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Hepatic steatosis in humans is associated with preserved glucagon action on amino acid metabolism

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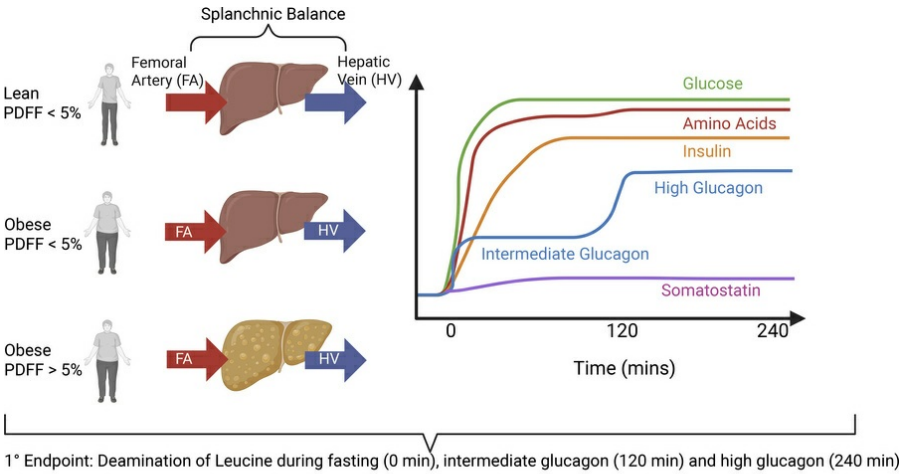
Graphical abstract



1° Endpoint: Deamination of Leucine during fasting (0 min), intermediate glucagon (120 min) and high glucagon (240 min)

2° Endpoint: Splanchnic balance of Amino Acids during fasting (0 min), intermediate glucagon (120 min) and high glucagon (240 min)

Both endpoints were unaffected by hepatic fat

Hepatic steatosis in humans is associated with preserved glucagon action on amino acid metabolism

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Dr. Vella has consulted for Boehringer-Ingelheim, Neurotronics and Rezolute. None of the other authors declare conflict of interests related to this study.

Abstract

Background: Amino acid (AA) concentrations are increased in prediabetes and diabetes. Since AA stimulate glucagon secretion which should then increase hepatic AA catabolism, it has been hypothesized that hepatic resistance (associated with hepatic fat content) to glucagon's actions on AA metabolism leads to hyperglucagonemia and hyperglycemia.

Methods: To test this hypothesis, we therefore studied lean and obese individuals, the latter group with and without hepatic steatosis as defined by Proton Density Fat Fraction (PDFF) > 5%. After an overnight fast, femoral vein, femoral artery, and hepatic vein catheters were placed. [3-^{3}H] glucose and L-[1-^{13}C,^{15}N]-leucine were used to measure glucose turnover and leucine oxidation respectively. During a hyperglycemic clamp, an amino acid mixture was infused together with insulin and glucagon (1.5 ng/kg/min 0 – 120 min; 3.0 ng/kg/min 120 – 240 min). Tracer-based measurement of hepatic leucine oxidation in response to rising glucagon concentrations and splanchnic balance (measured using arterio-venous differences across the liver), of the other AA were the main outcomes measured.

Results: The presence of hepatic steatosis did not alter hepatic glucose metabolism and leucine oxidation in response to insulin and rising concentrations of glucagon. Splanchnic balance of a few amino acids, and related metabolites differed amongst the groups. However, across-group differences of AA splanchnic balance in response to glucagon were unaffected by the presence of hepatic steatosis.

Conclusion: The action of glucagon on hepatic amino acid metabolism is unaffected by hepatic steatosis in humans.

Trial registration: This study was registered at Clinical Trials.Gov: NCT05500586.

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Introduction

It is estimated that 35-40% of the adult US population has obesity (1). Obesity-related metabolic dysfunction-associated steatotic liver disease (MASLD) is reported to have a prevalence of 24% (2). Both obesity and MASLD (3-5) independently increase the risk of type 2 diabetes (T2DM), a disease that increases morbidity and mortality while costing > $170 billion/year (6), making prevention important. T2DM is characterized by insulin secretion that is inadequate for the prevailing insulin action and also by glucagon concentrations inappropriate for the prevailing hyperglycemia. α-cell dysfunction (7), elevated fasting amino acids (AA) (8) and AA metabolites e.g. α-aminoadipic acid (9) are markers of T2DM risk.

In rodents, impaired hepatic glucagon signaling results in hyperglucagonemia and increased α-cell mass – an effect mediated by increased in circulating AA concentrations (10). Thus, the elevated concentrations of branched-chain AA and other AA metabolites that arise from impaired glucagon signaling (8) would further contribute to α-cell dysfunction. If impaired glucagon induced hepatic AA catabolism is the cause of hyperglycemia in MASLD, this would require selective preservation of the effects of glucagon on hepatic glucose metabolism (11). This could be explained by the different signaling pathways for glucagon actions on AA as opposed to other macronutrients (12, 13).

Recent reports suggest that obese humans with MASLD are resistant to the acute stimulation of hepatic amino acid catabolism by glucagon (14, 15). This is important because α-cells respond to elevated AA concentrations (16) by secreting glucagon (17, 18). A failure to stimulate hepatic AA clearance could result in glucagon secretion inappropriate for the prevailing glucose concentrations. In a prior human study addressing this question, most participants with obesity also had MASLD so that it is unclear if the failure of glucagon to stimulate AA clearance

and ureagenesis was caused by obesity, hepatic steatosis or both (14). There are other technical limitations to this prior experiment that may affect the generalizability of its conclusions (see discussion).

Previously, we have shown that α-cell dysfunction occurs early in the pathogenesis of prediabetes and predicts a longitudinal decline in glucose tolerance (7, 19). It is possible that rather than being due to intrinsic α-cell dysfunction, this is an appropriate response to (abnormally elevated) AA. AA concentrations in the circulation represent a balance between appearance (whether from endogenous sources or ingestion) and clearance. To accurately measure hepatic extraction of AAs under fasting and postprandial conditions, we studied subjects after placement of the appropriate intravascular catheters to facilitate measurement of AA arterio-venous differences across the liver.

To specifically address whether the presence of hepatic fat alters the effects of glucagon on hepatic AA metabolism, we studied lean and obese individuals in whom hepatic fat was quantified using MRI. In this way we were able to recruit obese individuals with a wide range of hepatic fat content. Subjects were studied after an overnight fast when femoral vein, femoral artery, and hepatic vein catheters were placed (Supplementary Figure 1). Insulin was infused at 0.8 mU/kg/min to mimic postprandial conditions, while peripheral glucose concentrations were maintained at ~9.5 mmol/L. Glucagon was infused at two concentrations ("intermediate" and "high") to mimic early postprandial concentrations observed after ingestion of a high protein meal. A mixture of AAs, used clinically for total parenteral nutrition was also infused to mimic postprandial conditions.

We report that while hepatic steatosis was associated with impaired insulin action, there was no effect on hepatic glucose metabolism in response to rising glucagon concentrations.

Tracer-based measurement of leucine oxidation in response to rising glucagon concentrations showed no effect of obesity alone or obesity with hepatic steatosis on leucine metabolism in the liver. Subtle differences in the splanchnic balance of some AA and related metabolites were identified and, in a few examples, correlated with hepatic fat content. However, the response to glucagon did not differ significantly across groups. Overall, this data suggests that hepatic glucagon resistance does not play a major role in the handling of AA by the liver.

Results

Subject Characteristics (Table 1)

A total of 20 subjects were studied. By design, subjects in the obese group had higher total body weight and BMI than the lean subjects (Table 1). The subgroup recruited because of the presence of high hepatic fat had a proton density fat fraction (PDFF > 5%), which, again by design, differed significantly from that in the other 2 groups. The increase in hepatic fat content was not accompanied by any differences in hepatic stiffness or Liver Iron Content. Fasting glucose concentrations and HbA1c values were higher in the groups with obesity but did not differ in those with and without increased hepatic fat. Despite increased β-cell responsivity to glucose (Φ), compared to the lean group, when expressed as a function of the prevailing insulin action (S_i), Disposition Index (DI) was decreased in subjects with obesity. These indices, estimated by the oral minimal model (20) did not differ in the subjects with obesity with and without increased hepatic fat.

