# Return of First-phase Insulin Secretion in Type 2 Diabetes is Associated With Depletion of Pancreas Lipid

Study Protocol & Statistical Analysis Plan

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## Methods

*Participants and Study Design*

Participants were AA and EA adults, determined by self-reported race, with T2D and were recruited based on the following: Inclusion criteria were diagnosis within the past 10 years; treated with diet, metformin, SGLT2-inhibitors, DPP-IV inhibitors, or GLP-1 RAs; adult men and women aged 35-65 yr; HbA1c $\leq$8.0; and with a BMI of 25-50 kg/m$^2$. Exclusion criteria were a change in body weight of >5 kg in the past 6 months; use of glucocorticoids; and tobacco or recreational drug use. Medication was discontinued 1 week prior to baseline testing for metformin and SGLT2-inhibitors, and 4 weeks prior to testing for DPP-IV-inhibitors and GLP-1 receptor agonists. Fasting glucose was monitored throughout the study, and any patient who demonstrated fasting glucose >200 mg/dL on 3 consecutive days was returned to medication. Routine physical activity was recorded by questionnaire at screening, and all participants were asked to maintain their usual level of physical activity throughout the study.

The study design was a 12-week randomized clinical trial with two arms: carbohydrate restricted (CR) and higher carbohydrate (HC). Both diets were developed by a Registered Dietitian who met with the study participants weekly. Participants were given detailed instructions on daily meal plans. Groceries were delivered to each participant weekly by a local delivery service. Participants prepared their own meals and snacks, at an energy level calculated to be weight-maintaining, based on the instructions provided by the study dietitian. Participants reported their body weight to the dietitian weekly, and their diet prescription was altered if their weight changed by more than 1 kg. The CR diet was composed of ~9% energy from carbohydrate, 26% energy from protein, and 65% energy from fat, whereas the HC diet was

composed of ~55% energy from carbohydrate, 25% energy from protein, and 20% energy from fat. The UAB Institutional Review Board approved the study.

*Procedures*

At baseline and after 12 weeks of diet therapy, participants underwent a standard, 75 g oral glucose tolerance test (OGTT) and a hyperglycemic clamp. The OGTT was 3 hours in length, with blood sampled at -30, -15, -5, 10, 20, 30, 60, 90, 120, 150 and 180 min relative to the start of glucose ingestion for measurement of glucose, insulin, and C-peptide.

The clamp protocol involved three stages: a 30-min stage to evaluate first-phase beta-cell response, a 2-hour phase to evaluate insulin sensitivity, and a final, 30-min phase to evaluate maximal beta-cell response to arginine. The first phase was conducted by administering a bolus of glucose over 2 min at time zero followed by an intravenous administration of glucose (as D20) to achieve a blood glucose concentration 50 mg/dL above that morning's fasted glucose concentration (G + 50). Formulas were used based on the RISE protocol (18) to determine both the bolus amount [weight (kg) x 50 mg/dL x 1.1 / 180] and to estimate the glucose infusion rate [weight (kg) x 5 x 60 / 180]; this rate was adjusted as necessary to achieve G + 50. Blood was sampled at -20 and -5 min prior to the start of glucose administration; then at 2, 4, 6, 8, 10, 15, 20, 25, and 30 min after the start of glucose administration. Glucose was measured at bedside, and sera were stored for later assessment of glucose, insulin, and C-peptide. After the 30-min first-phase stage, blood glucose was brought to 200 mg/dL and maintained there for the next 2 hours to determine insulin sensitivity. During this time, blood was sampled every 5 minutes. After the 150-min blood sample was collected, a second bolus of D20 was administered over 2 min [weight (kg) x 100 mg/dL x 1.1 / 180]. Blood glucose was brought to 300 mg/dL and

maintained there with a variable infusion of D20. Blood was sampled every 5 minutes to minute 180. After the 180-min blood collection, 5 g arginine (10% in saline) was infused over 5 min. Blood was sampled every 2 min from 182 to 190 min to capture maximal C-peptide secretion.

