

The Effects of Glucagon on Hepatic Metabolism

NCT05500586

23January2025

MAYO CLINIC

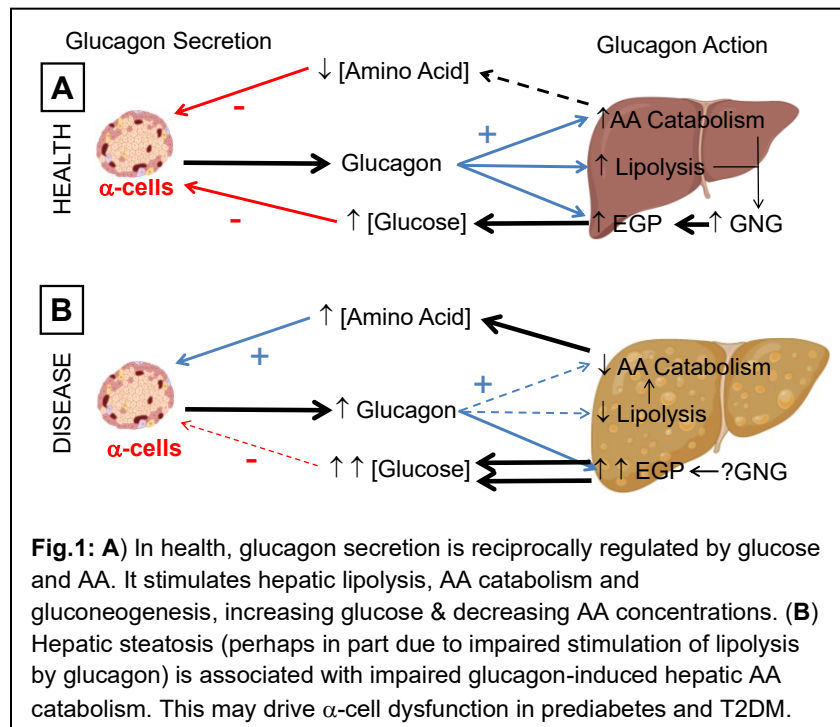
The Effects of Glucagon on Hepatic Metabolism – Version 7

IRB 22-000113: 1/23/2025

Dr. Adrian Vella

Abstract

We have shown that abnormal glucagon secretion occurs early in prediabetes and type 2 diabetes (T2DM)^{1,2} and predicts a decline in β -cell function³. It also contributes to hyperglycemia in T2DM^{4,5}. Glucagon stimulates amino acid (AA) catabolism^{7,8}. In turn, protein and AA stimulate glucagon secretion^{9,10}. Elevated AA^{11,12} & their metabolites¹³ are markers of T2DM risk. This is congruent with a recent report by Suppli et al. that glucagon fails to stimulate (hepatic) AA catabolism¹⁴ in obese humans with hepatic steatosis, itself a marker of T2DM risk¹⁵⁻¹⁷. Glucagon receptor antagonists or knockdown^{18,19} cause hepatic steatosis while agonists decrease it^{20,21}. Perry et al. have shown that glucagon activates hepatic adipose triglyceride lipase, promoting intra-hepatocyte triglyceride hydrolysis²². These observations suggest that defective glucagon action with respect to hepatic AA and lipid metabolism may contribute to T2DM pathogenesis while simultaneously exacerbating the impaired postprandial suppression of glucagon, increasing postprandial endogenous glucose production (EGP)^{4,5,23} (**Fig.1**). Do defects in hepatic glucagon action also apply to glucose metabolism? This experiment will examine how hepatic steatosis with and without T2DM alters glucagon's actions on hepatic metabolism.



I. Hypothesis and Specific Aims

It is estimated that 35-40% of the adult US population is obese²⁴. Obesity-related NonAlcoholic Fatty Liver Disease (NAFLD) is reported to have a prevalence of 24%²⁵. Both obesity and NAFLD¹⁵⁻¹⁷ increase risk of T2DM, a disease that increases morbidity and mortality while costing > \$170 billion/year²⁷, making prevention important. Recent evidence suggests that obese humans with NAFLD are resistant to the acute stimulation of hepatic AA catabolism by glucagon^{14,28-30}. This is important because α -cells

respond to elevated AA concentrations³³ by secreting glucagon^{10,34,35}. A failure to stimulate hepatic AA clearance could result in a vicious cycle of islet dysfunction (**Fig.1**). Indeed, elevated fasting AA¹¹ and AA metabolites e.g. α -amino adipic acid¹³ are markers of T2DM risk. In keeping with these observations, we reported that elevated fasting glucagon predicts a subsequent decline in β -cell function^{36,37} as quantified by the minimal model-derived Disposition Index³⁸.

Whether impaired postprandial glucagon suppression in prediabetes³⁸ and T2DM⁵ is an attempt to overcome resistance to glucagon's actions on hepatic AA catabolism, a defect in α -cell function, or a combination of both are important, unanswered questions. NAFLD is associated with T2DM risk¹⁵⁻¹⁷ and impaired insulin action³². Unfortunately, most of the obese subjects studied by Suppli et al. had NAFLD so that it is unclear if glucagon resistance is caused by obesity, hepatic steatosis or both¹⁴. **The experiments outlined will determine if glucagon's actions on hepatic amino acid catabolism and EGP interact with hepatic lipid metabolism**

in lean and obese subjects with and without T2DM (and with varying degrees of hepatic steatosis).

We will therefore address the following specific aim: -

Specific Aim: Determine glucagon's actions on hepatic amino acid, glucose and lipid metabolism in lean and obese subjects with and without T2DM and varying degrees of hepatic steatosis.

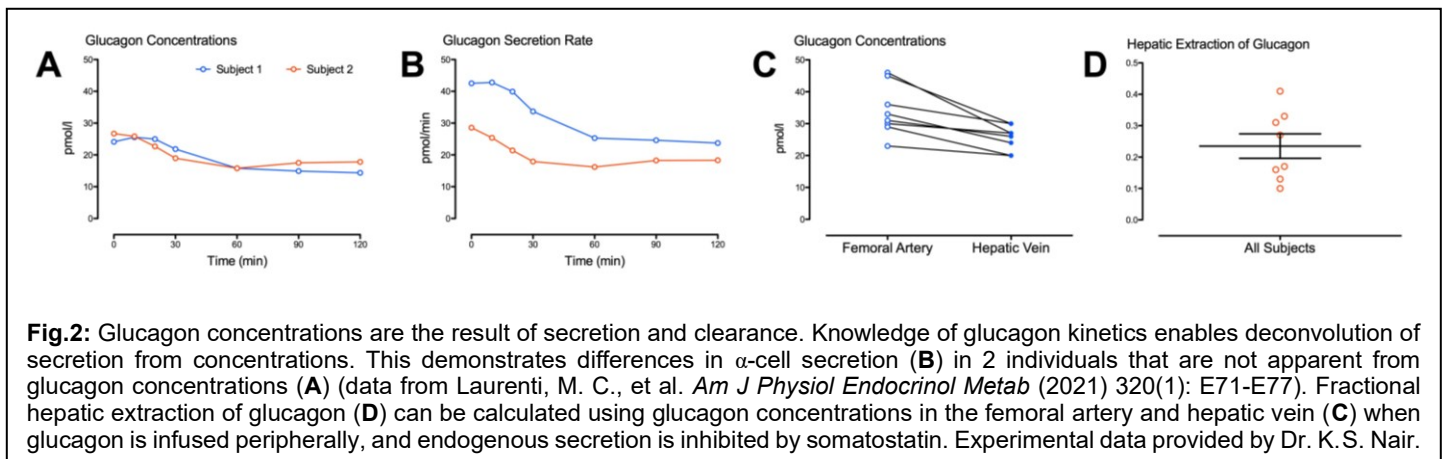
1° Hypothesis: Obesity impairs glucagon-induced hepatic transamination of leucine

2° Hypotheses:

- i. T2DM in obese subjects impairs glucagon-induced splanchnic extraction of amino acids to a greater extent than in obese individuals without T2DM
- ii. Obesity decreases the contribution of gluconeogenesis to EGP in response to glucagon
- iii. Obesity impairs splanchnic tissue palmitate uptake in response to glucagon