Concentrations of Glucose, Insulin, C-Peptide and Glucagon during fasting and then during the hyperglycemic clamp (Figure 1)

Prior to the start of the clamp, fasting glucose concentrations did not differ significantly amongst the groups (Panel A: 4.8 ± 0.3 vs. 5.6 ± 0.2 vs. 5.4 ± 0.02 mmol/L, lean vs. obese PDFF < 5% vs. obese PDFF > 5% respectively, $p = 0.07$). By design, glucose concentrations during the clamp did not differ amongst the groups during intermediate and high glucagon infusion (120 and 240 minutes respectively – Panel A).

Fasting insulin concentrations were significantly higher in subjects with obesity (Panel B: 14 ± 1 vs. 41 ± 5 vs. 48 ± 7 pmol/L, $p < 0.01$). There was no difference in fasting insulin concentrations in obese subjects with and without increased hepatic fat. Insulin concentrations during the clamp did not differ between groups ($p = 0.07$ – Panel B).

Fasting C-peptide concentrations mirrored the differences in fasting insulin concentrations (Panel C: 0.40 ± 0.04 vs. 0.81 ± 0.08 vs. 0.93 ± 0.11 nmol/L, $p < 0.01$) and remained slightly, but significantly ($p < 0.01$) higher in the subjects with obesity after the initiation of somatostatin infusion during the clamp.

Glucagon concentrations prior to the initiation of the clamp did not differ between groups (Panel D). At 120 min glucagon infused at 1.5 ng/kg/min (intermediate) resulted in concentrations (22 ± 2 vs. 26 ± 2 vs. 24 ± 2 pmol/L) that did not differ between groups ($p = 0.42$). At 240 min glucagon infused at 3.0 ng/kg/min (high) resulted in concentrations (45 ± 2 vs. 48 ± 3 vs. 46 ± 5 pmol/L) that also did not differ between groups ($p = 0.86$ – Panel D).

Rates of Glucose Infusion, Glucose Disappearance and Endogenous Glucose Production during fasting and then during the hyperglycemic clamp (Figure 2)

The amount of glucose infused to maintain the hyperglycemic clamp was greater in the lean subjects compared to those with obesity ($p < 0.01$ – Panel A). During intermediate glucagon

infusion (90 to 120 min) there were no significant differences in the glucose infusion rate (GIR) between obese subjects with and without increased hepatic fat. However, during the high glucagon infusion (210 to 240 min), *post hoc* testing showed significant differences in GIR between the two groups with obesity (Panel A: 7.5 ± 0.8 vs. 3.1 ± 0.5 mg/kg/min, obese PDFF < 5% vs. obese PDFF > 5% respectively, $p = 0.02$).

The rate of glucose disappearance (Rd) exhibited a similar pattern to GIR with no differences observed in the fasting state (-30 to 0 min) amongst the 3 groups (Panel B: $p = 0.15$). However, Rd was higher in the lean subjects compared to those with obesity ($p < 0.01$ – Panel B) during the clamp. During the high glucagon infusion (210 to 240 min), *post hoc* testing showed significant differences in Rd between the two groups with obesity (Panel B: 53 ± 7 vs. 26 ± 3 μmol/kg/min, obese PDFF < 5% vs. obese PDFF > 5% respectively, $p = 0.02$).

Rates of endogenous glucose production (EGP) during the fasting state did not differ between groups (Panel C: 15.7 ± 0.6 vs. 13.1 ± 0.9 vs. 13.8 ± 1.2 μmol/kg/min, lean vs. obese PDFF < 5% vs. obese PDFF > 5% respectively, $p = 0.15$). Also, no differences in EGP were apparent during the intermediate ($p = 0.88$) and high ($p = 0.57$) glucagon infusion rates.

Net Splanchnic Glucose Balance, Splanchnic Extraction Ratio, Splanchnic Glucose Uptake and Splanchnic Glucose Production during fasting and then during the two stages of the hyperglycemic clamp (Supplementary Figure 3 and Supplementary Table 1)

Net splanchnic glucose balance (NSGB), splanchnic extraction ratio (SER), splanchnic glucose uptake (SGU) and splanchnic glucose production (SGP) were calculated as previously described ((21) Supplementary Figure 2). NSGB was negative in the fasting state i.e., glucose concentrations were higher in the hepatic venous circulation (Supplementary Figure 3, Panel A).

This did not differ between groups (Supplementary Table 1). On the other hand, during the clamp, mean NSGB became positive in response to the conditions present. At the end of the intermediate glucagon infusion (120 min) there were small differences in NSGB across the groups although there were no significant differences between the two groups with obesity (Supplementary Table 1). No differences were apparent during the high glucagon infusion (240 min). SER, SGU and SGP did not differ across the groups studied during fasting and during the clamp studies (Supplementary Table 1 and Panels B, C, D of Supplementary Figure 3 respectively).

Splanchnic Leucine Uptake, KIC Release, Reamination and Leucine Breakdown during fasting and then during the two stages of the hyperglycemic clamp (Figure 3 and Supplementary Figures 4, 5)

Leucine uptake (Figure 3, Panel A) by the splanchnic tissues was calculated (Supplementary Figure 4). During fasting it was unaffected by obesity with or without increased hepatic fat. In response to insulin and glucagon, leucine uptake increased but did not differ significantly across the groups during both rates of glucagon infusion. When the correlation of leucine uptake with hepatic fat as a continuous variable was examined, no relationship was observed (Supplementary Figure 5, Panels A-C).

During fasting, α-ketoisocaproic acid (KIC) concentrations were higher in the arterial circulation compared to the hepatic vein i.e. KIC was extracted by the liver (Please see (22) and Supplementary Figure 4). There was no statistically significant difference across groups (1.6 ± 0.9 vs. 1.9 ± 0.5 vs. 0.6 ± 0.2 mmol/min, $p = 0.08$ – Figure 3, Panel B). In response to insulin and glucagon, KIC was released by the liver but no statistically significant across group differences

were apparent during intermediate glucagon infusion (-0.8 ± 0.2 vs. -0.8 ± 0.5 vs. -0.1 ± 0.2 mmol/min, $p = 0.06$– Figure 3, Panel B). During high glucagon infusion there were no significant across group differences ($p = 0.61$). However, when the correlation of leucine uptake with hepatic fat as a continuous variable was examined, a relationship was observed during intermediate glucagon infusion (Supplementary Figure 5, Panels D-F). Note that KIC release increased as hepatic fat increased.

In the model previously described by Cheng et al (22), reamination of KIC to leucine during steady-state conditions is equal to (therefore serving as a surrogate for) loss of ^{15}N from L-[1-^{13}C,^{15}N]-Leucine during conversion to KIC (Supplementary Figure 4). The rate of reamination did not differ across groups during fasting (17 ± 2 vs. 26 ± 5 vs. 20 ± 6 mmol/min, $p = 0.22$ – Figure 3, Panel C). In response to insulin and glucagon there was a tendency towards higher reamination rates in the groups with obesity (independent of hepatic fat) but this was not significant during intermediate (12 ± 1 vs. 19 ± 2 vs. 22 ± 7 mmol/min, $p = 0.15$) and high glucagon infusion (10 ± 1 vs. 16 ± 2 vs. 14 ± 4 mmol/min, $p = 0.39$ – Figure 3, Panel C). Again, when the correlation of reamination with hepatic fat as a continuous variable was examined, a relationship was observed during intermediate glucagon infusion (Supplementary Figure 5, Panels G-I). Reamination increased as hepatic fat increased.

Net leucine breakdown across the splanchnic tissues (Supplementary Figure 4) did not differ significantly across groups during fasting (7 ± 3 vs. 20 ± 3 vs. 19 ± 7 mmol/min, $p = 0.09$ – Figure 3, Panel D). During the subsequent two stages of the clamp, no significant differences were observed across the 3 groups (Figure 3, Panel D). There was a weak positive correlation of leucine breakdown with hepatic fat at all stages of the experiment (Supplementary Figure 5, Panels J-L).

Splanchnic Balance of Essential Amino Acids during fasting and then during the two stages of the hyperglycemic clamp (Figure 4)

The Splanchnic balance for individual essential AAs was calculated (Supplementary Figure 6). Positive values imply higher concentrations in the arterial circulation i.e., hepatic uptake and negative values imply the opposite i.e., hepatic release. No significant across-group differences were apparent in the branched chain AA (BCAA – Panels A-C).

