Role of Hyperinsulinemia in Non-Alcoholic Fatty Liver Disease (NAFLD) Pathogenesis: Diazoxide Pilot & Feasibility Study

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[redacted]

Protocol Signature Page

I confirm that I have read this protocol, I understand it, and I will work according to this protocol and to the ethical principles of Good Clinical Practice and the applicable federal, state, and local laws, rules, and regulations relating to the conduct of the protocol. Once the protocol has been approved by the IRB, I understand that any modification made during the course of the study must first be approved by the IRB, prior to implementation except when such modification is made to remove an immediate hazard to the participant. I certify that I, and the study staff, have received the requisite training to conduct this research protocol. I agree to maintain adequate and accurate records in accordance with Columbia University and NewYork-Presbyterian Hospital policies; and federal, state and local laws and regulations. I agree to maintain the confidentiality of all information received or developed in connection with this protocol.

Instructions to Principal Investigator: Sign and Date this signature page and print your name. Return the original, completed and signed to the Clinical Trials Office. Retain a copy in the regulatory binder.

[redacted]

Signature of Principal Investigator

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Columbia University
Name of Institution

05/22/2025
Date

PROTOCOL SYNOPSIS

Title	Role of Hyperinsulinemia in NAFLD Pathogenesis: Diazoxide Pilot & Feasibility Study
Short Title	Diazoxide P&F
Protocol Number	AAAU2570
ClinicalTrials.gov ID	NCT05729282
Phase	Phase I
Methodology	Prospective, double blind, randomized, controlled clinical trial
Study Period	Actual date of first participant enrolled: 08/01/2023 Estimated date of last participant enrolled: 08/01/2024
Study Center(s)	Single-center: Columbia University Irving Medical Center
Objectives	To evaluate the effect of diazoxide on hyperinsulinemia in NAFLD
Number of Participants	Up to 72 enrolled to obtain 18 evaluable participants (*i.e.*, completing all study procedures)
Diagnosis and Main Inclusion Criteria	*Diagnoses:* Overweight/obese, insulin resistance (prediabetes, impaired fasting glucose, or elevated HOMA-IR score, with fasting hyperinsulinemia), ± NAFLD *Inclusion criteria:* Age 18-70, speaking English or Spanish, untreated prediabetic dysglycemia (prediabetes or impaired fasting glucose, with fasting hyperinsulinemia), BMI 27-50 kg m ^{2}, ± uncomplicated NAFLD
Study Product, Dose, Route, Regimen	*Investigational:* • Diazoxide oral suspension 1 mg kg ^{1} x 27 doses over 14 days • Diazoxide oral suspension 2 mg kg ^{1} x 27 doses over 14 days • Placebo by mouth x 27 doses over 14 days *Non-investigational:* • FreeStyle Libre Pro continuous glucose monitor (CGM) • Deuterated water (deuterium oxide), 70% ($^{2}H_2O$, D_2O), 50-mL aliquots, 32 doses over 14 days
Duration of administration	27 doses over 14 days
Reference therapy	Placebo
Statistical Methodology	*Primary endpoint:* Changes in fasting insulin and glucose levels in response to diazoxide *vs.* placebo *Significance:* Differences between groups at individual time points tested using the Kruskal-Wallis test. Differences based on static measures or time-integrated (area-under-the-curve) data by *t* test. All analyses will be two sided with a significance threshold of $p < 0.05$.

I. STUDY DESIGN

This is a single-center, randomized, double blinded clinical trial to provide **pilot and feasibility** data on the use of diazoxide to ameliorate hyperinsulinemia in subjects with insulin-resistant (IR) prediabetic dysglycemia with, or at high risk for, non-alcoholic fatty liver disease (NAFLD) (together, IR-NAFLD). Specifically, we will assess the effects of diazoxide on fasting insulin and blood glucose levels. This is prerequisite proof of principle for a larger trial of using diazoxide to investigate the specific impact of hyperinsulinemia on the development and/or progression of IR-NAFLD to non-alcoholic steatohepatitis (NASH). We will recruit participants with a history of overweight/obesity and prediabetic state (*i.e.*, prediabetes or impaired fasting glucose, with fasting hyperinsulinemia), with a diagnosis of, or clinically judged to be at high risk for, uncomplicated non-alcoholic fatty liver disease (NAFLD). Participants will be randomized either to placebo or to diazoxide oral suspension at one of two dose levels (1 mg kg^{-1} or 2 mg kg^{-1}), which will be taken for 27 doses over a 14-day period while consuming a standardized, weight-maintaining diet. Levels of insulin, glucose, and serum lipids will be compared before, during, and after receipt of treatment. The primary outcomes will be changes in insulin and glucose levels in response to diazoxide *vs.* placebo. Up to 72 participants with IR-NAFLD will be randomized at Columbia University Irving Medical Center (CUIMC) to diazoxide 1 mg kg^{-1} *vs.* diazoxide 2 mg kg^{-1} *vs.* placebo in order to obtain data for 18 evaluable participants (*i.e.*, complete all study visits and receive all doses of study product).

The general study design is schematized in Figure I, below. Participants will be recruited from the NAFLD clinic and other outpatient clinical practices of CUIMC. At a Screening Visit (SV-0), following informed consent, recruits will have fasting screening labs drawn, including hemoglobin A_{1c} (HbA_{1c}), fasting plasma glucose (FPG) and insulin levels, and a standard lipid profile. Recruits aged 18-70 will be enrolled if they have a body mass index of 27-50 kg m^{-2} and evidence of insulin resistance. Those whose screening visits do not allow a blood draw before 10:00 will be scheduled for a second, phlebotomy-only screening visit. We define IR for the purposes of the study as prediabetic dysglycemia (FPG 100-125 mg dL^{-1} and/or HbA_{1c} 5.7-6.4%) and/or Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) score of ≥ 2.73; in both cases, recruits must have fasting insulin levels ≥ 13 μIU mL^{-1}. (See also full list of inclusion and exclusion criteria in Section 5). Upon satisfying inclusion and exclusion criteria, participants will be randomized to one of three groups: placebo, DZ1 (diazoxide 1 mg kg^{-1} per dose), or DZ2 (diazoxide 2 mg kg^{-1} per dose). All participants will be instructed to abstain from alcohol consumption for the full duration of the study, beginning 48 h prior to the first study visit. If found ineligible for this study, the screening data may indicate eligibility for inclusion in related protocols, IRB-AAAU3014/U9636/U9702/V2481. In that case, we will invite the enrollee to discuss the other studies and undergo informed consent. Data obtained for screening for this study will double count as screening for the others.