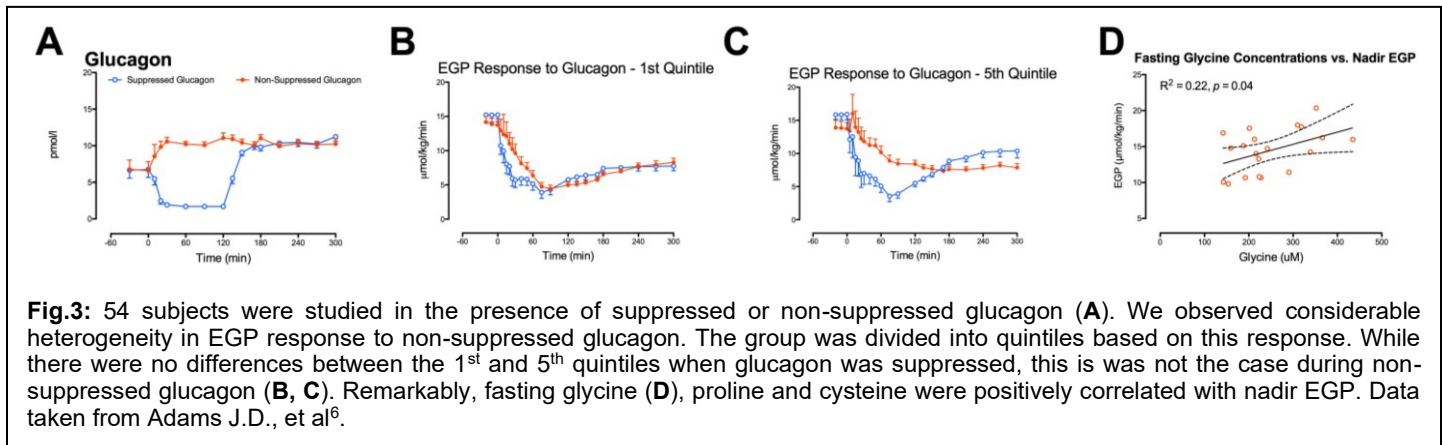
II. Background and Significance

T2DM and prediabetes are characterized by abnormal post-prandial suppression of glucagon, which contributes to postprandial hyperglycemia by increasing EGP^{4,5,39}. Although these effects are magnified by decreased and delayed insulin secretion, they are also apparent when insulin secretion is intact⁴. In rodents, altered glucagon signaling changes α -cell function and mass – an effect mediated by changes in circulating AA concentrations⁴⁰. Are the elevated concentrations of branched-chain AA and other AA metabolites¹¹ in T2DM a cause or an effect of global α -cell dysfunction (**Fig.1**)? Could altered glucagon signaling precipitate a vicious cycle resulting in T2DM? To measure glucagon secretion, as well as glucagon action, we have developed a population model of glucagon kinetics allowing us to deconvolve secretion from glucagon concentrations⁴¹ in a manner similar to Van Cauter's model for insulin secretion from C-peptide⁴². This (**Fig.2a,b**), together with hepatic glucagon extraction (**Fig.2c,d**) – to be measured in this experiment – enables better characterization of α -cell function in humans. Prior to this data (**Fig.2c,d**), glucagon extraction had only been measured in dogs^{43,44}.



In this experiment, we will measure hepatic extraction of AA^{7,8} in response to physiologic post-prandial concentrations of glucagon. In addition to changes in essential AA, we will also measure the extraction of other AAs, and their metabolites, across the liver. While measuring the effect of glucagon on AA metabolism, we will also examine how EGP²⁶ and hepatic lipid

metabolism⁴⁵ respond to these conditions. While early reports have focused on hepatic steatosis¹⁴, we will ascertain if its presence is necessary to alter glucagon's actions on hepatic



metabolism in obesity.

III. Progress Report and Preliminary Studies

- 1. Subject population:** Utilizing the Mayo Biobank as previously^{1,26,46}, subjects with varying degrees of steatosis^{47,48} have already been identified. In addition, >700 prior participants^{1,26,49,50}, will provide a suitable source of recruitment. The Olmsted County population (location of Rochester, MN) and surrounding communities are motivated to participate in research (~85% participated in a 2nd experiment²⁶ after a prior experiment¹).
- 2. Experience with study design / tracer methodology:** We have prior experience with the tracer methods^{26,45,51} utilized, clamp studies⁵²⁻⁵⁴ and hepatic vein catheterization⁵⁵⁻⁵⁷ (inc. clinical use⁵⁸⁻⁶¹).
- 3. Heterogeneity of EGP response to glucagon:** See Fig.3. We have published these data showing that heterogeneity of glucagon response is not explained by hepatic insulin action or anthropometric characteristics⁶.

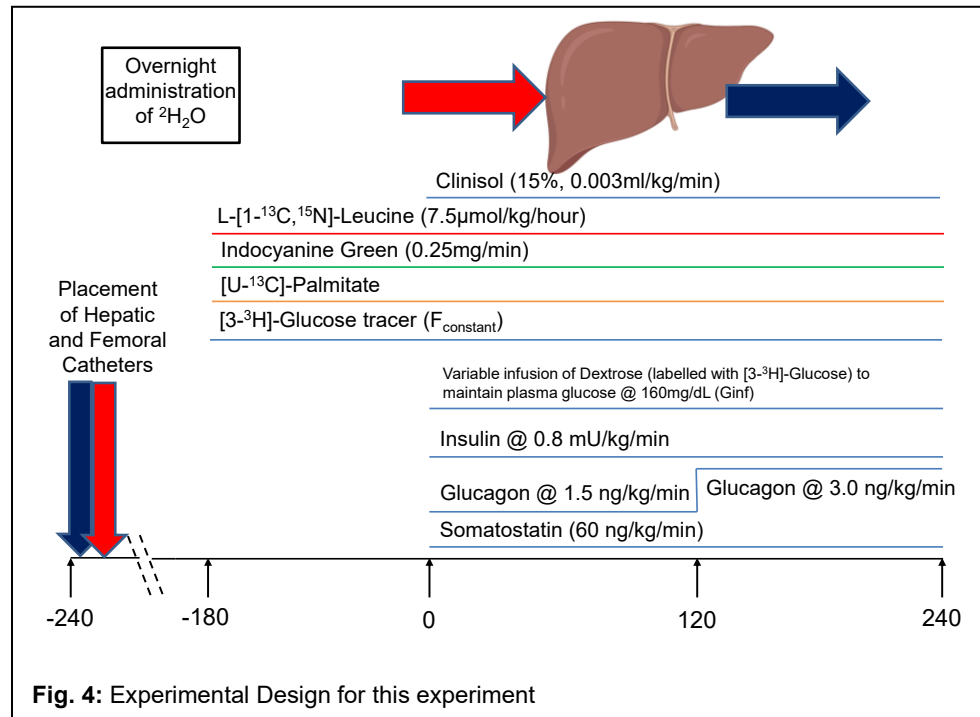
IV. Research Design and Methods

Study Subjects: After approval from the Mayo Clinic Institutional Review Board, we will recruit 20 weight-stable, subjects with type 2 diabetes mellitus (T2DM – BMI ≥ 28 Kg/M²) using local advertising, EHR search tools and the Mayo Biobank as previously¹ (if necessary). To ensure that at least half the cohort has hepatic steatosis we will use the Fatty Liver Index (FLI) to identify subjects at increased risk of hepatic steatosis⁶². We will exclude subjects with significant changes to their weight ($\geq 10\%$) or treatment regimen in the prior 3 months. They will have no micro- or macrovascular complications of T2DM.

In addition, we will recruit 20 age-, weight- and sex-matched subjects without diabetes using similar methods to ensure that at least half have hepatic steatosis.

Finally, we will also recruit an additional 20 age and sex matched subjects, without T2DM and with a BMI < 25Kg/M² (and no evidence of hepatic steatosis – see below) to serve as non-diabetic, non-obese controls. Because some lean individuals may have a BMI > 25, we will also recruit subjects who have low % body fat (<20% in males and < 30% in females).

The intention is that subjects also participate in a separate experiment to measure glucagon responsiveness to amino acids. The screening process will be common to both.



Screening Visit: After an overnight fast of 8 hours, subjects will present to the Clinical Research Trials Unit (CRTU) at approximately 0700. Subjects will provide written, informed consent. To ensure they are otherwise healthy, they will then undergo a history and physical examination; blood collection for complete

blood count, fasting glucose, HbA_{1c} , sodium, potassium, creatinine and urine collection to exclude pregnancy. An ECG will also be performed. The Minnesota Leisure-Time Physical Activity questionnaire will be used to assess habitual activity levels⁶³. The Alcohol Use Disorders Identification Test (AUDIT) questionnaire⁶⁴ will be used to screen for alcohol excess. Subjects consuming > 2 alcoholic drinks per day will be excluded (See Eligibility Criteria).

Body Composition: After informed written consent is obtained, % body composition will be measured using dual-energy X-ray absorptiometry (iDXA scanner; GE, Wauwatosa, WI). Liver fat will be measured by MRI using the proton density fat fraction (PDFF) technique⁶⁵. A cutoff $\geq 5.2\%$ implies $\geq$ Grade 1 steatosis. All subjects will be instructed to follow a weight-maintenance diet (55% carbohydrate, 30% fat and 15% protein) for at least 3 days prior to the study visit.

OGTT: After an 8 hour overnight fast, subjects without T2DM will ingest 75g glucose. Arterialized venous blood will be sampled over 2 hrs. at frequent intervals for glucose, insulin, C-peptide and glucagon in order to calculate indices of insulin action and insulin and glucagon secretion as previously described³⁶. Fasting and 2hr glucose will be used to categorize glucose tolerance status in non-diabetic subjects. This can be done during the screen visit or on a separate day.

Medication withdrawal: Participants on T2DM pharmacotherapy will discontinue this 3 weeks prior to the overnight study and then continue off medication for the duration of the study. While off medication, they will be asked to self-monitor their fasting glucose at least twice daily. Participants who take insulin or those likely to develop hyperglycemia (e.g. taking 2 or more oral medications), insulin therapy will be initiated under the close supervision of the PI. Subjects taking long and short acting insulin will be asked to discontinue long-acting insulin 48 hours prior to the study. During this period goal glucose is < 150mg/dL fasting and < 200mg/dL 2 hours after a meal..