In the case of aromatic AA (AA – Panels D-F), differences in tyrosine balance were apparent during intermediate glucagon infusion but not at other stages of the experiment. No correlation with hepatic fat content was noted (Supplementary Table 2).

The splanchnic balance of sulfur-containing AA (Panels G-I) differed significantly during fasting (methionine only) and during intermediate, but not high glucagon infusion. No correlation with hepatic fat content was noted (Supplementary Table 2).

Of the other essential AA (Panels J-L), the splanchnic balance of threonine differed during intermediate glucagon infusion but no correlation with hepatic fat content was noted (Supplementary Table 2).

Splanchnic Balance of Non-Essential Amino Acids during fasting and then during the two stages of the hyperglycemic clamp (Figure 5)

During the fasting state (Panel A) the splanchnic balance of glutamate differed significantly across groups ($p = 0.03$). However, these differences were no longer significant during both rates of glucagon infusion (Panels B and C respectively). The baseline splanchnic balance of glutamate correlated with weight but not hepatic fat (Supplementary Table 2).

Differences in the splanchnic balance of glycine and arginine became apparent during the experiment and (unlike those for alanine, glutamine, and serine) persisted to the end (Panels B

and C). Of these, only the splanchnic balance of arginine correlated with PDFF but not with weight (Supplementary Table 2).

Splanchnic Balance of Amino Acid Metabolites during fasting and then during the two stages of the hyperglycemic clamp (Figure 6)

The splanchnic balance of several metabolites did not differ significantly during the fasting state (Panel A), but differences became apparent during the experiment (Panels B and C). Of these, splanchnic balance of β-alanine correlated with weight but not PDFF (Supplementary Table 3). The splanchnic balance of allo-isoleucine and hydroxylysine correlated with PDFF but this was inconsistent throughout the experiment (Supplementary Table 3).

α-aminoadipic acid, α-amino-N-butyric acid, β-amino-isobutyric acid and γ-amino-N-butyric acid all exhibited differences in splanchnic balance although this was only consistent in the case of the latter 2 (Panels D-F). β-amino-isobutyric acid and γ-amino-N-butyric acid correlated with PDFF but not with weight (Supplementary Table 3).

Differences in the splanchnic balance of citrulline (Panel G) became significant during the experiment when clamp conditions decreased net hepatic release (Panels H and I). These differences correlated with PDFF (Supplementary Table 3). The differences for ethanolamine were less consistent over the duration of the experiment (Panel H and Supplementary Table 3).

Of the remaining metabolites analyzed (Panels J-L) no differences in splanchnic balance were noted.

Splanchnic Extraction Ratio of selected Amino Acids and Metabolites during fasting and then during the two stages of the hyperglycemic clamp (Supplementary Figures 7 and 8).

The splanchnic extraction ratio (SER – Supplementary Figure 7) was calculated for AA and metabolites whose splanchnic balance correlated with weight (glutamic acid, glutamine,

glycine and β-alanine) and with PDFF (arginine, β-amino-isobutyric acid, citrulline, ethanolamine (not shown) and γ-amino-N-butyric acid – Supplementary Figure 8).
SER (which unlike splanchnic balance is independent of splanchnic blood flow) did not differ consistently across groups but the differences, if any, were often driven by differences present at baseline and the change from baseline in response to insulin and glucagon did not differ across groups.

Discussion

Glucagon secretion that is inappropriate for the prevailing glucose concentrations is increasingly recognized early in prediabetes (7). Amino acids are potent glucagon secretagogues. In turn, glucagon stimulates the catabolism of AA by the liver (16). The observation that prediabetes and T2DM are associated with increased circulating concentrations of some AAs (as well as relative or absolute hyperglucagonemia) has led to the hypothesis that impaired glucagon action on hepatic AA catabolism results in hyperglucagonemia and its attendant adverse effects on glucose metabolism (23). This hypothesis has been bolstered by the observation that hyperglucagonemia and higher circulating AA are present in MASLD (24). In accordance with this hypothesis, Suppli et al. reported that hepatic steatosis impaired ureagenesis and AA catabolism in response to glucagon (14).

Glucagon signals through its 7-transmembrane helix receptor activating $G_{\alpha s}$-coupled proteins that increase cAMP and activate cAMP response element binding protein (CREP) and protein kinase A (PKA) which act in concert to decrease glycolysis and increase gluconeogenesis and glycogenolysis (25). Glucagon receptor activation also stimulates intra-hepatic lipolysis (26).

However, although hepatic steatosis has been proposed as a surrogate for hepatic glucagon resistance with a bifurcation in hepatic glucagon action on lipids and carbohydrates in rodents (13), this has not been observed in humans to date (27). Indeed several mechanisms for selective resistance of AA metabolism to glucagon have been proposed (28), but these have not been directly tested in humans.

We sought to address this question using an experimental design (Supplementary Figure 1) that enabled direct measurement of leucine oxidation across the splanchnic bed. In addition, the measurement of arterial and hepatic vein concentrations of all other AAs enabled assessment of their handling by the splanchnic tissues in experimental conditions intended to mimic post-cibal glucose, AA, insulin, and glucagon concentrations. More importantly, we designed an experiment to overcome the technical limitations of prior work (14) while approximating postprandial conditions.

The first priority was to ensure that we could differentiate the effects of obesity from those of MASLD. To do so, we studied lean subjects without any evidence of hepatic steatosis together with subjects with obesity with and without hepatic steatosis. Lower insulin infusion rates resulting in concentrations similar to those encountered during fasting may exacerbate the effects of glucagon on metabolism (14). However, the conclusions would have little relevance to normal postprandial physiology especially in patients with MASLD where insulin rises significantly in response to food ingestion. Therefore, we infused insulin at rates that resulted in peripheral concentrations similar to those observed in the postprandial period. This enabled us to maintain a hyperglycemic clamp and ensure that glucose concentrations did not differ across the groups studied as was the case with lower insulin concentrations (14).

At the time of screening, an oral glucose tolerance test (OGTT) was used to measure β-cell function and insulin action. Although no differences in these parameters between the obese subjects with and without hepatic steatosis were noted (Table 1), during the experiment it was apparent that peripheral glucose disposal was further impaired in the group with a PDFF > 5%, compared to the group with obesity and a PDFF < 5% (Figure 2, Panels A & B). On the other hand, hepatic responses to the experimental conditions did not differ across all groups (Figure 2, Panel C, Supplementary Figures 2 & 3, Supplementary Table 1). This implies that hepatic carbohydrate metabolic responses to rising glucagon are preserved in the presence of hepatic steatosis.

We used doubly-labelled leucine and the model previously described by Cheng et al (22) to estimate leucine uptake, KIC release, leucine reamination and leucine breakdown (Figure 3 and Supplementary Figure 4). As before (29), leucine reamination was used as a surrogate for the rate of transamination (loss of ^{15}N) and conversion to KIC, enabling measurement of the first step of leucine catabolism in response to glucagon (30, 31). This did not differ across groups (Figure 3). To ensure that we did not miss an effect of hepatic fat with glucagon responses, we examined the relationship of these fluxes with hepatic fat (Supplementary Figure 5). If anything, the (positive) correlations observed would suggest an enhanced response to glucagon in the presence of increased hepatic fat. This would tend to refute the hypothesis that hepatic fat is a marker of hepatic glucagon insensitivity. It could also suggest that decreased insulin action in people with MASLD ‘permits’ greater effects of glucagon on leucine metabolism at a given insulin concentration. This is certainly true of glucose metabolism with overt steatohepatitis associated with metabolic dysfunction (32) but whether it applies to amino acid metabolism will require further study.

We subsequently used splanchnic balance (Supplementary Figure 6) to screen for differences in metabolism of other AAs. Some across-group differences were identified, although these differences mostly correlated with weight and not hepatic fat. This was not the case for arginine where splanchnic balance correlated with hepatic fat (Supplementary Table 2). However, the changes in SER (which are independent from alterations in blood flow) from baseline in response to rising insulin and glucagon (120 min) and further increases in glucagon (240 min) followed the same pattern as in lean subjects (Supplementary Figure 8) and did not differ across groups.