Within 60 d of eligibility determination (although all reasonable efforts will be made for within 30 d), participants will present for Study Visit 1 (SV-1, Day 1). They will be asked to avoid alcohol for 2 d and strenuous physical activity for 1 d prior to SV-1, and to fast after 20:00 the previous evening. Participants will undergo a fasting morning blood draw at roughly 09:00. Investigational agent doses (IAD) and D_2O doses (DW) will be labeled alphanumerically as *DT*, where *D* is the Study Day on which the dose is taken and *T* is the time of day – "A" for AM, "M" for midday (only for D_2O doses on Days 1-4), and "P" for PM. At SV-1, participants will be provided with the first two IAD (both labeled IAD-1P, for "Day 1, PM") – diazoxide 1 mg kg^{-1} each, diazoxide 2 mg kg^{-1} each, or placebo. They will also be given general dietary instructions for the study and be asked to fast for the remainder of the day except for lunch around 13:00 and dinner around 18:00, and then to fast overnight, and not to consume alcohol or sugary beverages for the duration of the study. Next, they will be fitted with a FreeStyle Libre Pro continuous glucose monitor (CGM) while the device is commercially available. (CGM reading will be double-blind until the completion of study procedures for each participant.) Finally, after collection of saliva using a salivette (SC-1), they will

be given three 50-mL aliquots of deuterated water ($^2H_2O/D_2O$): one administered at the visit (DW-1A, for "Day 1, AM") and the others to be taken home to be consumed later that day (DW-1M,P). At 13:00, they will drink DW-1M (for "Day 1, midday"). At 21:00, they will self-administer both IAD-1P syringes simultaneously, for total diazoxide doses of 2 mg kg^{-1} (DZ1 group) or 4 mg kg^{-1} (DZ2 group). This will allow us to evaluate the effect of a single, undivided total daily dose of diazoxide on laboratory outcomes of interest. They will also ingest the third aliquot of D_2O (DW-1P) at that time.

Participants will return for Study Visit 2 (SV-2) on the morning of Day 2. At roughly 09:00, they will have a fasting blood draw (BD-2), collect a second salivette (SC-2) and consume another aliquot of D_2O (DW-2A). Participants will then be provided with 13 investigational agent doses (IAD-2P through -8P) and another 16 aliquots of D_2O (DW-2M through -8P). They will also be furnished with further study diet instructions and will begin the study diet thereafter. At 13:00 on Day 2 they consume DW-2M followed at 21:00 by IAD-2P and DW-2P. They will continue to take investigational agent doses every 12 hours (*i.e.*, at 09:00 for "A"-labeled doses and 21:00 for "P"-labeled doses) thereafter. On Days 3-4, participants continue consume D_2O aliquots three times daily, at 09:00 (DW-3A, 4A); 13:00 (DW-3M, 4M); and 21:00 (DW-3P, 4P). From Days 5 onward, D_2O aliquots are consumed only twice a day, at the same times as the corresponding IAD, at 09:00 (A) and 21:00 (P). On Day 8, mirroring the procedure of Day 1, participants will be asked to fast except for discrete meals at 09:00, 13:00, and 18:00. Study Visit 3 (SV-3) will then take place on the morning of Day 9; fasting labs will be drawn (BD-3) and saliva collected (SC-3) at approximately 09:00 and IAD-9A + DW-9A will be administered immediately thereafter. Participants will then be provided with IAD-9P through -14P and DW-9P through -14P. They and will continue to take doses of their assigned investigational agent and D_2O aliquots every 12 hours, with IAD-9P and DW-9P taken as usual that day at 21:00. This pattern will repeat as shown in Figure I.i again on Days 14-15: fasting except for three discrete meals on Day 14, followed by Study Visit 4 for morning blood draw (BD-4) and saliva collection (SC-4) on Day 15. CGM will be removed on Day 15 at SV-4. Study completion is defined as having reached the point of completing the SV-4 blood draw on Day 15.

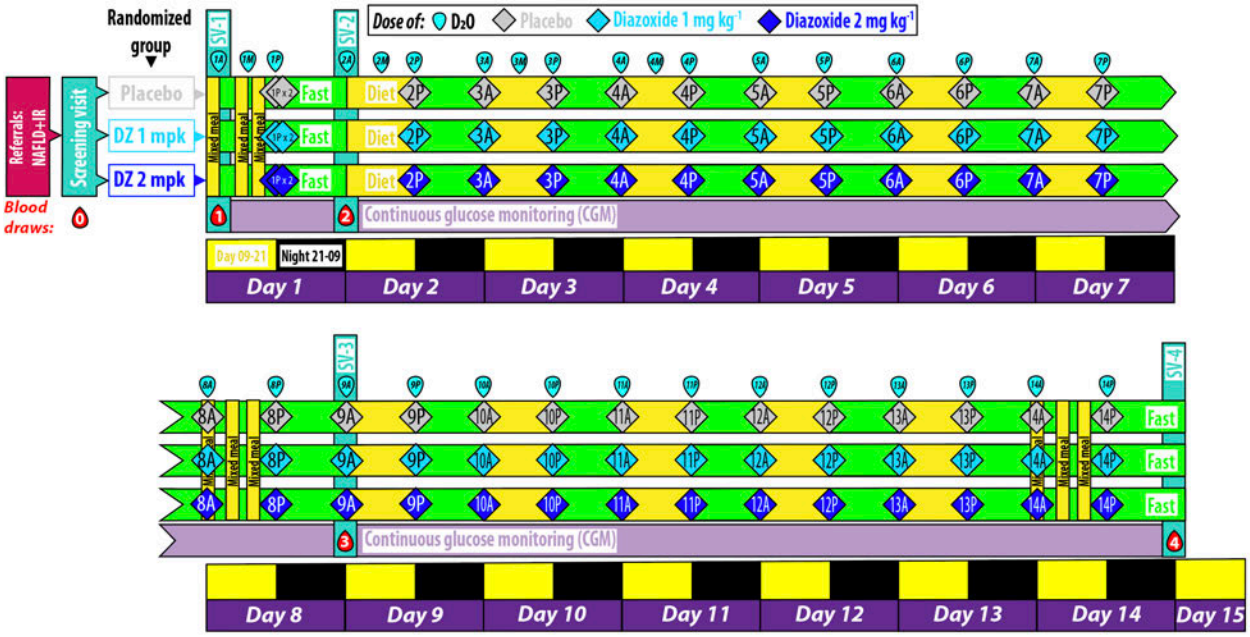


Figure I.i: Schematic of study design. DZ = diazoxide, mpk = mg per kg, SV = study visit

We paused the study after three participants completed each of the three arms (*i.e.*, completion of 3 of the block-randomized cohorts of 3 participants each) to perform an unblinded interim analysis to determine the possibility of discontinuing one of the two diazoxide arms. The interim data were inconclusive (due to variability from the small sample size) and so we decided to continue the study without a protocol change.