Experimental Design (Fig.4): We will study 60 subjects on one occasion. All subjects will be admitted to the Mayo Clinic CRTU at approximately 1700 hours the evening before the study. Women who could become pregnant will have urine point of care pregnancy testing. Following ingestion of a standard 10 kcal/kg caffeine-free meal, subjects will fast overnight. Blood will then be sampled for baseline enrichment and $^2\text{H}_2\text{O}$ (1.5g/kg of body water) will be given in 3 doses at 2200, 2400 and 0200⁶⁶. They will consume sips of enriched water (2.5g of $^2\text{H}_2\text{O}$ per 500ml of water) when thirsty. The following morning at approximately 0530 (-270 min), a forearm vein will be cannulated to allow for reinfusion of 'waste' blood obtained at the time of arterial, venous and hepatic venous sampling. In addition, an ipsilateral and a contralateral forearm vein will be cannulated to allow for nutrient and hormone infusions. A urinary catheter will also be placed at this time. At approximately 0600 (-240 min) subjects will be moved to the radiology suite where a hepatic vein catheter will be placed via the femoral vein under fluoroscopic guidance^{67,68}. A femoral artery catheter will also be placed. Subjects will then return to the CRTU where at approximately 0700 (-180 min), a primed, (10 μCi prime, 0.1 $\mu\text{Ci}/\text{min}$ continuous) infusion containing trace amounts of glucose labeled with $[3\text{-}^3\text{H}]$ glucose will be started and continued till approximately 1000 (0 min). Subsequently, the infusion will be varied so as to mimic the anticipated pattern of fall of EGP to minimize changes in Specific Activity⁵¹. Infusion of L-[1- ^{13}C , ^{15}N]-Leucine (7.5 $\mu\text{mol}/\text{kg}$ prime, 7.5 $\mu\text{mol}/\text{kg}/\text{hour}$ continuous) will also commence at approximately 0700 and continue till the end of the study. Infusion of indocyanine green (0.25mg/min), and [U- ^{13}C]-palmitate (300 nmol/min continuous) will commence at 0900 (-60min) and in the case of palmitate continue till the end of the study. Indocyanine green will be infused from -60 to 0 min and then from 60 to 120 min and from 180 to 240 min. At approximately 1000 (0 min) another glucose infusion, also labeled with $[3\text{-}^3\text{H}]$ glucose will commence, and the infusion rate varied to produce peripheral glucose concentrations of $\sim 160\text{mg}/\text{dL}$. 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) will be infused at this time. Also, at 1000, insulin (0.8 mU/kg/min) and glucagon (1.5 ng/kg/min) will be infused together with somatostatin (60 ng/kg/min, to inhibit endogenous islet hormone secretion). The insulin infusion rate will be kept constant for the remainder of the study but at approximately 1200 (120 min) the glucagon infusion rate will be doubled to 3.0 ng/kg/min. Blood samples from the femoral vein, hepatic vein and femoral artery will be obtained at -30 to 0 (baseline); 90 to 120 (low glucagon); 210 to 240 min (high glucagon). At the end of the study approximately (1400 – 240 min) all vascular catheters will be removed. Site care will be performed as per institutional guidelines with the application of localized pressure at the sites of femoral vascular access. Participants will consume a late lunch but remain supine in bed till approximately 1800. If there are no site concerns, they will leave the CRTU once they are able to do so safely.

They will subsequently resume their prior glucose lowering medication.

Analytical Techniques: All blood will be immediately placed on ice, centrifuged at 4°C , separated and stored at -80°C until assay. Glucose will be measured using a Yellow Springs glucose analyzer. Glucagon will be measured using an ELISA (Mercodia, Winston-Salem, NC⁶⁹). C-peptide will be measured using EMD Millipore (Billerica, MA) reagents. Insulin will be measured using a chemiluminescence assay with reagents obtained from Beckman (Access Assay, Beckman, Chaska, MN). $[3\text{-}^3\text{H}]$ glucose specific activity will be measured by liquid scintillation counting following deproteinization⁷⁰. C2/C5 ratio: The method of Jones et al.⁷¹ will be used to measure and analyze deuterium enrichment on the 2nd and 5th carbon of plasma glucose. Indocyanine Green concentrations in the serum will be measured using a spectrophotometer at the 805 nm wavelength⁷². Plasma palmitate concentrations, free fatty acids (FFA) and [U- ^{13}C]palmitate enrichment will be measured by ultra-performance liquid chromatography-mass spectrometry as previously^{73,74}. Amino acids, their metabolites and acyl-carnitines will be measured using derivatized standards and samples analyzed on a triple quadrupole mass

spectrometer coupled with an Ultra Pressure Liquid Chromatography system as previously described⁷⁵. Blood gases will be measured as previously described⁷⁶.

Calculations: The rate of leucine catabolism to α -keto-isocaproic acid (α -KIC) will be calculated from α -KIC enrichment multiplied by tracer infusion rate during all three phases of the experiment. Leucine carbon flux and leucine nitrogen flux will be calculated by multiplying the rate of tracer infusion by the ratio of tracer enrichment to plasma enrichment⁷⁷. The hepatic extraction for a given amino acid will be calculated from the arterio-venous difference of [amino acid] across the liver, multiplied by splanchnic blood flow. Splanchnic plasma flow will be calculated by dividing the indocyanine green infusion rate by the arterial-hepatic venous concentration gradient of the dye. Leg plasma flow will be calculated by dividing the indocyanine green infusion rate by the arterial-femoral venous concentration gradient of the dye. Dividing the plasma flow by 1-hematocrit will provide blood flow⁷². Endogenous glucose production (EGP) and glucose disposal (Rd) will be calculated as before^{51,78}. The contribution of gluconeogenesis to EGP will be calculated by multiplying EGP by the C2/C5 ratio; glycogenolysis will be calculated by subtracting gluconeogenesis from EGP as before²⁶. In addition to fasting, peak and nadir hormone concentrations, we will calculate area above basal (AAB) or area under the curve (AUC) during the experiment using the trapezoidal rule⁷⁹. S_i and β -Cell responsivity indices will be calculated⁸⁰ from the plasma glucose, insulin and C-peptide concentrations during the screening OGTT⁸¹. Splanchnic (largely hepatic) palmitate extraction will be calculated by multiplying splanchnic plasma flow by $(([\text{Palmitate}]_{\text{HA}} \times \text{Enrichment}_{\text{HA}}) - ([\text{Palmitate}]_{\text{HV}} \times \text{Enrichment}_{\text{HV}}))$ during the baseline, low and high glucagon steps of the experiment⁸² (HA = Hepatic Artery; HV = Hepatic Vein). Steady-state plasma FFA concentrations and enrichment together with leg and splanchnic plasma flow will be used to calculate regional FFA uptake and release⁴⁵. The contribution of visceral lipolysis to hepatic FFA delivery will be calculated using the values of splanchnic FFA release multiplied by the fractional reduction in hepatic vein FFA enrichment relative to hepatic artery FFA enrichment as before^{82,83}.

Statistical Analysis: One-way ANOVA will examine differences in 1° and 2° endpoints necessary to test our hypotheses (See **Table 1**) between lean, obese and T2DM groups. The outcome measures will be compared at baseline, low glucagon and high glucagon conditions. A Tukey's *post-hoc* test will be used to determine between-group differences when appropriate. A paired, 2-way student *t*-test (parametric) or Wilcoxon matched-pairs signed rank test (non-parametric) will examine temporal changes (e.g.: baseline to low glucagon) within each group. Relationships of the outcome measures with selected participant characteristics (e.g.: age, sex and weight; liver fat content) will be tested by regression analysis^{3,38}. As before⁴⁹, where necessary to correct for multiple testing in our 2° hypotheses, we will use the Benjamini-Hochberg approach⁸⁴. Data will be presented as mean $\pm$ SEM. All statistical tests will be two-sided and a *p*-value < 0.05 will be considered significant.

n = 20 / 20 / 20	Mean ± SD	Detectable between group difference	Detectable within group difference
1° - Leucine transamination (μmol/kg/min) ⁸	40 ± 6	6 (15%)	4 (10%)
2° - (i) Amino acid concentrations (μmol/L) ¹⁴	2666 ± 209	190 (7%)	138 (5%)
Leucine (mg/dL) ⁸	235 ± 47	43 (18%)	31 (13%)
2° - (ii) Gluconeogenesis (μmol/kg/min) ²⁶	7.4 ± 2.7	1.4 (18%)	1.8 (24%)
EGP (μmol/kg/min) ²⁶	13.9 ± 2.7	2.5 (18%)	1.8 (13%)
2° - (iii) Palmitate (μmol/min) ³¹	81 ± 30	15 (19%)	16 (20%)
Intra-Hepatic TG content (%) ³²	2.3 ± 1.0	1 (40%)	0.7 (30%)

Table 1: Means ± SD used to estimate power

Power Calculation:
Assuming similar variation to prior experiments, $n=20/20/20$ would provide 80% power, to detect small differences between (Table 1, Col. 3) and within groups (Table 1, Col.

4). For other analyses e.g. changes attributable to high vs. low glucagon, an ANCOVA model, incorporating respective baseline values and adjusting for confounding variables (age, weight and sex), will provide similar power to detect even smaller differences.

Interpretation: We will use L-[1-¹³C, ¹⁵N]-Leucine to determine the rate of transamination (loss of ¹⁵N) and conversion to α-keto-isocaproic acid (α-KIC) by examining their relative enrichment across the splanchnic bed. In this way we can directly measure the first step of leucine catabolism, previously shown to be stimulated by glucagon^{7,8}. This occurs in the liver in the presence of insulin concentrations similar to those proposed in our experiment⁷⁷. We can also determine whether a defect in glucagon action occurs distal to transamination (α-KIC to Isovaleryl-CoA). If glucagon's action on leucine transamination is impaired in people with obesity regardless of the presence or absence of T2DM, we will accept our 1° hypothesis. However, we will also use the AA panel⁷⁵ developed in our metabolomics core to simultaneously measure 80 analytes (Aas and their metabolites – Fig.3d) during fasting and in response to physiologic postprandial glucagon concentrations across the three groups studied. Our experimental design enables measurement of the arterio-venous difference of a given [AA] across the liver, multiplied by splanchnic blood flow to compare clearance between groups⁷². We will also use similar techniques to measure the rate of ureagenesis as a surrogate of AA catabolism. Our focus is on essential AA, although we will examine splanchnic balance for all AA and their metabolites as detected by our panel⁷⁵. We will compare the absolute and incremental changes in response to rising glucagon concentrations, thereby accounting for baseline differences in fasting glucagon concentrations across the three groups³⁸. It is possible that differences in hepatic extraction of a given AA are only apparent in the fasting state, implying that defects in glucagon action are subtle and overcome by glucagon concentrations encountered in the early postprandial period. More marked defects in glucagon action would be expected to result in decreased hepatic extraction during the lower glucagon infusion alone, or both, depending on the severity of the defect. We will also undertake regression analyses (adjusted for covariates as described above) comparing the rate of AA extraction with hepatic fat content to determine the contribution, if any, to hepatic glucagon action.