In addition to AA, we measured the splanchnic balance of metabolites (some AA-derived) that are part of our standard AA panel (see methods). We again used splanchnic balance (Supplementary Figure 6) to screen for differences in their metabolism (Figure 6). The splanchnic balance of β-aminoisobutyric acid and γ-amino-N-butyric acid was associated with PDFF rather than weight (Supplementary Table 3). This reflected significant differences in their arterial and hepatic venous concentrations in obese subjects with a PDFF > 5% but the change (or lack thereof) in SER from fasting during hyperinsulinemia and hyperglucagonemia did not differ from that in the other groups (Supplementary Figure 8). Similar patterns were observed for citrulline and ethanolamine (not shown).

Coincidentally a metabolite whose splanchnic balance differed across groups was α-aminoadipic acid which is associated with increased conversion of prediabetes to T2DM (9). This seems to be released by the liver to a greater extent in obesity. β-aminoisobutyric acid (thought to improve insulin action (33)) also seems to be released by the liver but at lower rates in people with obesity and hepatic steatosis (Supplementary Figure 6, Panel F). SER does not change appreciably from baseline during the experiment (as is the case for lean subjects and

people with obesity and a PDFF < 5%). γ-amino-N-butyric acid (a compound with unclear metabolic effects) is extracted by the splanchnic bed to a lesser extent in people with obesity and hepatic steatosis (Supplementary Figure 8, Panel H). Whether these metabolites may serve as useful markers of hepatic steatosis – and the metabolic abnormalities associated with this phenotype – remains to be ascertained.

The experiment has significant strengths including the placement of arterial and hepatic venous catheters allowing the comprehensive measurement of AAs and other metabolites of interest across the liver and the tracer-based measurement of hepatic metabolism. This together with the tests at screening allowed detailed phenotypic characterization of the subjects, as well as recruitment of obese subjects, otherwise matched for metabolic and anthropometric characteristics, with or without hepatic steatosis. Fulfilling this requirement was necessary to enable us to address our primary hypothesis and differentiate the effect of obesity from that of hepatic steatosis – a limitation of prior studies (14). Although a PDFF > 5% has consistently been used as a marker for significant hepatic steatosis (34), it is possible that metabolic effects could be present at lower values. To overcome this, we examined the correlation between various endpoints and PDFF in a continuous fashion. This strengthened our conclusions.

As with all experiments, there are some limitations that need to be considered when interpreting the results of the experiment. The first is the relatively small sample size studied, a consequence of the complexity, expense, and invasiveness of the experimental design. On the other hand, the ability to measure the extraction of multiple AAs across the liver coupled with state-of the art techniques makes it unlikely that we failed to detect a physiologically significant defect in hepatic glucagon action. This is borne out by the overlapping distribution of various endpoints across all 3 groups studied and also by the *post hoc* correlation with PDFF. Another

consequence of the small sample size is the unbalanced sex distribution. Although we have not previously studied the response of AA metabolism to glucagon, we have not observed an effect of sex on responses of carbohydrate metabolism to glucagon (35).

Despite our efforts to suppress endogenous insulin secretion using somatostatin it is clear that some portal insulin secretion persisted in obese subjects and may have attenuated some of glucagon's effects on hepatic metabolism. This is unlikely, given the minor differences in C-peptide concentrations (especially in the setting of physiological hyperinsulinemia) and the degree of impaired insulin action present in the affected subjects. Finally, the constraints of the experimental design necessitated that macronutrients were delivered into the peripheral rather than the portal circulation. Due to hepatic zonation, it is possible that we missed effects due to zone-specific gradients in hormonal and nutrient exposure although this is unlikely given the duration of each stage of the experiment (36) allowing time for equilibration. In addition, in a prior human experiment after portal delivery of AA, equilibration of hepatic vein and portal vein concentrations occurred rapidly (37). From this series of experiments in humans, where we sought to mimic postprandial conditions with simultaneous (physiologic) hyperinsulinemia, hyperglycemia and hyperglucagonemia, we conclude that there is no evidence of resistance to the actions of glucagon on AA metabolism conferred by hepatic steatosis. Abnormalities of glucagon secretion have been associated with impaired insulin action and with obesity (38, 39) but based on current evidence are unlikely to be explained by selective hepatic glucagon resistance.

Methods

Sex as a biological variable

We studied male and female subjects. We did not report findings separately.

Screening

After approval from the Mayo Clinic Institutional Review Board, we recruited subjects using intramural and extramural advertising. To enhance our ability to recruit subjects with hepatic steatosis, we wrote to subjects previously identified in the Mayo Clinic Biobank (40, 41) as having hepatic steatosis. We also used the fatty liver index (FLI) to identify subjects outside of the biobank at increased risk of hepatic steatosis (42). To be eligible, subjects had no history of chronic illness (including diabetes), macro- or microvascular disease or upper gastrointestinal surgery. Additionally, they were not taking medications that could affect weight or glucose metabolism. The alcohol use disorders identification test (AUDIT) questionnaire (43) was used to screen for alcohol excess.

Potentially eligible subjects interested in participating were invited to the Clinical Research and Trials Unit (CRTU) for a screening visit. After written, informed consent was obtained, participants underwent a 2–hour, 7-sample (0, 10, 20, 30, 60, 90 and 120 min) 75g OGTT. This allows estimation of insulin secretion and action using the oral minimal model and classification of subjects' glucose tolerance status as previously described (44). All subjects were instructed to follow a weight-maintenance diet containing 55% carbohydrate, 30% fat and 15% protein for at least three days prior to the study. Body composition was measured at the time of screening using dual-energy X-ray absorptiometry (iDXA scanner; GE, Wauwatosa, WI). Liver fat was measured by MRI using PDFF (45) where a value ≥ 5 % implies $\geq$ Grade 1 steatosis. Liver stiffness was measured by MRI elastography as previously described (46, 47) where a value of < 2.5 kPa is considered normal and a value in the range of 3.0 to 3.5 kPa suggests stage 1-2 fibrosis.

Experimental Design (See Supplementary Figure 1)

Participants were admitted to the CRTU at 1700 on the day before the study. After consuming a standard 10 kcal/kg caffeine-free meal, they fasted overnight other than sips of water when thirsty. The following morning at 0700 (-210 min), two forearm vein catheters were placed to allow for nutrient and hormone infusions. A urinary catheter was also placed at this time. Prior to their departure from the CRTU to the interventional radiology suite at 0730 (-180 min), a primed (10 μCi prime, 0.1 μCi/min continuous) infusion containing trace amounts of glucose labeled with [3-^{3}H] glucose was started and continued at this rate till 1030 (0 min). In the interventional radiology suite a hepatic vein catheter was placed via the femoral vein under fluoroscopic guidance (21). A femoral artery catheter was also placed. Infusion of indocyanine green (0.25mg/min) and L-[1-^{13}C,^{15}N]-Leucine (7.5μmol/kg prime, 7.5μmol/kg/hour continuous) was also started at this time. [U-^{13}C]-palmitate (300 nmol/min) commenced at 0830 (-120 min) after their return from interventional radiology and continued till the end of the study.

At 1030 (0 min) another glucose infusion, also labeled with [3-^{3}H] glucose commenced, and the infusion rate varied to produce peripheral glucose concentrations of ~160mg/dL (9mmol/L). The infusion rate containing trace amounts of glucose labeled with [3-^{3}H] glucose decreased (0.03 μCi/min) to minimize anticipated increases in Specific Activity caused by suppression of EGP (48). To mimic ingestion of a protein load, Clinisol (15%, 0.003ml/kg/min; 51% essential AA, 18% branched-chain AA, 9% aromatic AA; Baxter, Healthcare, Deerfield, IL) was also infused at this time. In addition, insulin (0.8 mU/kg/min), glucagon (1.5 ng/kg/min – intermediate glucagon infusion rate) and somatostatin (60 ng/kg/min) were also infused. The insulin infusion was kept constant for the remainder of the study but at 1230 (120 min) the glucagon infusion rate doubled to 3.0 ng/kg/min (high glucagon infusion rate). Blood samples

from the femoral vein, hepatic vein and femoral artery were obtained at -30 to 0 (baseline); 90 to 120 (moderate glucagon); 210 to 240 min (high glucagon). At the end of the study (1430 – 240 min) all vascular catheters were removed. Site care was performed as per institutional guidelines with the application of localized pressure at the sites of femoral vascular access. Participants consumed a late lunch but remained supine in bed until 1730 when they were mobilized. In the absence of site concerns, subjects left the CRTU once they were felt to be safe to do so.