II. OBJECTIVES

Primary objective: To perform a **pilot and feasibility** study to determine the impact of two doses of diazoxide on **insulin and glucose levels** in service of a planning a larger study on the impact of hyperinsulinemia on hepatic *de novo* lipogenesis.

Secondary objectives: To investigate the impact of diazoxide on lipid/lipoprotein metabolism, including:
1. Total circulating lipid/lipoprotein levels:
   a. Serum triglyceride (TG) levels
   b. Serum free fatty acid (FFA) levels
   c. Serum apolipoprotein B (apoB) levels

Exploratory objectives: To investigate the impact of diazoxide on:
   d. Continuous glucose monitoring (CGM) profile
      - *Note:* CGM only to be performed on patients when the FreeStyle Libre Pro blinded continuous glucose monitor is commercially available.
   e. Hepatic *de novo* lipogenesis in VLDL-TG and/or total plasma TG
      - + D_2O enrichment in total body water
   f. Triglyceride levels in very low-density lipoprotein (VLDL-TG)
   g. Apolipoprotein B-100 levels in very low-density lipoprotein (VLDL-ApoB)
   h. Serum bile acid profile
   i. Serum adiponectin
   j. Serum or plasma diazoxide level

III. TARGET PARTICIPANT POPULATION

Adult men or women aged 18-70 years with evidence of insulin resistance – either untreated pre-diabetic dysglycemia, defined as hemoglobin A_{1c} (HbA_{1c}) of 5.7-6.4% and/or fasting plasma glucose (FPG) of 100-125 mg dL^{-1} or HOMA-IR ≥ 2.73 – as well as fasting hyperinsulinemia of ≥ 13 μIU mL^{-1}; body mass index of 27-50 kg m^{-2}; and with, or clinically judged to be at high risk for, uncomplicated non-alcoholic fatty liver disease (*i.e.*, non-cirrhotic and no decompensating events)

IV. MEDICINAL PRODUCT, DOSAGE, AND MODE OF ADMINISTRATION

Table IV.i. *Investigational medicinal products*

Agent	Manufacturer	Study group	Dosing regimen			
			Dose number	*Dose name*	*Study Days taken*	*Dosing interval*
Diazoxide [a,b] (50 mg mL^{-1} oral suspension)	e5 Pharma	Diazoxide 1 mg kg^{-1}	1+2	1P (x2)	Day 1	Simultaneous
			3-27	2P-14P	Days 2-14	Every 12 hours
		Diazoxide 2 mg kg^{-1}	1+2	1P (x2)	Day 1	Simultaneous
			3-27	2P-14P	Days 2-14	Every 12 hours
Placebo [c] (drug-free vehicle)	CUIMC RP [d]	Placebo (40 μL kg^{-1})	1+2	1P (x2)	Day 1	Simultaneous
			3-27	2P-14P	Days 2-14	Every 12 hours

[a] Drug suspended in vehicle of sorbitol, mint flavor, sodium benzoate, methylparaben, poloxamer 188, propylparaben, and water

[b] FDA has declared exempt from IND (#163465, see attached documentation)
[c] Comprises flat diet tonic water (Canada Dry®) mixed with peppermint extract (McCormick®) and thickened with xanthan gum (Carrington Farms®)
[d] CUIMC Research Pharmacy

V. DURATION OF TREATMENT

Participants will complete a one-day screening and enrollment visit. They will have screening labs done on that day and if no exclusion criteria are met, they will begin the study protocol within 30 d. The study protocol includes 27 doses of diazoxide or placebo over 14 d. Outpatient Study Visits will occur on Study Days 1, 2, 9, and 15.

VI. STATISTICAL CONSIDERATIONS

VI.1 – Sample size

Based on the accrual rate we have observed to this point, we will need to enroll up to 24 participants per group (total: 72) to obtain 6 evaluable participants per group (total: 18), allowing us to detect a decrease in fasting insulin of 25% at $\alpha < 0.05$, $\beta > 0.80$, comparable to the effect size in obesity trials of diazoxide at 2 mpk total daily dose (TDD) [1]. This is the magnitude of reduction in hyperinsulinemia that we aim to induce for later studies, and this degree of change in insulin levels has been shown to correlate with significantly lower rates of hepatic *de novo* lipogenesis [2-4].

VI.2 – Statistical Analyses

The primary endpoint will be the percent change (and standard error in the mean) in fasting insulin and glucose levels in response to treatment with diazoxide *vs.* placebo. Data will be presented as mean ± standard error in the mean for normally distributed variables and as median ± interquartile range for non-normally distributed variables. Data will be analyzed by mixed-effects model (to overcome missing-data limitation of repeated measures ANOVA) followed by Tukey's multiple comparisons test or, where applicable, by simple linear regression. Multivariate and partial regression analyses will be performed where needed by standard methods. All analyses will be two sided with a significance threshold of $p < 0.05$.

TABLE OF CONTENTS

PROTOCOL SIGNATURE PAGE ..... **III**

PROTOCOL SYNOPSIS ..... **IV**

I. STUDY DESIGN ..... **V**

II. OBJECTIVES ..... **VII**

III. TARGET PARTICIPANT POPULATION ..... **VII**

IV. MEDICINAL PRODUCT, DOSAGE, AND MODE OF ADMINISTRATION ..... **VII**

V. DURATION OF TREATMENT ..... **VIII**

VI. STATISTICAL CONSIDERATIONS ..... **VIII**

ABBREVIATIONS **4**

1. INTRODUCTION **6**

1.1 – BACKGROUND 6
1.2 – RESEARCH APPROACH: HYPERINSULINEMIA REDUCTION AS A STRATEGY FOR THE TREATMENT OF SELECTIVE IR 7
1.3 – RESEARCH HYPOTHESES 8

2. STUDY OBJECTIVES AND ENDPOINTS **9**

2.1 – PRIMARY OBJECTIVE 9
2.2 – SECONDARY OBJECTIVES 9
2.3 – EXPLORATORY OBJECTIVES 9

3. STUDY ENDPOINTS **10**

3.1 – PRIMARY ENDPOINT 10
3.2 – SECONDARY ENDPOINTS 10

4. STUDY DESIGN AND RATIONALE **10**

4.1 – SCHEMATIC OVERVIEW 10
4.2 – STUDY DESCRIPTION 11
4.3 – RATIONALE FOR RESEARCH STRATEGY 12
4.5 – ALTERNATIVE RESEARCH STRATEGIES 15