On the other hand, if a defect in glucagon-induced hepatic transamination of leucine is more marked in people with T2DM, compared to weight-matched controls, this would imply that defects in glucagon's hepatic actions¹⁴ occur late in the evolution of prediabetes and T2DM and are independent of weight. We would accept our 2° hypothesis (i). We will subsequently examine extraction of splanchnic AA for differences attributable to T2DM. Because defects in α-cell secretion occur early in prediabetes¹, this would suggest that a defect in glucagon action is a later event (perhaps an adaptation to higher glucagon concentrations). In addition to hepatic fat content, we will explore whether Si (measured using screening OGTT), glycemic control and β-cell function are associated with the hepatic actions of glucagon⁴⁶. Of course, given the experimental design we will be able to examine if there are reciprocal changes in AA

concentrations across the lower limbs^{45,77}. Data on the effect of AA on α -cell secretion (a separate experiment) will enable exploratory analyses of the relationship (if any) between α -cell function and hepatic glucagon action.

Abnormal postprandial secretion of glucagon contributes to post-prandial hyperglycemia in prediabetes and T2DM by increasing EGP^{4,5}. However, impaired glucagon actions on AA catabolism would be expected to decrease the supply of gluconeogenic precursors⁸⁵. To test our 2° hypothesis (ii), we will compare the % contribution of gluconeogenesis to EGP^{26,55} in response to physiologic elevation of glucagon. We will also examine how the gluconeogenic and overall EGP responses to glucagon compare with changes in hepatic extraction of essential AA in response to glucagon. After testing our 2° hypothesis (ii), we will examine whether T2DM alters the effect of glucagon on gluconeogenesis compared to weight-matched obese subjects. It is possible that in T2DM, although absolute rates are increased compared to controls, the % rise from an (elevated) baseline is blunted due to impaired glucagon action. We will also explore whether hepatic fat content modulates the response of EGP and gluconeogenesis to glucagon.

Glucagon receptor antagonism¹⁸ or knockdown¹⁹ leads to abnormal liver function tests and hepatic steatosis. Conversely agonists²⁰ ($\pm$ GLP-1 receptor agonists) increase energy expenditure²¹. Recent evidence suggests that glucagon increases hepatic adipose triglyceride lipase activity and hepatic fatty acid oxidation²². It is unknown whether hepatic steatosis is a manifestation of impaired response to glucagon. We hypothesize that glucagon-stimulated hepatocyte lipolysis will be accompanied by one or more of: increased splanchnic fatty acid oxidation, increased escape of acyl-carnitines because of incomplete fatty acid oxidation, increased splanchnic extraction of plasma palmitate. To test our 2° hypothesis (iii), we will use established techniques^{45,82,83,86} to measure the effects of glucagon on the splanchnic extraction of palmitate. If glucagon-stimulated hepatocyte lipolysis results in a compensatory increase in splanchnic fatty acid uptake, we hypothesize that the increase in the splanchnic extraction of palmitate will be reduced in obese and T2DM subjects. If splanchnic fatty acid oxidation is impaired, we expect a lesser decrease in the splanchnic RQ (determined by arterial and hepatic vein blood gases) compared to lean controls. We expect greater fat oxidation because this is needed to provide energy for the increased gluconeogenesis caused by glucagon²². If there is less net splanchnic release of acyl-carnitines⁸⁷ and β -hydroxybutyrate in obese / T2DM subjects compared to lean controls we would take this as evidence for an impaired response of hepatic lipid metabolism to glucagon. If none of these responses are observed, we will measure whether glucagon increases splanchnic release of VLDL-triglycerides by measuring their concentrations in stored serum from the hepatic vein as a marker of hepatic unloading of fatty acids. Changes in splanchnic oxygen uptake will be used as our index of thermogenic effects of glucagon, which can be used to place the other measures⁸⁸ in context. We will also assess whether the relative responses of splanchnic lipid metabolism to glucagon vary as a function of hepatic fat content and also with the responses of AA and carbohydrate metabolism^{89,90} to glucagon, thereby ascertaining if these defects occur in concert. If we accept our 2° hypothesis (iii), we will then examine if T2DM alters the effect of glucagon on lipid metabolism compared to weight-matched obese subjects. If none of these parameters change in response to glucagon, we will interpret this as evidence against the concept that there is a powerful effect of glucagon on hepatic lipid metabolism in lean, obese and obese T2DM humans.

A limitation to our current use of deconvolution to calculate glucagon secretion rates from glucagon concentrations (**Fig.2a,b**) is that glucagon extraction as it traverses the liver is unknown⁴¹. Experiments in dogs have suggested that this is relatively low (~10-15%) but is altered by diet and disease^{43,44,91}. In the present experiments we will measure glucagon clearance across the liver (**Fig.2c,d**). This information will be used to refine our current model of glucagon kinetics⁴¹ in a fashion similar to that for insulin secretion⁴².

Limitations / Precautions/ Additional or Alternative Analyses:

- i. At present we are proposing to use a single AA tracer because of prior evidence that glucagon increases Leucine catabolism^{7,8}. However, if early data suggests other AA may be of interest we would consider use of additional, specific, tracers.
- ii. The cross-sectional design of the experiment means that we cannot determine the relative importance of insulin and glucagon action on hepatic metabolism. Other experiments will examine the effects of weight loss by studying subjects with T2DM after caloric restriction, an intervention known to improve insulin action⁹² (and decrease hepatic fat)⁹³.

V. Human Subjects

Recruitment of Women, Minorities and Children For the proposed study, there are no exclusions based on gender, race or ethnicity. Longitudinal access to the laboratory data and medical records of the Mayo Clinic Medical Record has previously allowed identification and recruitment of subjects with impaired fasting glucose or impaired glucose tolerance or diabetes. In this research proposal, we plan to continue using the Mayo Clinic Biobank ($n \sim 50,000$) where nondiabetic subjects will be identified, contacted and invited to participate in our studies. This strategy has served us well in the past. In our experience, women are recruited with a greater frequency than men in these studies. Overall, based on enrollment figures from our ongoing cohort and the Biobank it is anticipated that ~ 60% of the subjects presenting for a screening visit will be female. Likewise, based on our current cohort, we predict that 55% of subjects will be Caucasian, 10% will be African American, 17% will be Asian, and 15% will be of Hispanic ethnicity.

Recruitment of Minorities The majority of residents in Rochester, Minnesota and surrounding counties are White; recent population estimates from over the past decade have indicated that minorities make up a significantly larger segment of our communities. According to census area data such cultural groups as the Somalis, Hispanic/ Latinos, and South East Asians have increased the percentage of minorities living in Rochester and the surrounding counties from 3% in 1990 to nearly 10% in 2000. We are actively working with the minority outreach specialists in the CTSA and representatives from the local minority populations, to develop community-based strategies for recruitment of minorities at Mayo Clinic Rochester. In addition, to obtaining minority participation in research, the investigator and his team are committed to providing continuing diabetes care and counseling to these patients. Such initiatives include participation in the Minority Outreach Specialist's cultural events that combine health, education and community-based resources at community sites chosen by the multicultural leaders. These events are held every quarter and provide facilities for examination and point of care testing. Interpreters from the language department are on hand to help with this process. Culturally sensitive education materials in various languages have been produced and are available. Other activities include participation in a health awareness program and a question / answer session transmitted on the local community TV channel.

Plan to Test for Differences in Effect among Sex or Ethnic Groups We plan to match the obese and T2DM cohorts for age, sex and weight. The lean cohort will be matched for age and sex. We will also attempt to balance sex in each subgroup. This will enable us to conduct analyses which can detect significant differences in study endpoints attributable to these variables.

In addition, we plan to undertake exploratory analyses of the effects of our interventions on ethnic subgroups. However, it is unlikely that we will be adequately powered to perform more than exploratory analyses despite our recruitment efforts.

Pregnant women and unborn fetuses The proposed studies utilize radiation for the purposes of body composition measurement, fluoroscopy, and tritiated glucose will be infused at low dosage to measure glucose metabolism. The radiation safety committee approves radiation use in clinical studies after appropriate dosimetry has been undertaken. All women of childbearing potential will have a negative pregnancy test within 48 hours prior to the study. Because of the potential effect of pregnancy on insulin secretion and glucose metabolism, pregnant females will not be enrolled.

Recruitment of Children and neonates Individuals younger than 25 will not be recruited for this study. This is because prediabetes and type 2 diabetes are typically diseases with onset in adult life. In individuals with diabetes under the age of 25 there is a strong possibility that atypical forms of diabetes are present. Indeed, most people presenting with diabetes in Olmsted County below age 25 have type 1 not type 2 diabetes.

Recruitment of Prisoners: Prisoners will not be recruited for these studies.

Eligibility

After approval from the Mayo Clinic Institutional Review Board, we will utilize a combination of intramural and extramural advertising as well as EHR search tools, the Mayo Biobank (if necessary). And from a list of individuals who previously expressed interest in participation. Interested subjects will then be contacted and invited to come to the Clinical Research Trials Unit for a screening visit.

Inclusion criteria – Obese Subjects with Type 2 Diabetes

We will recruit up to 20 weight-stable, subjects with type 2 diabetes who have a BMI ≥ 28 Kg/M² and are managed by diet alone or a combination of metformin and / or sulfonylurea. Subjects must be willing to stop glucose-lowering medication for 3 weeks prior to participation and self-monitor their fasting glucose during this time. We will ensure that half of the subjects recruited will have $>5\%$ liver fat content as determined by MRI using the proton density fat fraction (PDFF) technique.