Analytic Techniques

All blood was immediately placed on ice after collection, centrifuged at 4^{o}C, separated and stored at -80^{o}C until assay. Plasma glucose concentrations were measured using a Yellow Springs glucose analyzer. Glucagon was measured using an ELISA (Mercodia, Winston-Salem, NC (49)). C-peptide was measured using EMD Millipore (Billerica, MA) reagents. Insulin was measured using a chemiluminescence assay with reagents obtained from Beckman (Access Assay, Beckman, Chaska, MN). [3-^{3}H] glucose specific activity was measured by liquid scintillation counting following deproteinization (50). Indocyanine Green concentrations in the serum were measured using a spectrophotometer at the 805 nm wavelength (51). Amino acids and their metabolites were measured using derivatized standards and samples analyzed on a triple quadrupole mass spectrometer coupled with an Ultra Pressure Liquid Chromatography system as previously described (52).

Calculations

Endogenous glucose production (EGP) and glucose disposal (Rd) were calculated as before (48, 53). Insulin action (S_i) and β-Cell responsivity (Φ) indices were calculated (54) from the plasma glucose, insulin and C-peptide concentrations during the screening OGTT (55).

The mean values of isotope enrichment during fasting (-30 to 0 min) during intermediate glucagon infusion (90 to 120 min) and during high glucagon infusion (210 to 240 min) were used for all tracer-based calculations of AA kinetics. The calculation of leucine carbon and nitrogen flux and leucine transamination KIC reamination to leucine under steady-state conditions (29)) utilized the model previously described by Cheng et al (22).

Splanchnic plasma flow was calculated by dividing the indocyanine green infusion rate by the arterial-hepatic venous concentration gradient of the dye. Where necessary, dividing the plasma flow by 1-hematocrit provided splanchnic blood flow (51). The splanchnic balance for a given AA was calculated from the arterio-venous difference of amino acid concentration across the liver, multiplied by splanchnic blood flow while the SER was calculated from the arterio-venous difference of AA concentration across the liver, divided by the arterial concentration (Supplementary Data).

Statistical Analysis

All continuous data are summarized as means ± SEM. Area under the curve (AUC) and area above basal (AAB) were calculated using the trapezoidal rule. One-way ANOVA and a Tukey's *post-hoc* test was used to determine between-group differences (parametric data). A Kruskal-Wallis test followed by Dunn's *post-hoc* test was used for non-parametric data. When necessary, linear regression was performed using BlueSky Statistics software v. 7.10 (BlueSky Statistics LLC, Chicago, IL, USA) and Prism 5 (GraphPad Software, San Diego, CA). A *p*-value

<0.05 was considered statistically significant. Although no data existed for our experimental conditions, Nygren et al. observed (Mean ± Standard Deviation) leucine reamination rate of 66.8 ± 9.5 mmol/min (29). Assuming a similar variability, 6 subjects per group would give us the ability to detect (80% power, $\alpha = 0.05$) a 20% difference in reamination rate attributable to hepatic steatosis.

Study Approval

The Mayo Clinic Institutional Review Board approved the study and associated study documents. It was subsequently registered at ClinicalTrials.gov. Somatostatin was infused under an IND approved by the FDA.

Data availability

All data reported in this paper is provided in an accompanying "Supporting Data Values" file available for download. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Author contributions

H.E.C., S.M. and A.M.E researched data and ran the studies, contributed to the discussion and reviewed/edited manuscript; F.B. and C.D.M. supervised the mathematical modeling, contributed to the discussion and reviewed/edited manuscript; S.M.T., M.J., C.J.F. and J.C.A.