Serum samples were stored at -70$^{o}$C until analyzed. Glucose was measured on a SIRRUS chemistry analyzer (Stanbio Laboratory, Boerne, TX) using glucose oxidase reagent; the minimum detectable value was 2 mg/dl; inter-assay CV was 4.48%; and intra-assay CV was 1.28%. Insulin was assayed using immunofluorescence technology (TOSOH AIA900, TOSOH Bioscience, South San Francisco, CA); assay sensitivity was 0.5 uU/ml; inter-assay CV was 3.95% and intra-assay CV was 1.49%. C-peptide was analyzed on the TOSOH; assay sensitivity was 0.04 ng/ml; inter-assay CV was 6.81% and intra-assay CV was 1.67%.

For this study, the lipid content of pancreas and liver was be assessed by MRI using the 3-point Dixon approach. This method provides high-resolution contiguous images of the abdomen in breathhold durations and its intrinsic 3D imaging capability allows whole organs and spatial variations in ectopic fat across any region of the imaged anatomy to be evaluated. The 3-point M Dixon fat fraction (FF) is computed from the separated water and fat signals on a voxel-by-voxel basis. The accuracy of the fat fraction metric from this multi-echo fat quantification method has been validated in several pancreatic/hepatic fat quantification studies using the following equation: Fat fraction3-point M Dixon = Signal Intensity (SI)FAT/(SIWATER+SIFAT) x 100%.

We will attempt to image both the head and tail of the pancreas, however all regions of the pancreas show similar associations with beta-cell function. Visceral fat volume also was assessed in a 10 cm region of interest. Trans-axial abdominal images was obtained using 3D volumetric T1-weighted magnetization prepared rapid acquisition gradient echo (MPRAGE).

The echo time, repetition time, and pulse flip angles will be selected to optimize the signal-intensity contrast between adipose and non-adipose tissue. Image acquisition was done at the Civitan International Neuroimaging Laboratory under the oversight of Dr. Bolding using a state-of-the-art Prisma 3.0-Tesla whole-body MRI scanner. Image analysis was done by Dr. Goss using Slice-O-Matic software for visceral fat volume, and Osirix MD on an iMAC computer for assessing the signal intensity of a region of interest on the pancreas and liver to determine the fat fraction. By quantifying visceral adipose, and pancreatic and hepatic lipid, we will be able to examine whether these depots change in tandem during the intervention, and which depot is most closely associated with improvements in insulin sensitivity and beta-cell function.

Body composition: Body composition was assessed using dual-energy X-ray absorptiometry (DXA) in the Metabolism Core Laboratory of UAB's Nutrition Obesity Research Center (NORC) with a Lunar iDXA instrument (GE Healthcare, Madison, WI) with the enCORE 2011 v15 (sp2) software package. Total body fat mass and lean body mass (LBM) in kg, as well as total body %fat, was recorded.

Resting energy expenditure (REE) and substrate utilization (respiratory quotient, RQ): REE and RQ was determined in the NORC Core Laboratory by indirect calorimetry (TrueOne 2400, Parvo Medics, Sandy, UT). Participants were tested in the fasted (10h) condition following a 30-min supine rest.

*Calculations*

OGTT: Fasting values were determined from the average of the -30, -15, and -5 minute time points. The first-phase C-peptide index (CPI) was calculated as the difference in C-peptide from 0 to 30 minutes divided by the difference in serum glucose from 0 to 30 minutes ([C-peptide$_{30min}$ - C-peptide$_{0min}$] / [glucose$_{30min}$ – glucose$_{0min}$]). The Matsuda Insulin Sensitivity Index

(ISI) was used as a measure of whole-body insulin sensitivity due to its ability to capture both hepatic and skeletal muscle-derived insulin sensitivity. The Disposition Index (DI), an index of beta-cell function, was calculated as the product of C-peptide secretion and insulin sensitivity, as reported: DI = CPI x ISI.

Hyperglycemic clamp: The RISE protocol was used to calculate first-phase C-peptide response as the mean incremental response above baseline over minutes 2-10, and the maximal C-peptide response as the mean incremental response during minutes 184-190 above the mean at min 170-180. Insulin sensitivity was calculated as described for the hyperglycemic clamp.