We will also exclude subjects with treatment change or significant weight change ($\geq 10\%$) in the prior 3 months. Subjects will have no history of micro- or macrovascular complications of T2DM.

Exclusion Criteria – Obese Subjects with Type 2 Diabetes: -

- (a) Age < 25 or > 65 years (to avoid studying subjects who could have latent type 1 diabetes, or the effects of age extremes in subjects with normal or impaired fasting glucose).
- (b) HbA_{1c} $\geq 8.5\%$
- (c) BMI ≤ 28 Kg/M²
- (d) For female subjects: positive pregnancy test at the time of enrollment or study
- (e) History of prior upper abdominal surgery such as adjustable gastric banding, pyloroplasty and vagotomy.
- (f) Active systemic illness or malignancy.
- (g) Symptomatic macrovascular or microvascular disease.
- (h) Known contraindications to MRI
- (i) Hematocrit $< 35\%$
- (j) History of uncontrolled thyroid disease
- (k) Consumption of > 2 alcohol drinks per day or > 14 per week or a positive AUDIT questionnaire.
- (l) Allergy to iodine
- (m) Thrombocytopenia, history of bleeding disorder or use of anticoagulation

Inclusion criteria – Obese subjects without Type 2 Diabetes

We will recruit up to 20 weight-stable, obese subjects who do not have type 2 diabetes but are age, sex and weight-matched ($\text{BMI} \geq 28 \text{ Kg/M}^2$) with subjects who have T2DM. We will ensure that half of the subjects recruited will have $>5\%$ liver fat content, as determined by MRI using the proton density fat fraction (PDFF) technique.

Exclusion Criteria – Obese subjects without Type 2 Diabetes: -

- (a) Age < 25 or > 65 years (match subjects with T2DM).
- (b) $\text{HbA}_{1c} \geq 6.5\%$
- (c) $\text{BMI} \leq 28 \text{ Kg/M}^2$
- (d) Use of any glucose-lowering agents
- (e) For female subjects: positive pregnancy test at the time of enrollment or study
- (f) History of prior upper abdominal surgery such as adjustable gastric banding, pyloroplasty and vagotomy.
- (g) Active systemic illness or malignancy.
- (h) Symptomatic macrovascular or microvascular disease.
- (i) Known contraindications to MRI
- (j) Hematocrit $< 35\%$
- (k) History of uncontrolled thyroid disease.
- (l) Consumption of > 2 alcohol drinks per day or > 14 per week or a positive AUDIT questionnaire.
- (m) Allergy to iodine
- (n) Thrombocytopenia, history of bleeding disorder or use of anticoagulation

Inclusion criteria – Lean subjects without Diabetes

We will recruit up to 20 weight-stable, subjects who do not have type 2 diabetes but are age and sex but are not weight-matched ($\text{BMI} \leq 25 \text{ Kg/M}^2$) with subjects who have T2DM.

Exclusion Criteria – Lean subjects without Diabetes: -

- (a) Age < 25 or > 65 years (match subjects with T2DM).
- (b) $\text{HbA}_{1c} \geq 6.5\%$
- (c) $\text{BMI} \geq 25 \text{ Kg/M}^2$ unless % body fat is $< 20\%$ in males and $< 30\%$ in females.
- (d) Use of any glucose-lowering agents
- (e) For female subjects: positive pregnancy test at the time of enrollment or study
- (f) History of prior upper abdominal surgery such as adjustable gastric banding, pyloroplasty and vagotomy.
- (g) Active systemic illness or malignancy.
- (h) Symptomatic macrovascular or microvascular disease.
- (i) Known contraindications to MRI
- (j) Hematocrit $< 35\%$
- (k) History of uncontrolled thyroid disease.
- (l) Consumption of > 2 alcohol drinks per day or > 14 per week or a positive AUDIT questionnaire.
- (m) Liver fat content $\geq 5\%$ as determined by MRI using the proton density fat fraction (PDFF) technique.
- (n) Allergy to iodine
- (o) Thrombocytopenia, history of bleeding disorder or use of anticoagulation

Retention

Subjects will be in the study for a relatively short period of time. After providing informed written consent and completing the screening process, the inpatient study will follow... During that time there will be the opportunity for contact and follow-up with the study team. It is our practice to confirm participation with the volunteer a week and then 48 hours prior to study.

Population: Subjects will be recruited from the Mayo Clinic and from Olmsted County residents and surrounding counties, who have previously indicated a willingness to participate in research – and who meet entry criteria. The racial composition of the county is outlined in the table below (%) using data from the 2000 population census. No children or prisoners will be recruited.

	American Indian or Alaskan Native	Asian or Pacific Islander	Black, not of Hispanic Origin	Hispanic	White, not of Hispanic Origin	Other	Total
Female	0.15	2.8	1.8	1.5	43.8	0	51.5
Male	0.15	2.8	1.8	1.5	43.7	0	48.5
Total	0.3	5.6	3.6	3.0	87.5	0	100.0

Research Materials: We will be obtaining blood samples to measure hormone and glucose concentrations in response to rising glucose concentrations.

Protection of Human Research Participants

The key personnel identified in this grant application have completed the required education on the protection of human research participants. The institution has established a formal program entitled the Mayo Investigator Training Program or MITP. The MITP is a web based educational course designed to provide all personnel involved in human subject research with training about human subject protection. All Mayo personnel engaged in human subject research are required to complete the course.

Adequacy of Protection against Risk through Informed Written Consent

The protocol and all the techniques to be used will be approved by the Mayo Clinic Institutional Review Board prior to initiation of any studies. Informed written consent will be obtained from subjects who meet inclusion criteria and pass the screening procedure, after the nature and possible consequences of the study have been explained. As detailed in the consent form, the **expected risks** include: -

- a) **Blood sampling.** Blood samples are collected by venipuncture for this study. Bruising can occur with venipuncture, as can fainting, etc. Risk Monitoring / Risk Reduction: The samples are collected using aseptic technique in designated venipuncture areas of the Clinic where facilities are available should untoward reactions (fainting, etc.) occur. Given the aseptic nature of the sample collection and the small risk of bruising, the monitoring plan is focused on advising volunteers to call the investigators should they have unusual pain or discomfort from the venipuncture site. Blood drawn within a 12-week period will not exceed 550mL (one pint).
- b) **Peripheral vascular catheter placement.** Catheter insertion, intravenous infusion and blood withdrawal are associated with a small risk of phlebitis. Risk Monitoring / Risk Reduction: This will be minimized by careful attention to sterile technique. If phlebitis occurs, it will be treated conservatively with heat and when appropriate, with antibiotics. The catheters will be cared for by experienced CRTU nurses in order to minimize the risk of these complications. In this protocol, “arterialized – venous” blood will be obtained by placing a hand in which a catheter has been inserted in a heated box during the study. The temperature inside the box is maintained at ~48.9°C. With prolonged exposure to continuous heat, there is a potential risk of local skin irritation or a minor burn. If this occurs, it will be treated appropriately. Catheter risks will be discussed with the volunteers prior to obtaining consent for the study.
- c) **Deuterated Water ingestion.** Deuterated water will be given in 3 divided doses. Risk Monitoring / Risk Reduction: Deuterated water is sometimes associated with dizziness and

vertigo which is reduced by giving the tracer in divided doses. The fact that it is given late at night while the patient is in bed will reduce the risk of falls. Prior experiments using deuterated water have suggested that the occurrence of this uncommon.

- d) **Femoral vein, hepatic vein and femoral artery catheter placement.** Risks associated with catheter placement include bleeding, swelling, pain or discomfort at the puncture site. Other potential risks include development of a pseudo-aneurysm, infection or decreased sensation in the leg of the puncture site. Risk Monitoring / Risk Reduction: These catheters be placed by a skilled, experienced interventional radiologist under fluoroscopic guidance in the interventional radiology suite. The study team is trained in the care of these catheters. After removal, pressure on the groin access site will be maintained as per a standard protocol to prevent hematoma or aneurysms. Participants will be instructed to lie flat with leg straight throughout the study and for 4-6 hours after study completion. Prolonged bedrest with restricted positioning may cause back pain or aggravate existing back pain. Participants will be given activity restrictions prior to discharge and the study team will maintain phone follow-up for 3-5 days after the study.
- e) **Placement of urinary catheter.** Risks associated with catheter placement include discomfort and the potential for urinary tract infection. Risk Monitoring / Risk Reduction: The urinary catheter will be placed by a skilled nurse or urology technician using strict aseptic technique. The study team is trained in the care of these catheters. The catheter will be in place only for the duration of the study to minimize infection risk.
- f) **Infusion of Glucose, tracers, amino acids, indocyanine green, somatostatin and insulin.** All infusions carry a risk of allergic reactions, bruising, discomfort at the site of infusion, and infection. Insulin can cause hypoglycemia and somatostatin can cause gastrointestinal distress. 50% Dextrose and amino acid infusions may cause phlebitis. Indocyanine Green contains sodium iodide and should be used with caution in patients who have a history of allergy to iodides. Risk Monitoring / Risk Reduction: All infusates are prepared by trained personnel from GMP grade material (under the supervision of a research pharmacist) in a laminar flow cabinet using aseptic technique. The PI holds an IND for somatostatin (**IND 116569**). To minimize the risk of phlebitis caused by 20 or 50% Dextrose and amino acid infusions, a large caliber vein will be used. If this is not possible a Peripherally Inserted Central Catheter (PICC Line) will be used. Participants allergic to iodine will be excluded. Access sites will be monitored closely for local discomfort, erythema and infiltration. Vital signs and intravenous infusion sites will be monitored hourly as per our standard practice during infusions in the CRTU.