placed the catheters necessary to complete the study, contributed to the discussion and reviewed/edited manuscript; K.R.B. supervised the statistical analyses; M.D.J. and K.S.N. contributed to the design and analysis of the study, contributed to the discussion and reviewed/edited manuscript; A.V. designed the study, oversaw its conduct, researched data and wrote the first draft of the manuscript. Dr. Adrian Vella is the guarantor of this work and, as such, has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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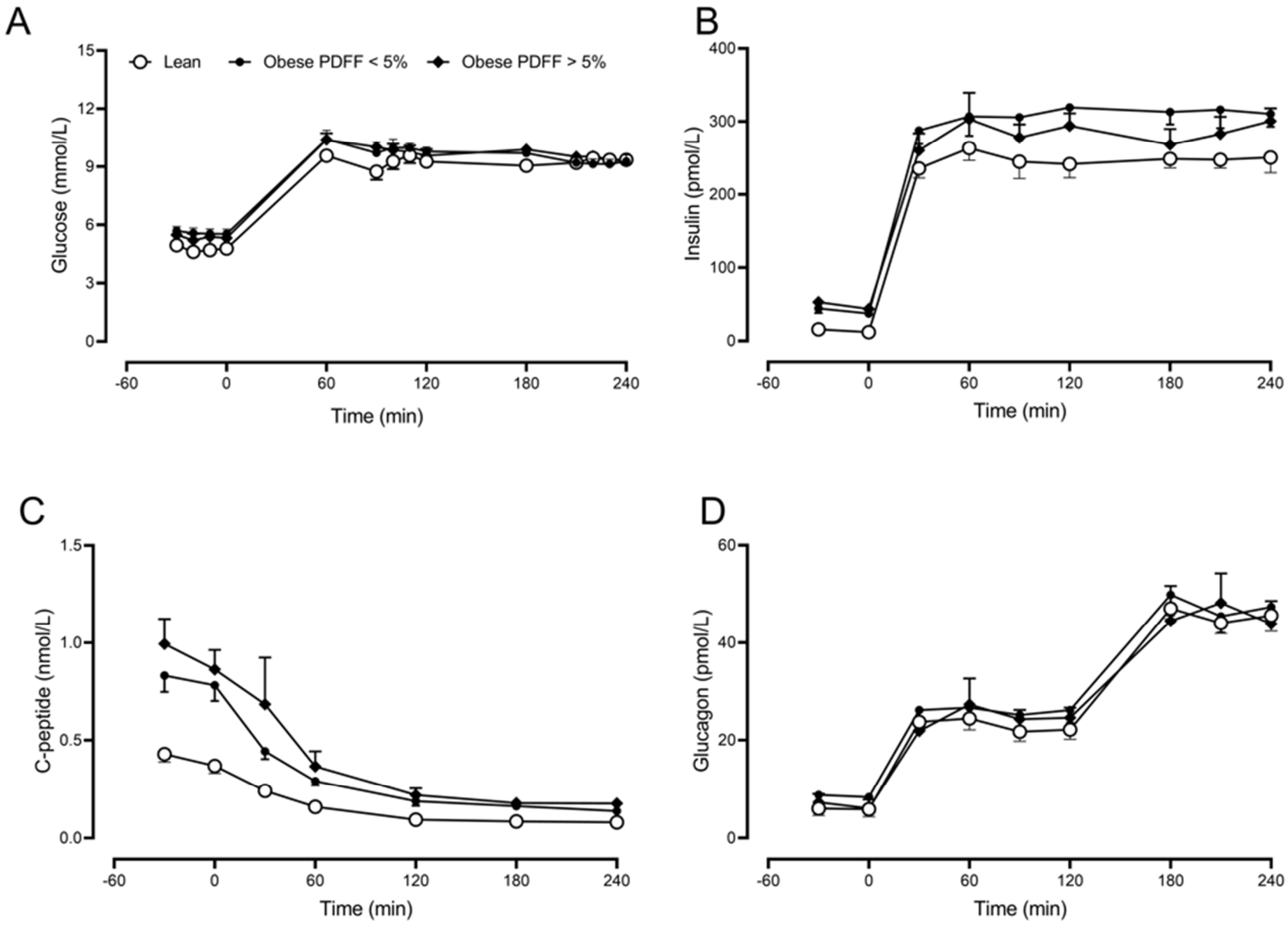
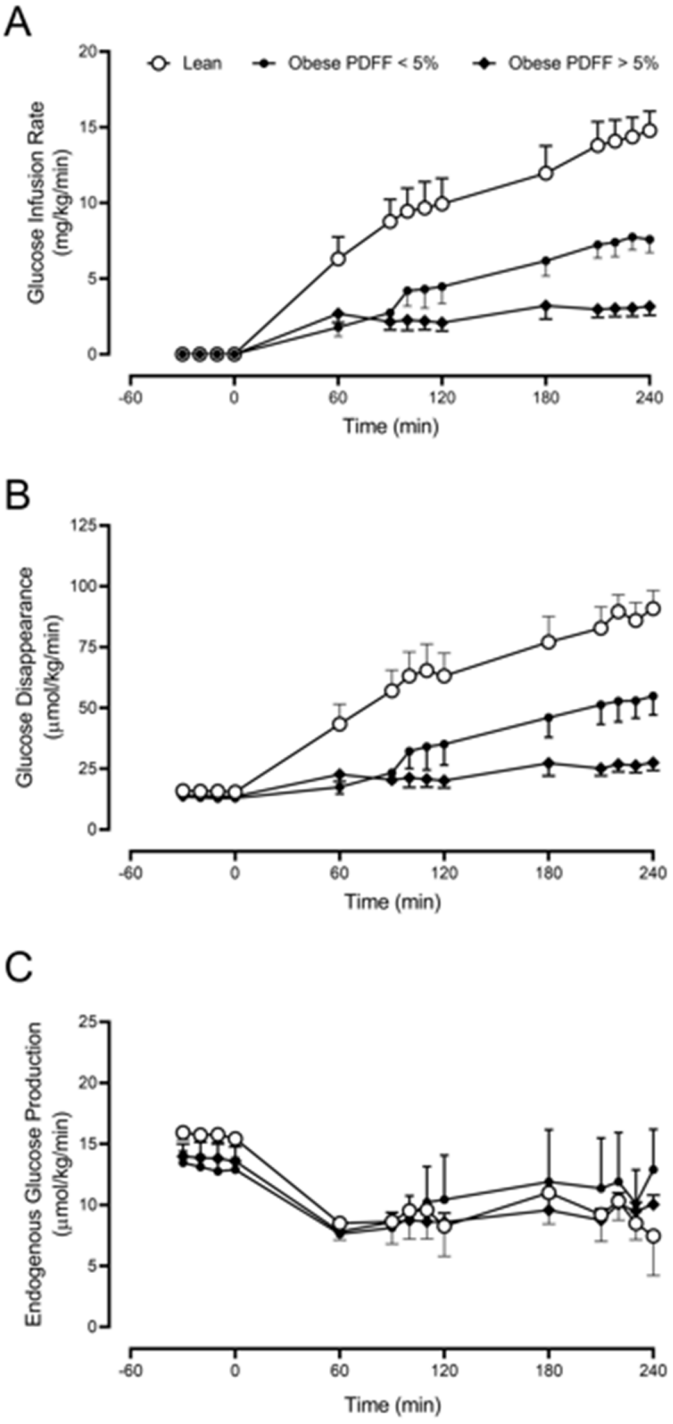
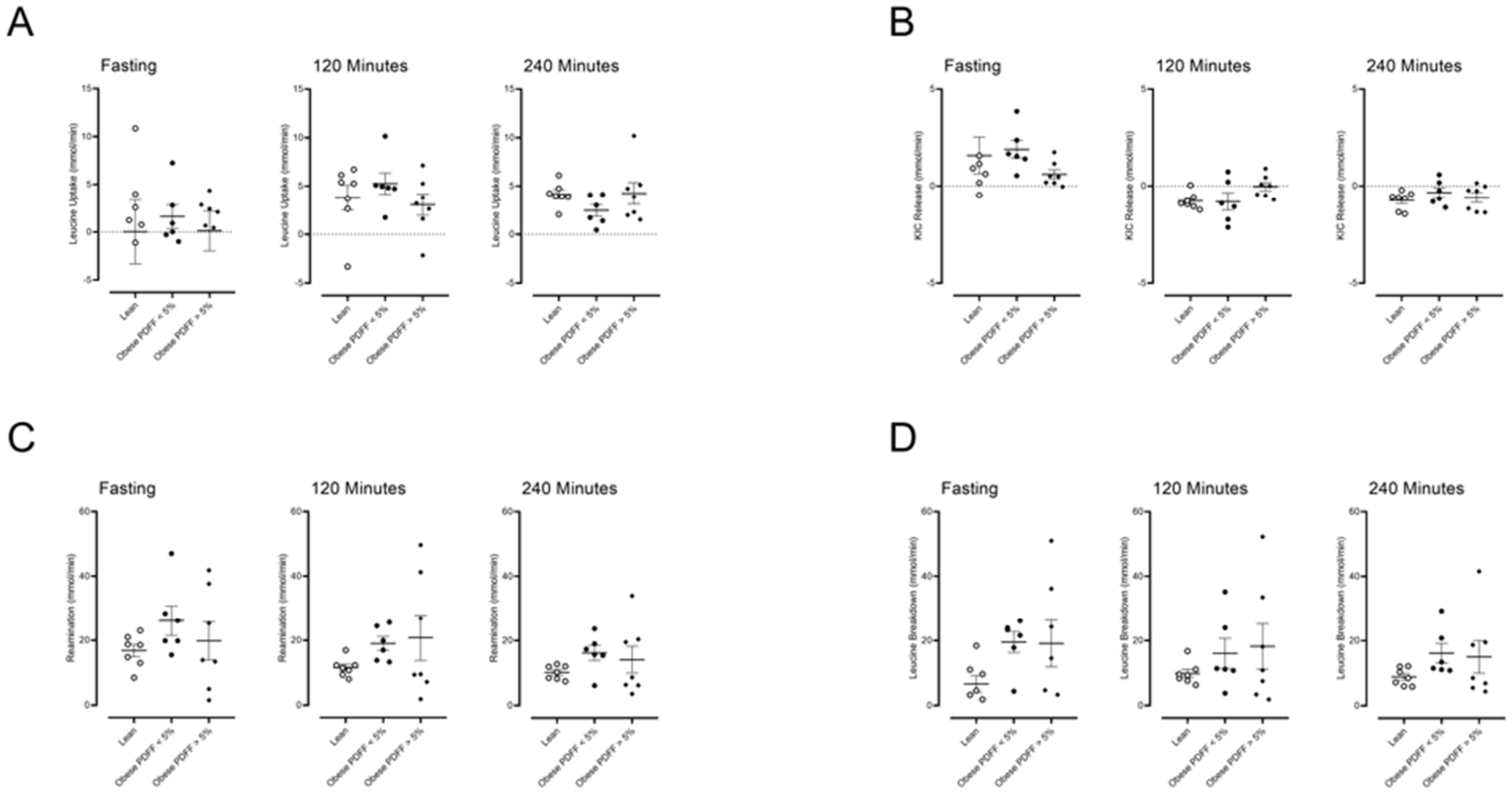
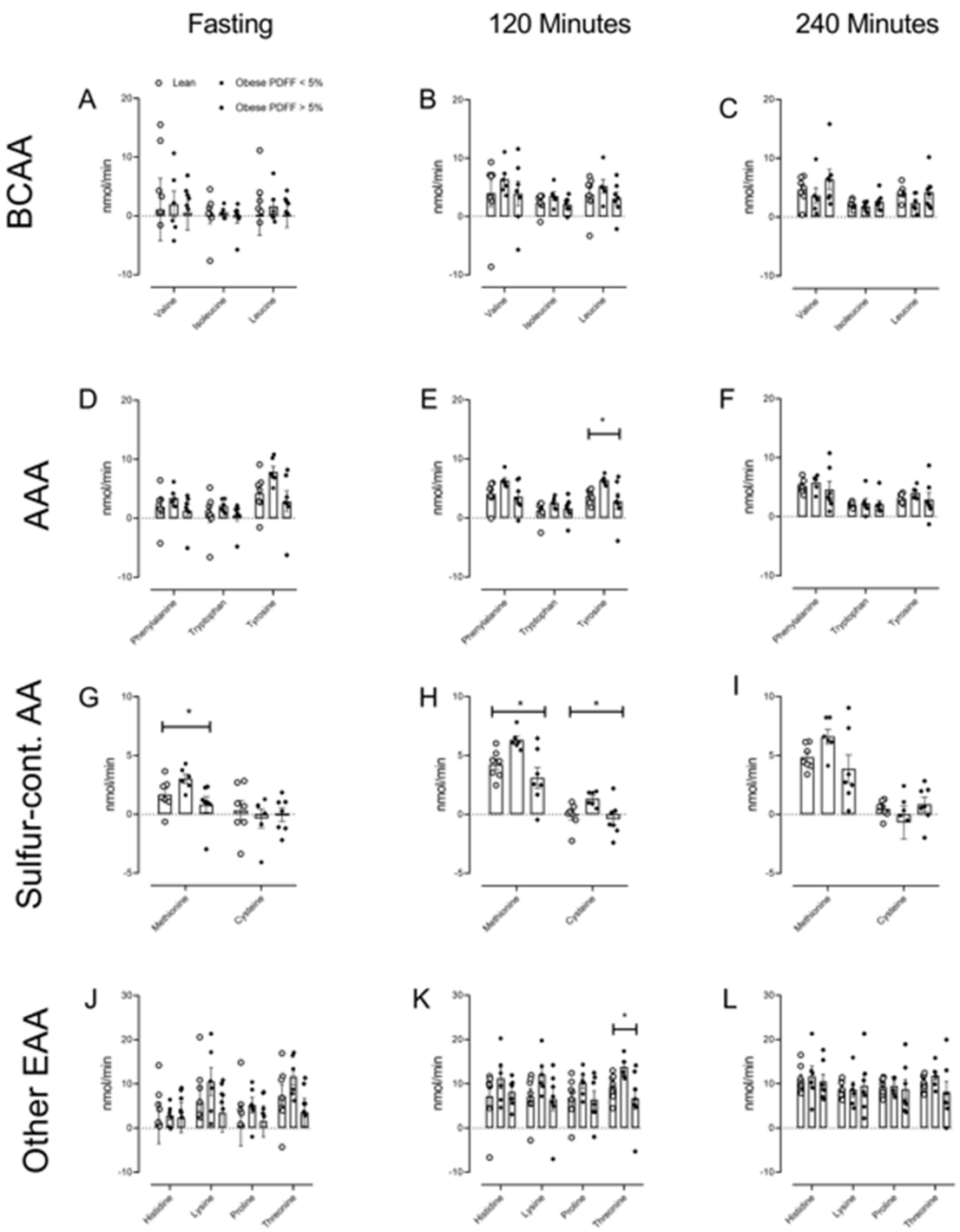
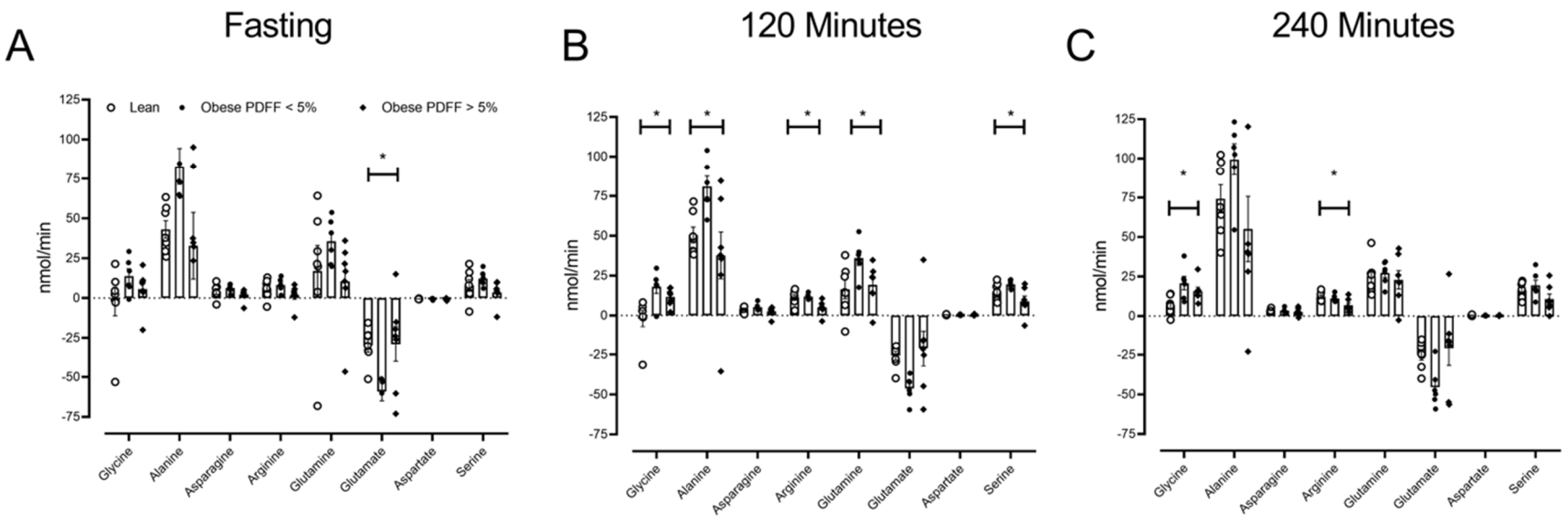