5. STUDY PARTICIPANTS **16**

5.1 – PARTICIPANTS 16
5.2 – INCLUSION CRITERIA 16
5.3 – EXCLUSION CRITERIA 17
5.4 – FOLLOW-UP OF ABNORMAL SCREENING LABORATORY RESULTS 20
5.5 – INCLUSION OF WOMEN AND MINORITIES 20
5.6 – VULNERABLE POPULATIONS 21
5.7 – PARTICIPANT RECRUITMENT 21

6. STUDY ONBOARDING AND WITHDRAWAL PROCEDURES **28**

6.1 – CUIMC RESEARCH PARTICIPANT ONBOARDING 28

6.2 – CLINICAL TRIAL DESIGN CONSIDERATIONS 29
6.3 – STUDY SAMPLE POLICIES 30
6.4 – STUDY WITHDRAWAL 31

7. STUDY CALENDAR 31

7.1 – SCREENING VISIT (SV-0) 31
7.2 – SCREENING PROCEDURES 32
7.3 – STUDY VISITS 35
7.4 – EXPLANATION OF STUDY PROCEDURES 39
7.5 – SCHEDULE OF BLOOD DRAWS 42

8. MEASUREMENT OF EFFECT 42

8.1 – BLOOD TESTS 42
8.2 – MEASUREMENT OF PRIMARY ENDPOINT 45
8.3 – MEASUREMENT OF SECONDARY ENDPOINTS 46
8.4 – MEASUREMENT OF EXPLORATORY ENDPOINTS 47

9. STATISTICAL CONSIDERATIONS 50

9.1 – GENERAL STATISTICAL CONSIDERATIONS 50
9.2 – SIZE AND ACCRUAL RATE 51

10. INVESTIGATIONAL STUDY PRODUCTS 51

10.1 – INVESTIGATIONAL STUDY PRODUCT REGULATORY POLICIES 51
10.2 – INVESTIGATIONAL PRODUCT HANDLING 52
10.3 – INVESTIGATIONAL PRODUCT INSPECTION 52
10.4 – DOSE PREPARATION STEPS 52
10.5 – DISPENSING DOSES OF INVESTIGATIONAL PRODUCT 53

11. NON-INVESTIGATIONAL MEDICINAL STUDY PRODUCTS 54

11.1 – DEUTERATED WATER/DEUTERIUM OXIDE ($^2H_2O/D_2O$), 70% (IN H_2O) 54

12. SAFETY AND ADVERSE EVENTS 55

12.1 – ADVERSE EVENTS AND UNANTICIPATED PROBLEMS 55
12.2 – SAFETY MATTERS RELATED TO BASIC STUDY PROCEDURES 57
12.3 – SAFETY MATTERS RELATED TO DIAZOXIDE 58
12.4 – SAFETY MATTERS RELATED TO INVESTIGATIONAL PLACEBO 61
12.5 – SAFETY-RELATED STUDY INTERRUPTION 61
12.6 – UNANTICIPATED RISKS OR ADVERSE EVENTS 62

13. PROTECTION OF HUMAN PARTICIPANTS 62

14. DATA COLLECTION, MAINTENANCE, AND REPORTING 63

14.1 – DATA HANDLING 63
14.3 – CONFIDENTIALITY 64
14.4 – SOURCE DOCUMENTS 65
14.5 – CASE REPORT FORMS 65
14.6 – RECORDS RETENTION 65
14.7 – SAMPLE AND DATA USE BY COLLABORATORS ON THIS STUDY 65
14.8 – SAMPLE RETENTION FOR INSULIN RESISTANCE BIOBANK (FUTURE USE OF BIOSPECIMENS) 67

15. STUDY FINANCES 68

15.1 – CONFLICT OF INTEREST 69
15.2 – PARTICIPANT COMPENSATION: STIPENDS AND REIMBURSEMENT 69

16. PUBLICATION PLAN 70

17. REFERENCES 71

ABBREVIATIONS

The following abbreviations and special terms are used throughout this clinical study protocol:

Abbreviation or special term	Explanation
ACE	Angiotensin converting enzyme
AE	Adverse event
ALT	Alanine aminotransferase
ApoB	Apolipoprotein B
ARB	Angiotensin receptor blocker
aq	Aqueous solution (*i.e.*, in water)
AST	Aspartate aminotransferase
AUC	Area under the curve
β-hCG	Human chorionic gonadotropin, β subunit
BMI	Body mass index (in kg m^{2})
BMP	Basic metabolic panel (a.k.a. basic chemistry, chem-7)
BMR	Basal metabolic rate
BP	Blood pressure
bpm	Beats per minute (*i.e.*, heart rate)
CBC	Complete blood count
CGM	Continuous glucose monitor(ing)
ChREBP	Carbohydrate response element binding protein
CRF	Case report form
CTMS	Clinical trial data management system
CUIMC	Columbia University Irving Medical Center
d	Day(s)
DBP	Diastolic blood pressure
DNL	*De novo* lipogenesis (hepatic, if not otherwise specified)
DPP4	Dipeptidyl peptidase-4
DW	Deuterated water dose/aliquot
DZ1	Group randomized to diazoxide at 1 mg kg^{1} per dose
DZ2	Group randomized to diazoxide at 2 mg kg^{1} per dose
ELISA	Enzyme-linked immunosorbent assay
EKG	Electrocardiogram
EMR	Electronic medical record (*i.e.*, NYP Epic and iNYP)
FDA	United States Food and Drug Administration
FFA	Free fatty acids (a.k.a. non-esterified fatty acids)
FPG	Fasting plasma glucose
FPLC	Fast protein liquid chromatography
FPLP	Fractionated plasma lipoproteins
GC	Gas chromatography
GCP	Good Clinical Practice
GLP-1	Glucagon-like peptide-1
GMP	Good Manufacturing Practice
h	Hour(s)
HbA_{1c}	Hemoglobin A_{1c} (*i.e.*, glycated hemoglobin)
HDL	High-density lipoprotein
HDL-C	High-density lipoprotein cholesterol
HOMA-IR	Homeostasis model assessment of insulin resistance
HR	Heart rate
IAD	Investigational agent dose

IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IICTR	Irving Institute for Clinical and Translational Research
InsR	Insulin receptor
IR	Insulin resistance
IRB	Institutional Review Board
IRS	United States Internal Revenue Service
IU	International units (prefixed *m-* for milli-)
IV	Intravenous(ly)
LDL	Low-density lipoprotein
LDL-C	Low-density lipoprotein cholesterol
LFT	Liver function tests (a.k.a. hepatic function panel)
LLN	Lower limit of normal range
min	Minute(s)
mo	Month(s)
mpk	Milligrams per kilogram of body weight
MPT	Microbiological and pyrogen tested
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NYP	NewYork-Presbyterian Hospital
OCRU	Outpatient clinical research unit
OGTT	Oral glucose tolerance test
PCSK9	Proprotein convertase subtilisin/kexin type 9
s	Second(s)
SBP	Systolic blood pressure
SGLT2	Sodium-glucose cotransporter 2
SID	Study identification number
SOP	Standard operating procedure
SST	Serum separator tube (“gold-top tube”)
t	Hyperglycemic clamp time point (in hours)
T2DM	Type 2 diabetes mellitus
TG	Triglyceride (a.k.a. triacylglycerol)
ULN	Upper limit of normal (reference range)
UP	Unexpected problem
USDA	United States Department of Agriculture
VLDL	Very low-density lipoprotein
VLDL-TG	Very low-density lipoprotein triglyceride
v/v	Volume per volume
WB	Western blot
WC	Waist circumference
w/v	Weight per volume
w/w	Weight per weight

1. INTRODUCTION

1.1 – Background

1.1.1. Epidemiologic association of type 2 diabetes and non-alcoholic fatty liver disease

Although high blood sugar defines type 2 diabetes mellitus (T2DM), it is, at its heart, a disorder of lipid metabolism [5]. This multifactorial breakdown of insulin's control of fat handling is most commonly considered in connection with cardiovascular disease [6], but T2DM lipid dysmetabolism is also more insidiously involved in the world's leading chronic liver disorder: non-alcoholic fatty liver disease (NAFLD) [7]. In fact, more than half of patients with T2DM have some degree of NAFLD, and a majority of those patients have progressed on to non-alcoholic steatohepatitis (NASH) [8]. Unchecked, NASH can progress to cirrhosis and hepatocellular carcinoma [9], risks that are further exacerbated by T2DM [10]. However, considerable mystery still shrouds the connection between these two common but deadly metabolic diseases, hindering the development of direly needed, rational treatments for T2DM-associated NAFLD.

1.1.2. Mechanisms linking insulin signaling to non-alcoholic fatty liver disease

The epidemiologic connection between T2DM and NAFLD implies a common pathogenic basis, although causality is likely bidirectional and mutually reinforcing [11, 12]. Insulin resistance appears to serve as the foundation for this nexus through perturbations in TG metabolism, chiefly high circulating TG (hypertriglyceridemia) and hepatic steatosis leading to NAFLD or NASH [2-4, 13-17]. Insulin signaling regulates multiple lipid-metabolic processes in the liver but it has become apparent that excessive *de novo* lipogenesis (DNL) is the hallmark of IR-associated NAFLD (IR-NAFLD) [2-4, 13, 18]. Thus, we will focus our research efforts in the long run chiefly on hepatic DNL. Perhaps surprisingly in light of the foregoing, insulin signaling actually stimulates liver DNL [19, 20], but despite much circumstantial evidence, the signaling route between the insulin receptor (InsR) and SREBP-1c has been only partially mapped.

1.1.3. Hyperinsulinemia as a metabolic bad actor

The most common model of garden-variety IR is *selective*: insulin progressively loses its ability to blunt hepatic glucose production while retaining some degree of sensitivity to insulin's stimulation of DNL [19, 21-23]. However, the hyperinsulinemia needed to compensate for IR drives hepatic DNL to excess as an unfortunate tradeoff in pursuit of euglycemia [21, 24]. Indeed, hepatic insulin exposure is significantly augmented in NAFLD, due both to increased insulin delivery to the liver and decreased fractional hepatic insulin clearance [16]. Congruently, diabetes treatment with basal insulin peglispro, which exhibits preferential action in the liver, significantly increases liver fat content [25], serum VLDL-TG, and VLDL-TG secretion rate [26], relative to comparable treatment with non-liver-selective insulin glargine.

Although this seemingly pro-steatotic hyperinsulinemia is, again, generally considered to compensate for poorly suppressible hepatic glucose production (among others), some have proposed alternative origins. For instance, chronic dysregulation of fatty acid metabolism [27] or simply obesity *per se* [28] may trump an underlying hyperglycemic drive as the principal stimulus for hyperinsulinemia. On the other hand, this hyperinsulinemia may not solely reflect a compensatory mechanism (*i.e.*, secondary hyperinsulinemia) but also may evolve non-adaptively, independently of glucose levels, as a *primary* hyperinsulinemia. Such primary hyperinsulinemia may then itself help propel the development of T2DM, for example by inducing further IR [19, 29, 30], promoting weight gain [31-33], and/or fostering inflammation [34-37]. The primary hyperinsulinemia hypothesis is not mutually exclusive with the concept of selective IR and, in fact, could even accentuate hepatic IR selectivity and its steatotic consequences.

This concept of hyperinsulinemia itself as a metabolic bad actor is not novel; influential thinkers in diabetology have espoused it for decades [5, 24, 38-42] based on a wealth of supporting evidence. At the epidemiologic level, hyperinsulinemia was found to be the most significant predictor of progression to diabetes in a longitudinal study of older adults with prediabetes, even after adjusting for body mass index and multiple other variables [43]. Another cohort study analyzed the statistical interaction of hyperinsulinemic-euglycemic clamp and glucose tolerance test data from over 1100 individuals to glean an "insulin hypersecretor" sub-phenotype that predicted a quicker deterioration of glucose tolerance

and less favorable lipid profile [44]. These associations might appear merely to reflect the consequences of IR itself, but considerable mechanistic work supports the biological plausibility of an IR-causal role for hyperinsulinemia [19, 20, 29, 31-37, 45-48]. Obesity has been associated with circulating insulin levels over 50% than in lean controls matched for insulin sensitivity by hyperinsulinemic-euglycemic clamp [28]. Congruently, adiposity was associated with increased insulin secretion independently of the degree of IR [49]. Insulin levels need not even be *absolutely* high to elicit IR; *relative* hyperinsulinemia – for example, unabating postprandial-grade insulinemia during fasting in overtreated type 1 diabetes – has also been demonstrated to desensitize insulin signaling [50, 51]. At a more granular level, chronic hyperinsulinemia itself can induce IR through downregulation of insulin receptor levels [29, 52, 53]. We have modeled this phenomenon in primary hepatocytes, demonstrating that insulin receptor downregulation spurs differential (*i.e., selective*) responses of the lipid *vs.* glucose arms to an acute insulin challenge [19].