Radiation. Subjects will be exposed to radiation in this study. Lean body mass and percent body fat will be measured at the time of screening using DEXA (dual energy x-ray absorptiometry). In addition, tritiated glucose will be infused at low dosage to measure glucose metabolism. Catheters will be placed by a skilled, experienced interventional radiologist under fluoroscopic guidance in the interventional radiology suite. There is no risk of exposure to radiation during an MRI procedure, assessment of potential contraindications to MRI will be conducted by the trained, clinical MRI staff prior to the scan in keeping with typical procedure at the Department of Radiology at Mayo Clinic Risk Monitoring / Risk Reduction: The amount of radiation that a volunteer will receive will be well below levels that result in significant risk of harmful effects. Proposed radiation exposure will be reviewed by the Mayo Clinic Radiation Safety Board and Radiology Operational Review Committee, prior to initiation of any study. Women who could become pregnant will be required to have a negative pregnancy test prior to

participation in each study to ensure that pregnancy does not confound our studies. The Mayo CRTU body composition core has a QDR4500 with fan scan technology which allows body composition to be performed in only a few minutes. The amount of infused tritiated glucose used will be the lowest dose that can be reliably counted. .

- g) **Ingestion of glucose.** Glucose ingested as a drink during an oral glucose tolerance test can cause nausea and abdominal cramps. Risk Monitoring / Risk Reduction: Subjects will be allowed to rinse out their mouth with a sip of water within 5 minutes of glucose ingestion. We will ensure that subjects can ingest / tolerate a meal prior to leaving the CRTU.
- h) **Confidentiality.** All studies expose participants to the psychosocial risks arising from any breach in confidentiality. The risks include anxiety, confusion, damage to family relationships or a compromised ability to obtain insurance or employment. These risks may not be confined to the individual but may extend to other family members. Risk Monitoring/Risk Reduction: The nature of the information obtained will be explained in detail to each participant – specifically none of this information will become part of the medical record and cannot impact the availability and nature of care or access to healthcare. Confidentiality of all medical records is strictly maintained by established procedures. The original study data are kept in the study facility and are entered into a computer under the direction of a biostatistician. Physical records are stored under lock and electronic records through security passwords. Violations of confidentiality will be immediately reported to the IRB and Data Monitoring Board. Study records will not identify subjects by name but will use a numeric code.
- i) **Pregnancy.** Inclusion criteria include the intention of female volunteers not to conceive for the period of study. All women of childbearing potential will have a negative pregnancy test within 48 hours prior to any study because of the potential effect of pregnancy on glucose metabolism.

Data and Safety Monitoring Plan (DSMP)

Brief Description of the Purpose of the Study – The study will ascertain whether impaired fasting glucose with or without impaired glucose tolerance alters the glucose threshold for basal insulin secretion and whether this is accompanied by defects in the insulin response to hyperglycemia.

Adherence Statement – The Data Safety Monitoring Plan (DSMP) outlined below will adhere to the protocol approved by the Mayo Clinic CTSA Research Review Committee and the Mayo Clinic IRB.

Protection of Subject Privacy –During this study, medical history and physical examination will be performed at baseline and at regular intervals for the various study interventions. All of the materials collected are for research purposes only, and data will be kept in strict confidence. No information will be given to anyone without permission from the subject. The consent form includes the informed consent statement required by Mayo Clinic for studies involving biospecimen. This statement guarantees confidentiality and identifies the subject as the owner of the information from the biospecimen analysis. Confidentiality is assured by use of identification codes. All data, whether generated in the laboratory or at the bedside, will be identified with an identification code unique to the subject.

Database Protection –The database is secured with password protection. The informatics manager receives only coded information which is entered into the database under those identification numbers. Electronic communication with outside collaborators involves only unidentifiable information. Confidentiality of all medical records is strictly maintained by

established procedures. The original study data are kept in the study facility and are entered into a computer under the direction of a biostatistician. Physical records are stored under lock and key.

Confidentiality During AE Reporting – AE reports and annual summaries will not include subject-identifiable material. Each will include the identification code only.

Adverse Event Information

Definition - An adverse event (AE) is any untoward medical occurrence in a subject temporally associated with participation in the clinical study or with use of the experimental agent being studied. An adverse finding can include a sign, symptom, abnormal assessment (laboratory test value, vital signs, electrocardiogram finding, etc.) or any combination of these.

A Serious Adverse Event (SAE) is any adverse event that results in one or more of the following outcomes:

- Death
- A life-threatening event
- Inpatient hospitalization or prolongation of existing hospitalization
- A persistent or significant disability/incapacity
- A congenital anomaly or birth defect
- Important medical event based upon appropriate medical judgment

Classification of AE Severity – AEs will be labeled according to severity which is based on their impact on the patient. An AE will be termed ‘mild’ if it does not have a major impact on the patient, ‘moderate’ if it causes the patient some minor inconvenience and ‘severe’ if it causes a substantial disruption to the patient’s wellbeing.

AE Attribution Scale – AEs will be categorized according to the likelihood that they are related to the study intervention. Specifically, they will be labeled either definitely, probably, possibly or unrelated to the study intervention.

Expected Risks – Expected risks are documented in the ‘Protection of Human Subjects’ Section of this application

SAE Reporting

SAEs that are unanticipated, serious, and/or possibly related to the study intervention will be reported to the Independent Monitor, IRB, CTSA, FDA (if applicable), and NIDDK within 7-15 calendar days depending on the severity of the event in accordance with requirements.

Anticipated SAEs or those unrelated to the study intervention will be reported to the same individuals/entities in accordance with requirements.

Data Quality and Safety Review Plan and Monitoring

Data Quality and Management

Description of Plan for Data Quality and Management – The PI will review all data collection forms on an ongoing basis for data completeness and accuracy as well as protocol compliance. A statement reflecting the results of the review will be sent to the NIDDK in the annual report (non-competing continuation).

Frequency of Data Review for this Study –The frequency of data review for this study differs according to the type of data and can be summarized in the following table.

Data type	Frequency of review	Reviewer
Subject accrual (adherence to protocol regarding demographics, inclusion/exclusion)	Weekly	Principal Investigator
Adverse event rates (injuries)	Weekly	Principal Investigator, Independent Monitor
Compliance with follow-up	Monthly	Principal Investigator, Independent Monitor
Out of range laboratory data	Weekly	Principal Investigator, Independent Monitor
Stopping rules report regarding statistical power implications of dropouts and missing data	Quarterly	Principal Investigator, Independent Monitor

Measurement and reporting of participant compliance to protocol – Data on compliance to the treatment protocol will be collected twice weekly by research staff and reviewed quarterly by the study investigator, the study statistician, and the safety officer.

Subject withdrawal – subjects will stop the study if they decide to withdraw consent. Moreover, discontinuation will occur if any change in the subject's general health or compliance is deemed by the investigator to alter the risks of participation in the study / interpretation of the study.

Justification of Sample Size – Previous data was used to determine a sample size necessary to detect a clinically meaningful difference between groups. Please refer to the power calculations.

Stopping Rules - This study will be stopped prior to its completion if: (1) study recruitment or retention becomes futile, (2) any new information becomes available during the trial that necessitates stopping the trial; and (3) other situations occur that might warrant stopping the trial. Although we do not anticipate problems with recruitment and retention, if necessary, The PI will consult with a biostatistician to assess the impact of significant data loss due to problems in recruitment, retention or data collection.

Designation of an Independent Monitor –The Independent Monitor for this study is Matthew T. Drake, M.D. Ph.D. Dr. Drake is an endocrinologist who serves as the independent monitor of the Mayo CTSA. He is not associated with this research project and thus works independently of the PI, Dr. Vella. Dr. Drake is not part of the key scientific personnel involved in this grant and his salary is paid by Mayo Clinic to ensure independence. He is qualified to review the patient safety data generated by this study because of his experience as a PI of several human studies and as a member of the IRB.

Safety Review Plan – Study progress and safety will be reviewed monthly by the PI with the study team (and more frequently if needed). Progress reports, including patient recruitment, retention/attrition, and AEs will be provided to the Independent Monitor following each of the

monthly reviews. An annual report will be compiled and will include a list and summary of AEs. In addition, the annual report will address (1) whether adverse event rates are consistent with pre-study assumptions; (2) reason for dropouts from the study; (3) whether all participants met entry criteria; (4) whether continuation of the study is justified on the basis that additional data are needed to accomplish the stated aims of the study; and (5) conditions whereby the study might be terminated prematurely. The annual report will be signed by the Independent Monitor and will be forwarded to the IRB and NIDDK. The IRB and other applicable recipients will review progress of this study on an annual basis.

Informed Consent

Written informed consent will be obtained from each subject at entry into the study. The recruitment process is described in the Specific Aims of the grant and has been previously used by us after IRB approval. The Institutional Review Board will approve all patient recruitment material. Informed consent is obtained by the following process:

- The subject will be asked to review the study consent form.
- The PI, Co-Investigator (Co-I) or designated member of the study team, will meet with the subject to review the form, to confirm the subject's understanding of the study, and to answer any questions that the subject might have; and
- Once the subject demonstrates understanding of the study and agrees to participate in the study, the consent will be signed in the presence of the study team member.

VI. Gender/Minority Mix

The majority of residents in Rochester, Minnesota and surrounding counties are White; recent population estimates from over the past decade have indicated that minorities make up a significantly larger segment of our communities. According to census area data such cultural groups as the Somalis, Hispanic/ Latinos, and South East Asians have increased the percentage of minorities living in Rochester and the surrounding counties from 3% in 1990 to nearly 10% in 2000. Despite the increases in the number of minorities within rural Rochester, Minnesota, it is likely that recruitment will fall short in the area of minority participation. However, we are actively working with the minority outreach specialist in the Center for Patient Oriented Research and Tribal Elders from the local minority populations, to develop complementary community-based strategies for recruitment of minorities at Mayo Clinic Rochester.