A
Lean
Obese PDFF < 5%
Obese PDFF > 5%
Glucose (mmol/L)
15
12
9
6
3
0
-60
0
60
120
180
240
Time (min)
B
Insulin (pmol/L)
400
300
200
100
0
Time (min)
C
C-peptide (nmol/L)
1.5
1.0
0.5
0.0
Time (min)
D
Glucagon (pmol/L)
60
40
20
0
Time (min)


A
Lean
Obese PDFF < 5%
Obese PDFF > 5%
Glucose Infusion Rate (mg/kg/min)
Time (min)
B
Glucose Disappearance (µmol/kg/min)
Time (min)
C
Endogenous Glucose Production (µmol/kg/min)
Time (min)


A
Fasting
120 Minutes
240 Minutes
Leucine Uptake (mmol/min)
B
Fasting
120 Minutes
240 Minutes
KIC Release (mmol/min)
C
Fasting
120 Minutes
240 Minutes
Reamination (mmol/min)
D
Fasting
120 Minutes
240 Minutes
Leucine Breakdown (mmol/min)
Lean
Obese PDFF < 5%
Obese PDFF > 5%


Fasting
120 Minutes
240 Minutes
BCAA
AAA
Sulfur-cont. AA
Other EAA
Lean
Obese PDFF < 5%
Obese PDFF > 5%
A
B
C
D
E
F
G
H
I
J
K
L
nmol/min
Valine
Isoleucine
Leucine
Phenylalanine
Tryptophan
Tyrosine
Methionine
Cysteine
Histidine
Lysine
Proline
Threonine