Statistics:

a. *Analyses*. The primary analysis for each aim will be a mixed model, using all available data and utilizing the estimated correlation between measurement times. The mixed model is less biased towards statistically significant results in the context of missing data, and/or when a moderate to high correlation exists between outcome measures. For Aim 1, the primary outcome will be pancreas lipid. Statistical models will include time, diet, and the time-by-diet interaction. Other potential covariates (e.g., gender, race, total body fat, adherence to diet) will be explored in preliminary analyses. Similar analyses will be conducted for hepatic lipid and visceral adipose tissue. For Aim 2, the primary outcome will be beta-cell function, as reflected in first phase C-peptide secretion from the hyperglycemic clamp. Statistical models will include time, diet, and the time-by-diet interaction. Other potential covariates (e.g., gender, race, total body fat, adherence to diet, insulin sensitivity) will be explored in preliminary analyses. Similar analyses will be conducted for PhiD from the MMTT, and insulin sensitivity from the hyperglycemic clamp. Food records will be coded to assess adherence as a dichotomous variable, and adherence

will be defined as +/- 10 percentage points of the targeted carbohydrate intake. For Aim 3, the primary variable of interest is the race-by-diet interaction term, which will be added to the models from Aims 1 and 2. If either of these interaction terms is significant, the data will be analyzed separately within each race group. Influential data points and outliers will be examined in a sensitivity analyses.

Correlative analyses will be conducted between changes (week 12 minus baseline) in measures of glucose metabolism (e.g., glucose AUC from liquid meal test, HbA1c), markers of inflammation, visceral fat, and hepatic/pancreatic lipid with Pearson's correlations coefficients (adjusting for relevant confounding variables). The correlation between change in pancreas lipid and change in first-phase C-peptide secretion will be examined, adjusting for insulin sensitivity. For all analyses, variables known to deviate from a normal distribution will be log transformed. All statistical tests will be two-sided and will be performed using an alpha level of 0.05. Statistical analyses will be performed using SAS Version 9.4®. Baseline descriptive characteristics will summarized as means (SD) or proportions to include demographic, clinical, and other measures.

b. *Power calculation.* Based on our published data, a sample size of 15 in each group will have 80% power to detect a difference in means of -89.700 (the difference between a Group 1 mean, μ1, of 6.400 and a Group 2 mean, μ2, of 96.100) in first-phase response at week 12 assuming that the common standard deviation is 84.639 using a two group t-test with a 0.050 two-sided significance level. Because we believe that the association between pancreas lipid and beta-cell function will be stronger in African-Americans, we have elected to target a final sample size of

15 participants per diet-race group (total of 60 participants with complete data). Using the Fisher r-to-z transformation, a value of z can be calculated that can be applied to assess the significance of the difference between two correlation coefficients, ra and rb, found in two independent samples. Given 30 subjects in each race group, we will be able to detect a difference of 0.3 with a one-sided alpha of 0.05. We will recruit 80 participants (20 per diet-race group) to account for missing data, potential drop-outs, or for participants who have to withdraw from the study due to problems with glycemic control.

c. *Randomization*

The study statistician (Dr. Desmond) will prepare a closed envelope randomization for the two treatment arms stratified by race group using Proc Plan in SAS Ver. 9.4®. Patients will receive their group assignment following baseline testing.