VII. References

1. Shah, M., *et al.* TCF7L2 Genotype and alpha-Cell Function in Humans Without Diabetes. *Diabetes* **65**, 371-380 (2016).
2. Karanth, S., *et al.* A Hepatocyte FOXN3-alpha Cell Glucagon Axis Regulates Fasting Glucose. *Cell Rep* **24**, 312-319 (2018).
3. Adams, J.D., *et al.* Fasting glucagon concentrations are associated with longitudinal decline of beta-cell function in non-diabetic humans. *Metabolism* **105**, 154175 (2020).
4. Shah, P., Basu, A., Basu, R. & Rizza, R. Impact of lack of suppression of glucagon on glucose tolerance in humans. *Am J Physiol* **277**, E283-290 (1999).
5. Shah, P., *et al.* Lack of suppression of glucagon contributes to postprandial hyperglycemia in subjects with type 2 diabetes mellitus. *J Clin Endocrinol Metab* **85**, 4053-4059 (2000).

6. Adams, J.D., *et al.* Insulin secretion and action and the response of endogenous glucose production to a lack of glucagon suppression in nondiabetic subjects. *Am J Physiol Endocrinol Metab* **321**, E728-E736 (2021).
7. Charlton, M.R., Adey, D.B. & Nair, K.S. Evidence for a catabolic role of glucagon during an amino acid load. *J Clin Invest* **98**, 90-99 (1996).
8. Charlton, M.R. & Nair, K.S. Role of hyperglucagonemia in catabolism associated with type 1 diabetes: effects on leucine metabolism and the resting metabolic rate. *Diabetes* **47**, 1748-1756 (1998).
9. Cherrington, A.D., *et al.* Insulin, glucagon, and glucose as regulators of hepatic glucose uptake and production in vivo. *Diabetes Metab Rev* **3**, 307-332 (1987).
10. Bock, G., *et al.* Effects of nonglucose nutrients on insulin secretion and action in people with pre-diabetes. *Diabetes* **56**, 1113-1119 (2007).
11. Wang, T.J., *et al.* Metabolite profiles and the risk of developing diabetes. *Nature medicine* **17**, 448-453 (2011).
12. Newgard, C.B., *et al.* A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. *Cell Metab* **9**, 311-326 (2009).
13. Wang, T.J., *et al.* 2-Aminoadipic acid is a biomarker for diabetes risk. *J Clin Invest* **123**, 4309-4317 (2013).
14. Suppli, M.P., *et al.* Glucagon Resistance at the Level of Amino Acid Turnover in Obese Subjects With Hepatic Steatosis. *Diabetes* **69**, 1090-1099 (2020).
15. Franch-Nadal, J., *et al.* Fatty liver index is a predictor of incident diabetes in patients with prediabetes: The PREDAPS study. *PLoS One* **13**, e0198327 (2018).
16. Balkau, B., *et al.* Nine-year incident diabetes is predicted by fatty liver indices: the French D.E.S.I.R. study. *BMC Gastroenterol* **10**, 56 (2010).
17. Zelber-Sagi, S., *et al.* Non-alcoholic fatty liver disease independently predicts prediabetes during a 7-year prospective follow-up. *Liver Int* **33**, 1406-1412 (2013).
18. Kazda, C.M., *et al.* Evaluation of Efficacy and Safety of the Glucagon Receptor Antagonist LY2409021 in Patients With Type 2 Diabetes: 12- and 24-Week Phase 2 Studies. *Diabetes Care* **39**, 1241-1249 (2016).
19. Berglund, E.D., *et al.* Hepatic glucagon action is essential for exercise-induced reversal of mouse fatty liver. *Diabetes* **60**, 2720-2729 (2011).
20. Gilon, P. The Role of alpha-Cells in Islet Function and Glucose Homeostasis in Health and Type 2 Diabetes. *J Mol Biol* **432**, 1367-1394 (2020).
21. Finan, B., *et al.* A rationally designed monomeric peptide triagonist corrects obesity and diabetes in rodents. *Nature Medicine* **21**, 27-36 (2015).
22. Perry, R.J., *et al.* Glucagon stimulates gluconeogenesis by INSP3R1-mediated hepatic lipolysis. *Nature* **579**, 279-283 (2020).
23. Jiang, G. & Zhang, B.B. Glucagon and regulation of glucose metabolism. *Am J Physiol Endocrinol Metab* **284**, E671-678 (2003).
24. Ogden, C.L., *et al.* Trends in Obesity Prevalence Among Children and Adolescents in the United States, 1988-1994 Through 2013-2014. *JAMA* **315**, 2292-2299 (2016).

25. Younossi, Z.M., *et al.* Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* **64**, 73-84 (2016).
26. Varghese, R.T., *et al.* Diabetes-Associated Variation in TCF7L2 Is Not Associated With Hepatic or Extrahepatic Insulin Resistance. *Diabetes* **65**, 887-892 (2016).
27. Albright, A.L. & Gregg, E.W. Preventing type 2 diabetes in communities across the U.S.: the National Diabetes Prevention Program. *American journal of preventive medicine* **44**, S346-351 (2013).
28. Wewer Albrechtsen, N.J., *et al.* Hyperglucagonemia correlates with plasma levels of non-branched-chain amino acids in patients with liver disease independent of type 2 diabetes. *Am J Physiol Gastrointest Liver Physiol* **314**, G91-G96 (2018).
29. Wewer Albrechtsen, N.J., *et al.* The Liver-alpha-Cell Axis and Type 2 Diabetes. *Endocr Rev* **40**, 1353-1366 (2019).
30. Winther-Sorensen, M., *et al.* Glucagon acutely regulates hepatic amino acid catabolism and the effect may be disturbed by steatosis. *Mol Metab* **42**, 101080 (2020).
31. Zhang, L., Hames, K.C. & Jensen, M.D. Regulation of direct adipose tissue free fatty acid storage during mixed meal ingestion and high free fatty acid concentration conditions. *Am J Physiol Endocrinol Metab* **320**, E208-E218 (2021).
32. Smith, G.I., *et al.* Insulin resistance drives hepatic de novo lipogenesis in nonalcoholic fatty liver disease. *J Clin Invest* **130**, 1453-1460 (2020).
33. Dean, E.D. A Primary Role for alpha-Cells as Amino Acid Sensors. *Diabetes* **69**, 542-549 (2020).
34. Robertson, R.P., *et al.* Assessment of beta-Cell Mass and alpha- and beta-Cell Survival and Function by Arginine Stimulation in Human Autologous Islet Recipients. *Diabetes* **64**, 565-572 (2015).
35. Robertson, R.P., *et al.* Arginine is preferred to glucagon for stimulation testing of beta-cell function. *Am J Physiol Endocrinol Metab* **307**, E720-727 (2014).
36. Sathananthan, A., *et al.* A concerted decline in insulin secretion and action occurs across the spectrum of fasting and postchallenge glucose concentrations. *Clin Endocrinol (Oxf)* **76**, 212-219 (2012).
37. Bock, G., *et al.* Pathogenesis of pre-diabetes: mechanisms of fasting and postprandial hyperglycemia in people with impaired fasting glucose and/or impaired glucose tolerance. *Diabetes* **55**, 3536-3549 (2006).
38. Sharma, A., *et al.* Impaired Insulin Action is Associated with Increased Glucagon Concentrations in Non-Diabetic Humans. *J Clin Endocrinol Metab* **103**, 314-319 (2018).
39. Butler, P.C. & Rizza, R.A. Contribution to postprandial hyperglycemia and effect on initial splanchnic glucose clearance of hepatic glucose cycling in glucose-intolerant or NIDDM patients. *Diabetes* **40**, 73-81 (1991).
40. Dean, E.D., *et al.* Interrupted Glucagon Signaling Reveals Hepatic alpha Cell Axis and Role for L-Glutamine in alpha Cell Proliferation. *Cell Metab* **25**, 1362-1373 e1365 (2017).
41. Laurenti, M.C., *et al.* Assessment of individual and standardized glucagon kinetics in healthy humans. *Am J Physiol Endocrinol Metab* **320**, E71-E77 (2021).