Whether deleterious hyperinsulinemia is primary or secondary/reactive, high insulin levels certainly seem to selectively augment DNL and thereby promote NAFLD [2, 17, 54-58]. Powerful though the evidence appears, ultimately it remains circumstantial. We aim ultimately to test this hypothesis directly and thereby provide the first proof in humans that hyperinsulinemia is a necessary condition for excessive DNL in common, selective IR (see Fig. 1.i).

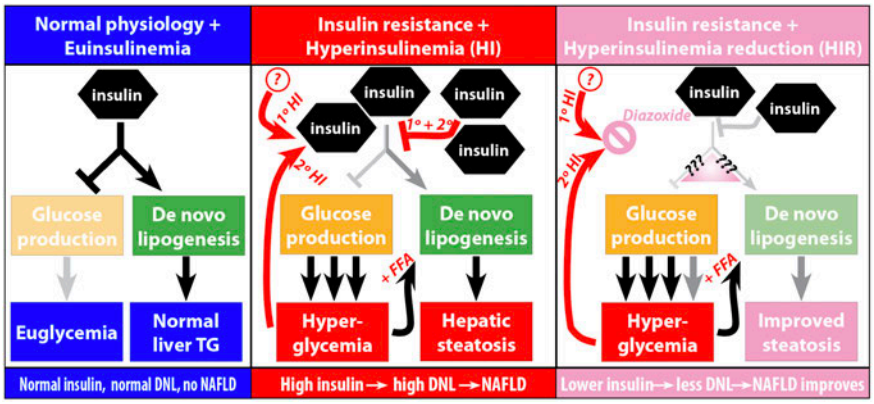


Figure 1.i. Central hypothesis *vis-à-vis* hepatic insulin resistance

1.2 – Research Approach: Hyperinsulinemia reduction as a strategy for the treatment of selective IR

1.2.1. Diazoxide for hyperinsulinemia reduction

Our long-term objective is to assess the impact specifically of directly lowering insulin levels on hepatic triglyceride metabolism; to our knowledge, no human studies have yet done this. Hyperinsulinemia reduction (HIR) can be achieved under special insulin- and glucose-controlled "pancreatic clamp" conditions [59-64] or with pharmacologic agents that block insulin secretion [65-67]. Such drugs include somatostatin or its analogues (*e.g.*, octreotide) [66, 68, 69] and – our selected agent – diazoxide, an activator of the ATP-sensitive potassium channel whose closure is prerequisite for β-cell insulin exocytosis [65]. Diazoxide has the advantages of oral administration, modulating endogenous insulin production [65, 70], maintaining the portal-systemic gradient of endogenous insulin levels [71], and ability to study the longer periods of time that are necessary to fully appreciate changes in hepatic DNL [3, 13]. Diazoxide has a long track record of off-label use in clinical trials. For instance, several studies – with varying success – have investigated diazoxide as a weight-loss medication [1, 31, 72-74] or as a preservative means of "resting" failing pancreatic β-cells [75-79].

Interestingly, diazoxide-induced HIR in obesity trials did not necessarily lead to proportional rises in blood glucose levels [73, 74, 80]. In fact, in some studies, the fall in fasting insulin levels did not significantly change fasting blood glucose at all, even after weeks to months of treatment [1, 31, 65, 72, 81]. Although improving insulin sensitivity in these trials may help to explain the relative glycemic tolerance to decreasing insulin levels, the apparent uncoupling of insulin and glucose levels has been observed even with acute or subacute diazoxide exposure [1, 65, 72, 82] and in the absence of weight loss [1]. This lends further, albeit indirect, evidence that hyperinsulinemia in common IR states such as obesity is not purely a compensatory mechanism for impaired insulin-simulated glucose disposal [27, 44]. This interpretation is complicated because the relative stability in glucose levels may be partially attributable to a separate, likely central, mechanism by which

diazoxide influences hepatic glucose production [82]. However, this extra-pancreatic glycemic effect of diazoxide dissipates in people with type 2 diabetes [83], and therefore may also be attenuated in insulin-resistant prediabetic dysglycemia. Furthermore, non-diazoxide-based HIR in obese animals is associated with a paradoxical improvement in dysglycemia [48] that is independent of body weight [37, 47]. Taken together, these human and animal studies provide further evidence that hyperinsulinemia in common IR states such as obesity is not solely a compensatory mechanism for an underlying hyperglycemic drive [27, 44]. The apparent ability to dissociate insulin from glucose levels in individuals with IR renders it particularly attractive as a tool for testing our hypothesis. In fact, as aforementioned, deintensification of target tissues' insulin exposure may allow for some degree of insulin re-sensitization, which is expected to improve both hyperglycemia *and* hepatic steatosis.

1.2.2. Need for preliminary pilot and feasibility study

The theoretical advantages of diazoxide as a safe and effective means of temporarily lowering insulin levels are clear. However, diazoxide presents certain disadvantages that we need to account for prior to launching a large, time- and resource-intensive clinical trial of its impact on hepatic TG metabolism. First, and most notably, the impact of single doses [65, 70, 82, 83] or short courses [1, 84, 85] of diazoxide on insulin levels has been rather variable in the published literature, likely due to differences in study populations, timing, feeding state, and concurrent interventions. Significantly, diazoxide's volume of distribution, and therefore the serum levels it achieves, vary considerably with adiposity, which likely also contributed to the observed heterogeneity in its insulin-lowering potency [72, 86]. We therefore need to assess the magnitude and time course of diazoxide-mediated HIR to expect in our IR-NAFLD population. To this end, we also would like to test the effect of two doses of diazoxide in order to evaluate the insulinostatic effectiveness of each in our hands and to weigh against any potential adverse effects that crop up.

Second, as discussed above, lowering of insulin levels in people with IR does not necessarily translate into clearly proportional rises in blood glucose [1, 31, 65, 73, 74, 80, 81]. Nevertheless, diazoxide treatment would likely raise blood glucose levels at least slightly in people with IR [73, 74, 80]. It is important for us to know what magnitude of change to expect because (a) we wish to avoid hyperglycemia in future studies from a risk perspective and (b) concomitant rises in blood glucose levels, if significant, may confound the interpretation of insulin-related changes in hepatic DNL [17, 87]. If diazoxide substantially raises blood glucose levels, we may need to include a concomitant hyperglycemic clamp atop diazoxide treatment in future studies in order to neutralize confounding by hyperglycemia. However, if diazoxide only minimally (or even indiscernibly) affects blood glucose levels despite clearly suppressing insulin secretion, we will be able to avoid the considerable technical burden that clamp studies entail.

