amino acids and lower levels of glycine compared to controls without...
NAFLD (32). However, in a sub-analysis of selected HOMA-IR-matched individuals from the two groups, the significant differences for all amino acids, except for alanine, disappeared (32). When comparing NAFLD with fibrosis to HOMA-IR-matched controls without NAFLD in the same sub-analysis, the only amino acids that remained altered in the group with NAFLD and fibrosis were glutamate (higher) and glycine (lower) (32). This suggests that NAFLD-associated hyperaminoacidemia may – at least partly – be driven by reduced insulin sensitivity (32). In the present study, most amino acids were similar between groups despite the T2D with NAFLD group appeared more insulin resistant based on HOMA-IR and Matsuda index. This may be attributed to the fact that both groups were indeed insulin resistant despite differences in estimates.

In the present study, the T2D with NAFLD group had a higher plasma concentration of isoleucine and glutamate and a lower concentration of serine and glycine than T2D without NAFLD. Low plasma glycine levels have consistently been reported in obesity and T2D (33, 34) and shown to predict the development of diabetes in healthy individuals (33). The combination of high circulating glutamate and low serine and glycine levels has previously been reported in non-diabetic individuals with NAFLD (diagnosed by MRS or liver biopsy, respectively) (35, 36) and in NAFLD with fibrosis (diagnosed by transient elastography) (32). Interestingly, these three amino acids are all involved in the synthesis of the antioxidant glutathione. Glutathione is produced in response to oxidative stress and reactive oxygen species during liver damage and excessive fat oxidation (35). High plasma levels of glutamate may, in turn, be explained by the accelerated production of glutathione, which is cleaved into glutamate and a glutathione remnant by the enzyme gamma glutamyltransferase when it leaves the hepatocyte, resulting in a net increase of glutamate (36). In support of this notion, individuals with NAFLD had increased hepatic uptake and degradation of glycine, serine and alanine and a net release of glutamate compared to controls in a study measuring splanchnic fluxes in the fasted state (37). These findings led to the proposal of an NAFLD-specific metabolic index consisting of the ratio between glutamate and the product of serine and glycine (glutamate/(serine + glycine)) (36).

We found a positive association between glucagon and the amino acids valine, leucine, isoleucine, methionine and phenylalanine (the three first being branched chain amino acids (BCAAs)). BCAAs are mainly metabolized in muscles where there are no glucagon receptors (38, 39). Moreover, BCAAs are not suspected to stimulate glucagon secretion (40) and we do not regard this as a causal association but believe that glucagon and BCAAs are increased independently of each other in parallel with general dysmetabolism in T2D. Glucagon upregulates glutaminase, the enzyme that deaminates glutamine into glutamate and ammonium (41), supporting our findings of a negative association between glucagon and glutamine. Further, glucagon increases glutamate uptake in the liver (42, 43). Finally, the observed negative association between glucagon and glycine has previously been explained by glucagon-induced upregulation of the glycine cleavage system (44, 45, 46).