42. Van Cauter, E., Mestrez, F., Sturis, J. & Polonsky, K.S. Estimation of insulin secretion rates from C-peptide levels. Comparison of individual and standard kinetic parameters for C-peptide clearance. *Diabetes* **41**, 368-377 (1992).
43. Ishida, T., *et al.* The effect of ingestion of meat on hepatic extraction of insulin and glucagon and hepatic glucose output in conscious dogs. *Metabolism* **32**, 558-567 (1983).
44. Ishida, T., Lewis, R.M., Hartley, C.J., Entman, M.L. & Field, J.B. Comparison of hepatic extraction of insulin and glucagon in conscious and anesthetized dogs. *Endocrinology* **112**, 1098-1109 (1983).
45. Basu, A., *et al.* Systemic and regional free fatty acid metabolism in type 2 diabetes. *Am J Physiol Endocrinol Metab* **280**, E1000-1006 (2001).
46. Varghese, R.T., *et al.* Mechanisms Underlying the Pathogenesis of Isolated Impaired Glucose Tolerance in Humans. *J Clin Endocrinol Metab* **101**, 4816-4824 (2016).
47. Allen, A.M., *et al.* Nonalcoholic fatty liver disease incidence and impact on metabolic burden and death: A 20 year-community study. *Hepatology* **67**, 1726-1736 (2018).
48. Peeraphatdit, T.B., *et al.* A Cohort Study Examining the Interaction of Alcohol Consumption and Obesity in Hepatic Steatosis and Mortality. *Mayo Clin Proc* **95**, 2612-2620 (2020).
49. Sathananthan, A., *et al.* Common genetic variation in GLP1R and insulin secretion in response to exogenous GLP-1 in nondiabetic subjects: a pilot study. *Diabetes Care* **33**, 2074-2076 (2010).
50. Smushkin, G., *et al.* Diabetes-associated common genetic variation and its association with GLP-1 concentrations and response to exogenous GLP-1. *Diabetes* **61**, 1082-1089 (2012).
51. Vella, A. & Rizza, R.A. Application of isotopic techniques using constant specific activity or enrichment to the study of carbohydrate metabolism. *Diabetes* **58**, 2168-2174 (2009).
52. Vella, A., *et al.* Effect of glucagon-like peptide-1(7-36)-amide on initial splanchnic glucose uptake and insulin action in humans with type 1 diabetes. *Diabetes* **50**, 565-572 (2001).
53. Vella, A., *et al.* Type I diabetes mellitus does not alter initial splanchnic glucose extraction or hepatic UDP-glucose flux during enteral glucose administration. *Diabetologia* **44**, 729-737 (2001).
54. Vella, A., *et al.* Effect of enteral vs. parenteral glucose delivery on initial splanchnic glucose uptake in nondiabetic humans. *Am J Physiol Endocrinol Metab* **283**, E259-266 (2002).
55. Laurenti, M.C., *et al.* Insulin Pulse Characteristics and Insulin Action in Non-diabetic Humans. *J Clin Endocrinol Metab* **106**, 1702-1709 (2021).
56. Laurenti, M.C., *et al.* Diabetes-associated genetic variation in TCF7L2 alters pulsatile insulin secretion in humans. *JCI Insight* **5**(2020).
57. Laurenti, M.C., *et al.* Assessment of pulsatile insulin secretion derived from peripheral plasma C-peptide concentrations by nonparametric stochastic deconvolution. *Am J Physiol Endocrinol Metab* **316**, E687-E694 (2019).
58. Thompson, S.M., Vella, A., Service, F.J. & Andrews, J.C. Selective Arterial Calcium Stimulation With Hepatic Venous Sampling in Immune-Mediated Hypoglycemia. *J Endocr Soc* **1**, 671-675 (2017).
59. Thompson, S.M., *et al.* Impact of variant pancreatic arterial anatomy and overlap in regional perfusion on the interpretation of selective arterial calcium stimulation with hepatic venous sampling for preoperative localization of occult insulinoma. *Surgery* **158**, 162-172 (2015).

60. Thompson, S.M., Vella, A., Service, F.J., Thompson, G. & Andrews, J.C. Selective Arterial Calcium Stimulation with Hepatic Venous Sampling in Patients with Recurrent Endogenous Hyperinsulinemic Hypoglycemia and Metastatic Insulinoma: Evaluation in Five Patients. *J Vasc Interv Radiol* **28**, 1745-1749 (2017).
61. Thompson, S.M., *et al.* Selective Arterial Calcium Stimulation With Hepatic Venous Sampling Differentiates Insulinoma From Nesidioblastosis. *J Clin Endocrinol Metab* **100**, 4189-4197 (2015).
62. Bedogni, G., *et al.* The Fatty Liver Index: a simple and accurate predictor of hepatic steatosis in the general population. *BMC Gastroenterol* **6**, 33 (2006).
63. Taylor, H.L., *et al.* A questionnaire for the assessment of leisure time physical activities. *J Chronic Dis* **31**, 741-755 (1978).
64. Saunders, J.B., Aasland, O.G., Babor, T.F., de la Fuente, J.R. & Grant, M. Development of the Alcohol Use Disorders Identification Test (AUDIT): WHO Collaborative Project on Early Detection of Persons with Harmful Alcohol Consumption--II. *Addiction* **88**, 791-804 (1993).
65. Moura Cunha, G., *et al.* Quantitative magnetic resonance imaging for chronic liver disease. *Br J Radiol* **94**, 20201377 (2021).
66. Adkins, A., *et al.* Higher insulin concentrations are required to suppress gluconeogenesis than glycogenolysis in nondiabetic humans. *Diabetes* **52**, 2213-2220 (2003).
67. Meier, J.J., Veldhuis, J.D. & Butler, P.C. Pulsatile insulin secretion dictates systemic insulin delivery by regulating hepatic insulin extraction in humans. *Diabetes* **54**, 1649-1656 (2005).
68. Service, G.J., *et al.* Hyperinsulinemic hypoglycemia with nesidioblastosis after gastric-bypass surgery. *N Engl J Med* **353**, 249-254 (2005).
69. Lund, A., *et al.* Evidence of Extrapaneacretic Glucagon Secretion in Man. *Diabetes* **65**, 585-597 (2016).
70. Basu, R., *et al.* Use of a novel triple-tracer approach to assess postprandial glucose metabolism. *Am J Physiol Endocrinol Metab* **284**, E55-E69 (2003).
71. Jones, J.G., *et al.* NMR Derivatives for Quantification of ²H and ¹³C-Enrichment of Human Glucuronide from Metabolic Tracers. *Journal of Carbohydrate Chemistry* **25**, 203-217 (2006).
72. Basu, A., *et al.* Effects of type 2 diabetes on the ability of insulin and glucose to regulate splanchnic and muscle glucose metabolism: evidence for a defect in hepatic glucokinase activity. *Diabetes* **49**, 272-283 (2000).
73. Miles, J.M., Ellman, M.G., McClean, K.L. & Jensen, M.D. Validation of a new method for determination of free fatty acid turnover. *The American journal of physiology* **252**, E431-438 (1987).
74. Persson, X.M., Blachnio-Zabielska, A.U. & Jensen, M.D. Rapid measurement of plasma free fatty acid concentration and isotopic enrichment using LC/MS. *J Lipid Res* **51**, 2761-2765 (2010).
75. Lanza, I.R., *et al.* Quantitative metabolomics by H-NMR and LC-MS/MS confirms altered metabolic pathways in diabetes. *PLoS One* **5**, e10538 (2010).
76. Jensen, M.D., Johnson, C.M., Cryer, P.E. & Murray, M.J. Thermogenesis after a mixed meal: role of leg and splanchnic tissues in men and women. *Am J Physiol* **268**, E433-438 (1995).

77. Nair, K.S., Ford, G.C., Ekberg, K., Fernqvist-Forbes, E. & Wahren, J. Protein dynamics in whole body and in splanchnic and leg tissues in type I diabetic patients. *J Clin Invest* **95**, 2926-2937 (1995).
78. Smushkin, G., *et al.* The effect of a bile acid sequestrant on glucose metabolism in subjects with type 2 diabetes. *Diabetes* **62**, 1094-1101 (2013).
79. Vella, A., *et al.* Effects of dipeptidyl peptidase-4 inhibition on gastrointestinal function, meal appearance, and glucose metabolism in type 2 diabetes. *Diabetes* **56**, 1475-1480 (2007).
80. Cobelli, C., *et al.* The oral minimal model method. *Diabetes* **63**, 1203-1213 (2014).
81. Breda, E., Cavaghan, M.K., Toffolo, G., Polonsky, K.S. & Cobelli, C. Oral glucose tolerance test minimal model indexes of beta-cell function and insulin sensitivity. *Diabetes* **50**, 150-158 (2001).
82. Nielsen, S., Guo, Z., Johnson, C.M., Hensrud, D.D. & Jensen, M.D. Splanchnic lipolysis in human obesity. *J Clin Invest* **113**, 1582-1588 (2004).
83. Jensen, M.D., Cardin, S., Edgerton, D. & Cherrington, A. Splanchnic free fatty acid kinetics. *Am J Physiol Endocrinol Metab* **284**, E1140-1148 (2003).
84. Hochberg, Y. & Benjamini, Y. More powerful procedures for multiple significance testing. *Stat Med* **9**, 811-818 (1990).
85. Hayashi, Y. Glucagon regulates lipolysis and fatty acid oxidation through inositol triphosphate receptor 1 in the liver. *J Diabetes Investig* **12**, 32-34 (2021).
86. Hames, K.C., Vella, A., Kemp, B.J. & Jensen, M.D. Free fatty acid uptake in humans with CD36 deficiency. *Diabetes* **63**, 3606-3614 (2014).
87. Lytle, K.A., *et al.* Hepatic Fatty Acid Balance and Hepatic Fat Content in Humans With Severe Obesity. *J Clin Endocrinol Metab* **104**, 6171-6181 (2019).
88. Guo, Z. & Jensen, M.D. Arterio-venous balance studies of skeletal muscle fatty acid metabolism: What can we believe? *Am J Physiol Endocrinol Metab* **305**, E925-930 (2013).
89. Bucci, M., *et al.* Enhanced fatty acid uptake in visceral adipose tissue is not reversed by weight loss in obese individuals with the metabolic syndrome. *Diabetologia* **58**, 158-164 (2015).
90. Iozzo, P., *et al.* Fatty acid metabolism in the liver, measured by positron emission tomography, is increased in obese individuals. *Gastroenterology* **139**, 846-856, 856 e841-846 (2010).
91. Ishida, T., *et al.* Differential effects of oral, peripheral intravenous, and intraportal glucose on hepatic glucose uptake and insulin and glucagon extraction in conscious dogs. *J Clin Invest* **72**, 590-601 (1983).
92. Kelley, D.E., *et al.* Relative effects of calorie restriction and weight loss in noninsulin-dependent diabetes mellitus. *J Clin Endocrinol Metab* **77**, 1287-1293 (1993).
93. Johansson, H.E., *et al.* Energy restriction in obese women suggest linear reduction of hepatic fat content and time-dependent metabolic improvements. *Nutr Diabetes* **9**, 34 (2019).