A
Fasting
B
120 Minutes
C
240 Minutes
Lean
Obese PDFF < 5%
Obese PDFF > 5%
nmol/min
125
100
75
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Asparagine
Arginine
Glutamine
Glutamate
Aspartate
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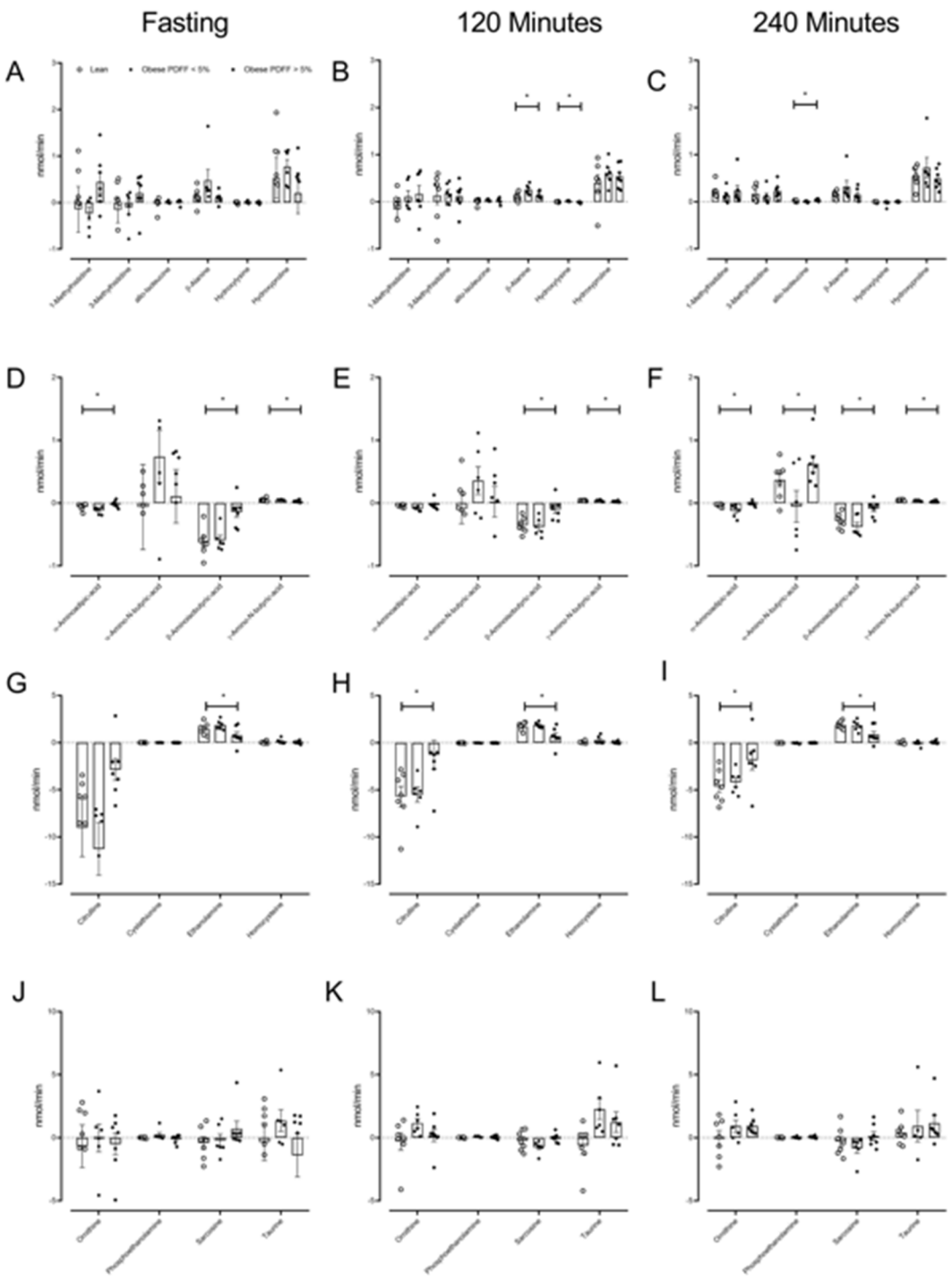
Fasting
120 Minutes
240 Minutes
A
B
C
D
E
F
G
H
I
J
K
L
Lean
Obese PDFF < 5%
Obese PDFF > 5%
nmol/min

Figure Legends

Figure 1. The mean (± SEM) Glucose (Panel A), Insulin (Panel B), C-peptide (Panel C) and Glucagon (Panel D) concentrations during fasting and then during the hyperglycemic clamp, for lean subjects (○), obese subjects with a PDFF < 5% (●) and obese subjects with a PDFF > 5% (♦). PDFF = Proton Density Fat Fraction. *n* = 7 in the Lean group, *n* = 6 in the obese group with PDFF < 5% and *n* = 7 in the obese group with PDFF > 5%.

Figure 2. The mean (± SEM) Glucose (Panel A), Insulin (Panel B), C-peptide (Panel C) and Glucagon (Panel D) concentrations during fasting and then during the hyperglycemic clamp, for lean subjects (○), obese subjects with a PDFF < 5% (●) and obese subjects with a PDFF > 5% (♦). PDFF = Proton Density Fat Fraction. *n* = 7 in the Lean group, *n* = 6 in the obese group with PDFF < 5% and *n* = 7 in the obese group with PDFF > 5%.

Figure 3. The mean (± SEM) together with the individual values of leucine uptake (Panel A), KIC release (Panel B), leucine reamination (Panel C) and leucine breakdown (Panel D) in lean subjects (○), obese subjects with a PDFF < 5% (●) and obese subjects with a PDFF > 5% (♦). KIC = α-keto-isocaproic acid; PDFF = Proton Density Fat Fraction. *n* = 7 in the Lean group, *n* = 6 in the obese group with PDFF < 5% and *n* = 7 in the obese group with PDFF > 5%.

Figure 4. The mean (± SEM) together with the individual values of splanchnic balance for branched-chain amino acids (BCAA), aromatic amino acids (AAA), sulfur-containing amino acids (Sulfur-cont. AA) and other essential amino acids (Other EAA), measured in lean subjects (○), obese subjects with a PDFF < 5% (●) and obese subjects with a PDFF > 5% (♦) during fasting (Panels A, D, G, J), during intermediate (120 Minutes – Panels B, E, H, K) and during the high glucagon infusion rates (240 minutes – Panels C, F, I, L). * *p*-value <

0.05 for a one-way analysis of variance (ANOVA) test; PDFF = Proton Density Fat Fraction. n = 7 in the Lean group, n = 6 in the obese group with PDFF < 5% and n = 7 in the obese group with PDFF > 5%.

Figure 5. The mean (± SEM) together with the individual values of splanchnic balance for non-essential amino acids during fasting (Panel A), during intermediate (120 Minutes – Panel B) and during high glucagon infusion rates (240 minutes – Panel C) in lean subjects (○), obese subjects with a PDFF < 5% (●) and obese subjects with a PDFF > 5% (♦). * p-value < 0.05 for a one-way analysis of variance (ANOVA) test; PDFF = Proton Density Fat Fraction. n = 7 in the Lean group, n = 6 in the obese group with PDFF < 5% and n = 7 in the obese group with PDFF > 5%.

Figure 6. The mean (± SEM) together with the individual values of splanchnic balance for metabolites measured during fasting (Panels A, D, G, J), during intermediate (120 Minutes – Panels B, E, H, K) and high glucagon infusion rates (240 minutes – Panels C, F, I, L) in lean subjects (○), obese subjects with a PDFF < 5% (●) and obese subjects with a PDFF > 5% (♦). * p-value < 0.05 for a one-way analysis of variance (ANOVA) test; PDFF = Proton Density Fat Fraction. n = 7 in the Lean group, n = 6 in the obese group with PDFF < 5% and n = 7 in the obese group with PDFF > 5%.

Table 1: Participant Characteristics

	Lean	Obese PDFF < 5%	Obese PDFF > 5%	*P*-value
n	7	6	7	
Age (Years)	38 ± 4	52 ± 5	46 ± 5	0.79
Sex (M / F)	0 / 7	3 / 3	0 / 7	
Total Body Mass (Kg)	63 ± 2	101 ± 4*	87 ± 3*$^{\#}$	< 0.01
BMI (Kg/M^2)	23 ± 1	32 ± 1*	32 ± 2*	< 0.01
LBM (Kg)	42 ± 1	56 ± 3*	47 ± 2*$^{\#}$	< 0.01
PDFF (%)	1.8 ± 0.2	3.4 ± 0.4*	15.6 ± 2.9*$^{\#}$	< 0.01
Stiffness (KPa)	1.8 ± 0.1	2.1 ± 0.1	2.0 ± 0.1	0.06
LIC (mgFe/g Liver)	0.75 ± 0.03	1.00 ± 0.18	0.83 ± 0.02	0.20
HbA1c (%)	4.9 ± 0.1	5.1 ± 0.1	5.3 ± 0.1*	0.04
Fasting glucose (mmol/l)	4.4 ± 0.2	5.3 ± 0.2*	5.1 ± 0.2*	< 0.01
120-minute glucose (mmol/l)	7.1 ± 0.5	7.4 ± 0.5	7.9 ± 0.9	0.69
S_i (10^{-4} dl/kg/min per μU/ml)	24 ± 5	4 ± 1*	6 ± 2*	< 0.01
Φ (10^{-9} min^{-1})	13 ± 1	20 ± 1*	24 ± 2*	< 0.01
DI (10^{-14} dl/kg/min/pmol)	489 ± 96	123 ± 35*	206 ± 48*	< 0.01

Participant characteristics at the time of screening characterized by their weight and hepatic fat content as measured by the Proton Density Fat Fraction (PDFF) using Magnetic Resonance Elastography. BMI = Body Mass Index; LBM = Lean Body Mass; LIC = Liver Iron Content; S_i = Insulin Action; Φ = β-cell responsivity to glucose; DI = Disposition Index. Data represent Mean ± SEM. *p*-values represent results of a one-way analysis of variance (ANOVA) test. **Post-hoc* Tukey's test suggests a significant difference ($p < 0.05$) for the Obese groups vs. the Lean group. $^{\#}$*Post-hoc* Tukey's test suggests a significant difference ($p < 0.05$) for the Obese group with a PDFF < 5% vs. the Obese group with a PDFF > 5%.