Although the primary goal of this P&F study is to assess diazoxide's impact on glucose and insulin, we will also use it as an opportunity to preliminarily gauge its effect on DNL non-invasively through labeling of body water with orally ingested D_2O. However, we consider this an exploratory endpoint because we expect the sample size to be too small to be able to discern a significant difference in DNL even with effective insulin lowering. Thus, although the DNL data produced will likely not be conclusive, this experience will help us become accustomed to the experimental procedures associated with D_2O labeling and DNL measurement.

1.3 – Research hypotheses

In our **pilot and feasibility** study, we plan to administer one of two doses of diazoxide *vs.* placebo to participants with IR in order to assess their impact on insulin and glucose levels.

1.3.1. Primary hypothesis

Diazoxide will produce a dose-dependent lowering of fasting insulin levels and will result in a mild rise in fasting blood glucose levels, reflected as well as fasting/overnight continuous glucose monitoring (CGM).

1.3.2. Secondary hypotheses

Decreasing insulin levels with diazoxide:

- Triglycerides: Expect that TG will slightly decrease over the course of the study due to inhibition of *de novo* lipogenesis and, perhaps, some improvement in insulin sensitivity. However, this effect may be counterbalanced by a loss of insulin's inhibition of hepatic secretion of VLDL.
    - Again, our ultimate goal is to specifically assess hepatic DNL, which may change independently of total serum TG levels [2, 4, 13, 88, 89].
- Apolipoprotein B: Expect to slightly increase serum ApoB levels due to loss of insulin's inhibition of VLDL secretion
- Free fatty acids: Will raise circulating free fatty acid levels, but likely not to a great degree given the exquisite insulin sensitivity of WAT lipolysis

1.3.3. Exploratory hypotheses:

- Continuous glucose monitoring (non-fasting): Diazoxide treatment will likely increase glucose levels across the day, particularly after meals.
- Hepatic *de novo* lipogenesis: Diazoxide treatment will likely decrease hepatic DNL, but may not have a statistically significant effect.
- Bile acids: Diazoxide treatment will likely increase the ratio of 12α-hydroxylated bile acids, or their synthetic intermediates, to non-12α-hydroxylated species
- Adiponectin: Diazoxide treatment will have no direct effect on adiponectin levels over the time course of the study, or may slightly increase
- Insulin-like growth factor binding protein-1 (IGFBP-1): IGFBP-1 levels will track in inverse proportion to insulin.

2. STUDY OBJECTIVES AND ENDPOINTS

2.1 – Primary Objective

To perform a **pilot and feasibility** study to determine the impact of a short course of two dosages of diazoxide on insulin and glucose levels in service of a planning a larger study on the impact of hyperinsulinemia on hepatic *de novo* lipogenesis.

2.2 – Secondary Objectives

To investigate the impact of diazoxide on lipid/lipoprotein metabolism, including:

a. Serum triglyceride (TG) levels
b. Serum free fatty acid (FFA) levels
c. Serum apolipoprotein B (apoB) levels

- To bank residual samples in our Insulin Resistance Biobank.

2.3 – Exploratory Objectives

To investigate the impact of diazoxide on:

d. Continuous glucose monitoring (CGM) profile during non-fasting hours
e. Hepatic *de novo* lipogenesis
f. Triglyceride levels in very low-density lipoprotein (VLDL)
g. Apolipoprotein B-100 levels in VLDL
h. Serum bile acid profile
i. Serum adiponectin
j. Serum or plasma insulin-like growth factor binding protein-1 (IGFBP-1)

3. STUDY ENDPOINTS

3.1 – Primary endpoint

Absolute and relative changes in fasting levels of glucose and insulin, including by continuous glucose monitoring (CGM).

3.2 – Secondary endpoints

Table 3.i *– Secondary endpoints*

2° endpoint component	Method	Assay units
a. Δ Serum TG levels	Direct measurement in serum, by automatic analyzer	mg dL^{-1}
b. Δ Serum FFA levels	Direct measurement in serum, by automatic analyzer	mmol L^{-1}
c. Δ Serum apolipoprotein B levels	Direct measurement in serum, by ELISA and/or by WB	mg dL^{-1}

3.3 – Exploratory endpoints:

Table 3.ii *– Exploratory endpoints*

Exploratory endpoint component	Method	Assay units
d. Continuous glucose monitoring (CGM) profile during non-fast times	FreeStyle Libre Pro continuous glucose monitor (while commercially available)	mg dL^{-1}
e. Hepatic *de novo* lipogenesis (DNL)	Mass spectrometric analysis of ^2H enrichment in VLDL-TG and/or total plasma TG	% FA in VLDL-/total plasma TG
f. Δ TG levels in VLDL	Colorimetric assay in plasma samples after VLDL fractionation	mg dL^{-1}
g. Δ ApoB levels in VLDL	ELISA/WB in plasma samples after VLDL fractionation	mg dL^{-1}
h. Δ Plasma levels of BA species and precursors	LC-MS/MS[a] in plasma (14 BA species + 2 precursors)	µmol L^{-1}
i. Δ Plasma adiponectin level and complex distribution	ELISA in plasma and/or WB	ng dL^{-1}
j. Δ Plasma or serum insulin-like growth factor binding protein-1 (IGFBP-1) level	ELISA in serum or plasma	ng mL^{-1}
k. Serum or plasma diazoxide level	LC-MS/MS[a] in serum or plasma	ng mL^{-1}

[a] Liquid chromatography-tandem mass spectrometry

4. STUDY DESIGN AND RATIONALE

4.1 – Schematic overview

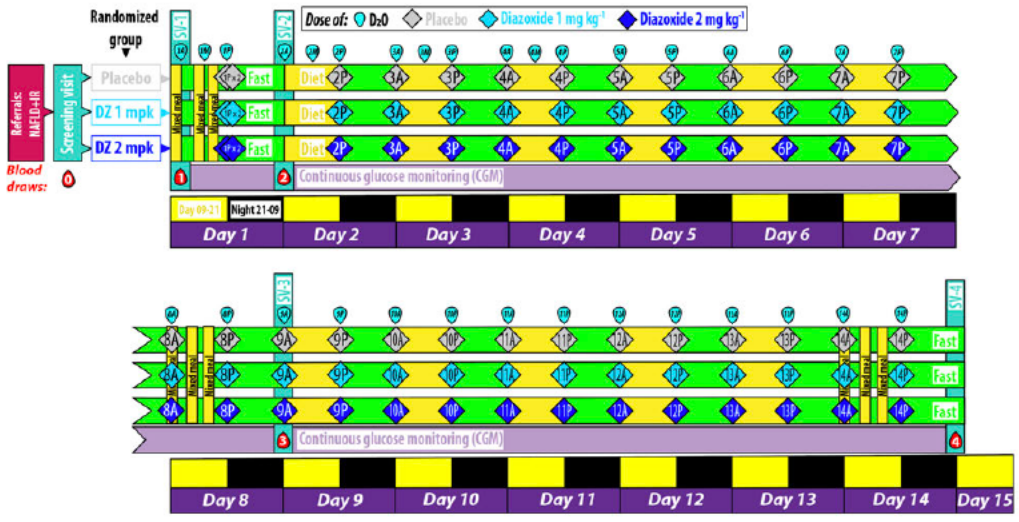


Figure 4.i. *Schematic overview of the study. DZ = diazoxide, MPK = mg per kg, SV = study visit*