### Table 2  Concentrations of individual amino acids.

<table>
<thead>
<tr>
<th></th>
<th>T2D without NAFLD</th>
<th>T2D with NAFLD</th>
<th>P</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>399 (362–494)</td>
<td>439 (360–517)</td>
<td>0.53</td>
<td>−50–30</td>
</tr>
<tr>
<td>Arginine</td>
<td>73 (64–81)</td>
<td>74 (62–83)</td>
<td>0.85</td>
<td>−8.0–6.0</td>
</tr>
<tr>
<td>Asparagine</td>
<td>43 (37–49)</td>
<td>40 (34–48)</td>
<td>0.083</td>
<td>−0.0–6.0</td>
</tr>
<tr>
<td>Aspartate</td>
<td>5.6 (4.0–7.5)</td>
<td>6.3 (5.0–8.2)</td>
<td>0.084</td>
<td>−1.7–0.1</td>
</tr>
<tr>
<td>Glutamate</td>
<td>88 (64–116)</td>
<td>112 (91–156)</td>
<td>0.003</td>
<td>−45 to −10</td>
</tr>
<tr>
<td>Glutamine</td>
<td>585 (496–633)</td>
<td>556 (470–647)</td>
<td>0.33</td>
<td>−24–67</td>
</tr>
<tr>
<td>Glycine</td>
<td>207 (181–235)</td>
<td>190 (169–217)</td>
<td>0.042</td>
<td>1.0–32</td>
</tr>
<tr>
<td>Histidine</td>
<td>77 (74–83)</td>
<td>76 (69–86)</td>
<td>0.43</td>
<td>−2.0–5.0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>73 (66–79)</td>
<td>79 (67–90)</td>
<td>0.047</td>
<td>−13 to −0.0</td>
</tr>
<tr>
<td>Leucine</td>
<td>130 (120–145)</td>
<td>134 (115–160)</td>
<td>0.41</td>
<td>−15–6.0</td>
</tr>
<tr>
<td>Lysine</td>
<td>187 (171–213)</td>
<td>188 (173–209)</td>
<td>0.82</td>
<td>−10–13</td>
</tr>
<tr>
<td>Methionine</td>
<td>22 (19–25)</td>
<td>22 (19–25)</td>
<td>0.61</td>
<td>−1.0–2.0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>57 (53–64)</td>
<td>57 (52–62)</td>
<td>0.37</td>
<td>−2.0–5.0</td>
</tr>
<tr>
<td>Proline</td>
<td>203 (168–241)</td>
<td>207 (178–259)</td>
<td>0.33</td>
<td>−33–11</td>
</tr>
<tr>
<td>Serine</td>
<td>105 (90–117)</td>
<td>95 (81–106)</td>
<td>0.020</td>
<td>2.0–16</td>
</tr>
<tr>
<td>Threonine</td>
<td>110 (99–124)</td>
<td>109 (89–126)</td>
<td>0.52</td>
<td>−6.0–13</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>55 (47–58)</td>
<td>58 (50–61)</td>
<td>0.14</td>
<td>−6.0–1.0</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>57 (49–64)</td>
<td>54 (48–62)</td>
<td>0.25</td>
<td>−2.0–6.0</td>
</tr>
<tr>
<td>Valine</td>
<td>246 (221–264)</td>
<td>267 (230–293)</td>
<td>0.13</td>
<td>−33–4.0</td>
</tr>
</tbody>
</table>

Fasting plasma concentrations of individual amino acids. Data are presented as median with IQR in parentheses. Group comparison between T2D without NAFLD (n = 58) and T2D with NAFLD (n = 52) with Kruskal–Wallis test.

IQR, interquartile range.
Strengths of the study include using MRS for evaluation of liver fat content, mass spectrometry-validated sandwich ELISA for analysis of glucagon, and targeted mass spectrometry for analysis of individual amino acids. We also believe that the well-characterized cohort of individuals with T2D recruited according to prespecified inclusion criteria, yielding a relatively homogeneous group of individuals with T2D with established or at high risk of cardiovascular disease and different degrees of liver fat content, is a strength for association studies like the present. Further, we included multivariate linear regression analyses aiming to adjust for confounders. However, the disadvantage of linear regression, in this case, is that it simplifies results by assuming a completely linear relationship between the data, which turned out to be a simplification in our study. We omitted including plasma insulin and HOMA-IR in the models as the insulin assay used fails to precisely measure some of the insulin analogues used in the cohort (47), thus questioning the reliability of the insulin concentrations in those participants on insulin treatment. Limitations of the study include that these secondary exploratory analyses were not predefined and that information on dietary protein intake, which may have affected concentration of plasma amino acids, were not available. Finally, it should be emphasized that we did not adjust for multiple comparisons due to the exploratory nature of these secondary analyses. Thus, type I errors may have occurred, although our positive findings correspond well to previous reports.

In conclusion, in the present cohort of well-characterized individuals with T2D, MRS-assessed liver fat content was positively associated with plasma glucagon concentrations. Only a few amino acids differed in plasma concentration between T2D with NAFLD compared to the T2D without NAFLD (higher glutamate and isoleucine and lower glycine and serine). It may be speculated that the hyperaminoacidaemia previously observed in NAFLD is driven by peripheral proteolysis.
due to insulin resistance independently of coexisting NAFLD. Future mechanistic studies are needed to investigate this and to delineate the relationship between glucagon, individual amino acids, and NAFLD. In addition, larger epidemiologic studies are warranted to investigate if certain amino acids, perhaps in combination with glucagon, may serve as biomarkers for NAFLD risk or established NAFLD.

Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/EC-23-0161.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the study reported.

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Data availability
The data supporting the findings of this study are not currently available in a public repository.

Author contribution statement
I.R. contributed to the conceptualization of the present secondary analysis, performed statistical analyses and drafted the manuscript. M.L.J contributed to study conduct and data collection. A.L. and M.P.S contributed to the conceptualization of the present secondary analysis and edited the draft manuscript.

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