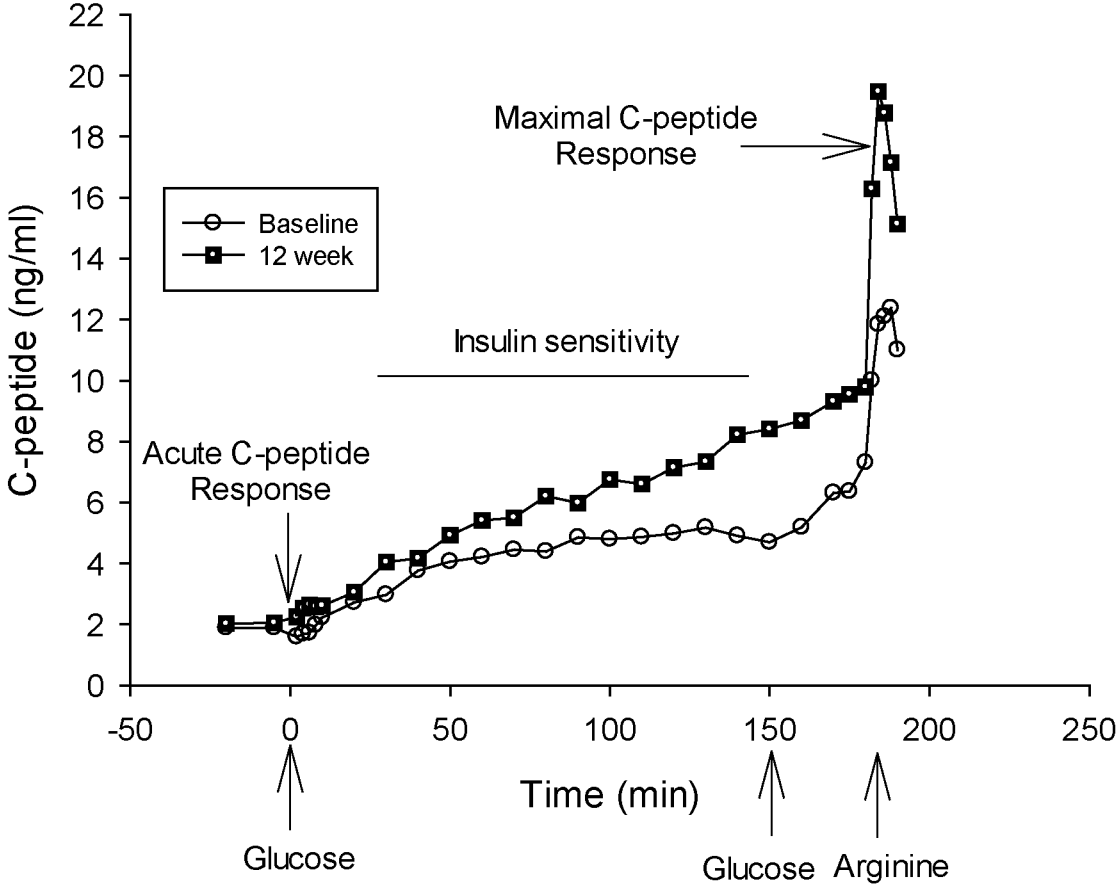
Patient management following the study:

Given that patients were removed from medication prior to study initiation, and were carefully monitored for glycemic control during the study, it is important to ensure that they make a smooth transition to a free-living situation. Following final testing, all study participants will be given access to continued diet/nutrition support, provided by the study team, to assure a smooth transition to a free-living diet. Patients will be able to choose their preferred diet (they may choose to stay on their assigned intervention diet, or to transition to a different diet). Medical management, including of medication type and dosage, will be available during this transition. All patients will demonstrate stable glycemic control on a given diet and medication regimen before exiting the study.

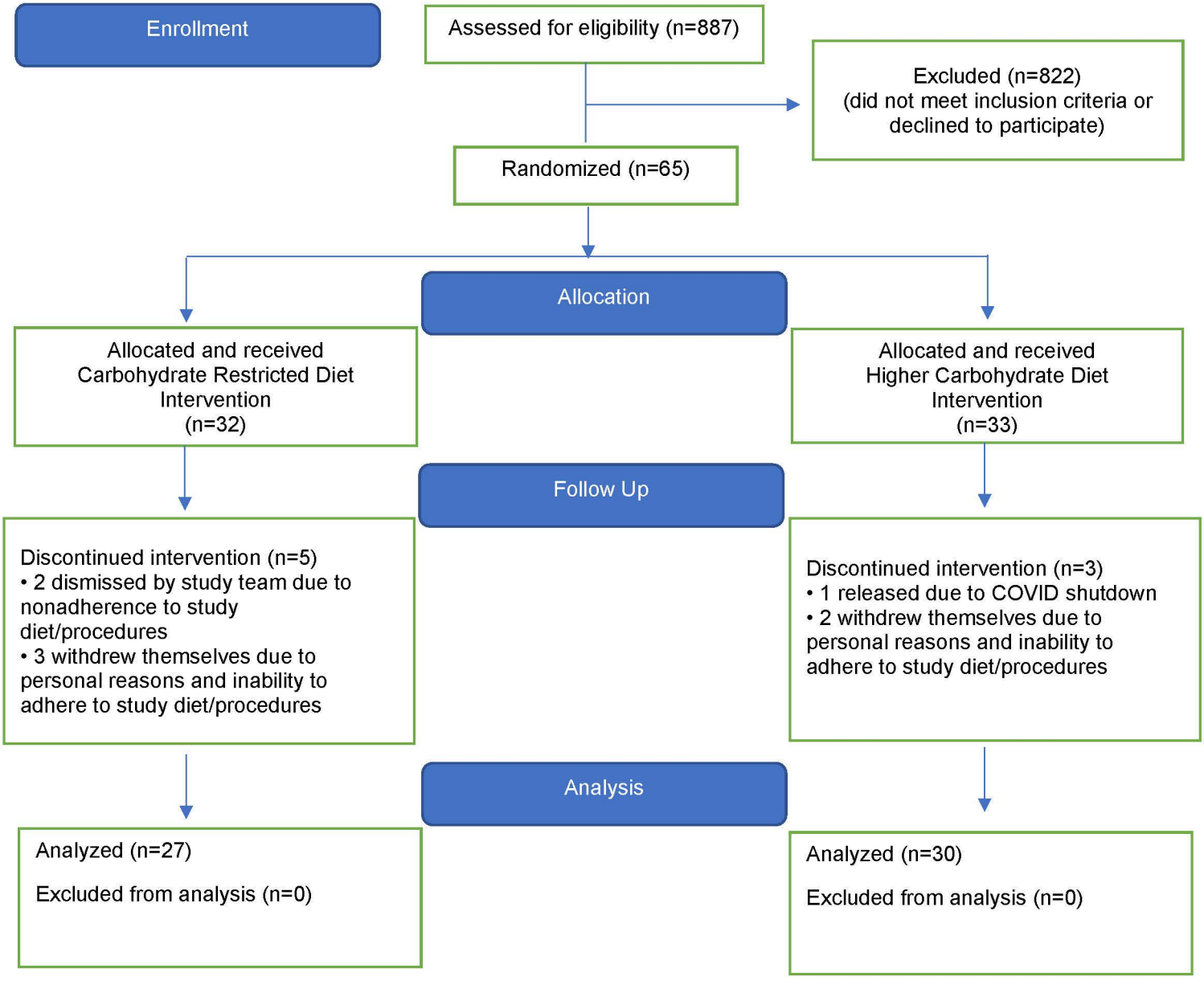
**Supplementary Table 1.** Insulin sensitivity from the hyperglycemic clamp and OGTT (Matsuda Index) by race and diet.

| **Insulin sensitivity measure** | **Baseline Mean (SD)** | **Week 12 Mean (SD)** | **Change Mean (SEM)** | **P for change** |
|---|---|---|---|---|
| Hyperglycemic clamp | | | | |
| EA HC | 9.13 (7.42) | 15.87 (16.65) | 5.31 (4.17) | 0.24 |
| EA CR | 6.33 (5.74) | 9.03 (6.06) | 2.3 (2.74) | 0.42 |
| AA HC | 6.65 (4.49) | 8.12 (4.77) | 1.28 (0.72) | 0.09 |
| AA CR | 10.24 (8.99) | 12.16 (19.96) | 4.07 (3.42) | 0.26 |
| Matsuda Index (OGTT) | | | | |
| EA HC | 2.46 (1.91) | 3.36 (2.27) | 0.89 (0.49) | 0.1 |
| EA CR | 2.08 (1.02) | 2.74 (1.35) | 0.66 (1) | 0.083 |
| AA HC | 2.18 (1.16) | 5.99 (2.98) | 0.66 (0.32) | 0.06 |
| AA CR | 3.11 (3.99) | 4.39 (2.94) | 1.28 (0.75) | 0.11 |

**Abbreviations:** EA: European American, AA: African American, CR: Carbohydrate restricted diet, HC: Higher carbohydrate diet.




**Supplementary Fig. 1.** Schematic of the hyperglycemic clamp. Glucose is injected as a bolus over 2 minutes at 0 and 150 minutes. Arginine is infused from 180-185 minutes. After the time 0 bolus, blood glucose is brought to 50 mg/dL above basal and held there until minute 30 using an infusion of 20% dextrose. After the time 150 bolus, blood glucose is brought to 300 mg/dL and held there until minute 180 using an infusion of 20% dextrose. Data from a representative participant are shown at baseline (open circles) and 12 weeks (filled squares).




Supplementary Fig 2. CONSORT diagram.