4.2 – Study description

This is a single-center, randomized, double blinded clinical trial to provide **pilot and feasibility** data on the use of diazoxide to ameliorate hyperinsulinemia in subjects with, or at high risk for, insulin-resistant (IR) prediabetic dysglycemia and non-alcoholic fatty liver disease (NAFLD) (together, IR-NAFLD). Specifically, we will assess the effects of diazoxide on fasting insulin and blood glucose levels. This is prerequisite proof of principle for a larger trial of using diazoxide to investigate the specific impact of hyperinsulinemia on the development and/or progression of IR-NAFLD to non-alcoholic steatohepatitis (NASH). We will recruit participants with a history of overweight/obesity and prediabetic state (*i.e.*, prediabetes or impaired fasting glucose, with fasting hyperinsulinemia), with a diagnosis of, or clinically judged to be at high risk for, uncomplicated non-alcoholic fatty liver disease (NAFLD). Participants will be randomized either to placebo or to diazoxide oral suspension at one of two dose levels (1 mg kg^{-1} or 2 mg kg^{-1}), which will be taken for 27 doses over a 14-day period while consuming a standardized, weight-maintaining diet. Levels of insulin, glucose, and serum lipids will be compared before, during, and after receipt of treatment. The primary outcomes will be changes in insulin and glucose levels in response to diazoxide *vs.* placebo. Up to 72 participants with IR-NAFLD will be randomized at Columbia University Irving Medical Center (CUIMC) to diazoxide 1 mg kg^{-1} *vs.* diazoxide 2 mg kg^{-1} *vs.* placebo in order to obtain data for 18 evaluable participants (*i.e.*, complete all study visits and receive all doses of study product).

The general study design is schematized in Figure I, below. Participants will be recruited from the NAFLD clinic or other outpatient clinical practices of CUIMC. At a Screening Visit (SV-0), following informed consent, recruits will have fasting screening labs drawn, including hemoglobin A_{1c} (HbA_{1c}), fasting plasma glucose (FPG) and insulin levels, and a standard lipid profile. Recruits aged 18-70 will be enrolled if they have a body mass index of 27-50 kg m^{-2} and evidence of IR. Those whose screening visits do not allow a blood draw before 10:00 will be scheduled for a second, phlebotomy-only screening visit. We define IR for the purpose of this study as prediabetic dysglycemia (FPG 100-125 mg dL^{-1} and/or HbA_{1c} 5.7-6.4%) and/or a Homeostasis Model of Assessment Insulin Resistance (HOMA-IR) score of ≥ 2.73; in both cases, recruits must have a fasting insulin levels ≥ 13 μIU mL^{-1}. (See also full list of inclusion and exclusion criteria in Section 5). Upon satisfying inclusion and exclusion criteria, participants will be randomized to one of three groups: placebo, DZ1 (diazoxide 1 mg kg^{-1} per dose), or DZ2 (diazoxide 2 mg kg^{-1} per dose). All participants will be instructed to abstain from alcohol consumption for the full duration of the study, beginning 48 h prior to the first study visit. If found ineligible for this study, the screening data may indicate eligibility for inclusion in related protocols, IRB-AAAU3014/U9636/U9702/V2481. In that case, we will invite the enrollee to discuss the other studies and undergo informed consent. Data obtained for screening for this study will double count as screening for the others.

Within 60 d of eligibility determination (although all reasonable efforts will be made for within 30 d), participants will present for Study Visit 1 (SV-1, Day 1). They will be asked to avoid alcohol for 2 d and strenuous physical activity for 1 d prior to SV-1, and to fast after 20:00 the previous evening. Participants will undergo a fasting morning blood draw at roughly 09:00,. Investigational agent doses (IAD) and D_2O doses (DW) will be labeled alphanumerically as *DT*, where *D* is the Study Day on which the dose is taken and *T* is the time of day – "A" for AM, "M" for midday (only for D_2O doses on Days 1-4), and "P" for PM. At SV-1, participants will be provided with the first two investigational agent (IAD) (both labeled IAD-1P, for "Day 1, PM") – diazoxide 1 mg kg^{-1} each, diazoxide 2 mg kg^{-1} each, or placebo. They will also be given general dietary instructions for the study and be asked to fast for the remainder of the day except for lunch around 13:00 and dinner around 18:00, and then to fast overnight, and not to consume alcohol or sugary beverages for the duration of the study. Next, they will be fitted with a FreeStyle Libre Pro continuous glucose monitor (CGM) while the device is commercially available. (CGM reading will be double-blind until the completion of study procedures for each participant.) Finally, after collection of saliva using a salivette (SC-1), they will be given three 50-mL aliquots of deuterated water ($^2H_2O/D_2O$): one administered at the visit (DW-1A, for "Day 1, AM") and the others to be taken home to be consumed later that day (DW-1M,P). At 13:00, they will drink DW-1M (for "Day 1, midday"). At 21:00, they will self-administer both IAD-1P syringes simultaneously, for total diazoxide doses of 2 mg kg^{-1} (DZ1 group) or 4 mg kg^{-1} (DZ2 group). This will allow us to evaluate the effect of a single, undivided total daily dose of diazoxide on laboratory outcomes of interest. They will also ingest the third aliquot of D_2O (DW-1P) at that time.

Participants will return for Study Visit 2 (SV-2) on the morning of Day 2. At roughly 09:00, they will have a fasting blood draw (BD-2) and consume another aliquot of D_2O (DW-2A). From this point on, IAD are labeled equivalently to the D_2O aliquots, as Day/Time (*e.g.*, 2P for Day 2, PM dose, *etc.*) Participants will then be provided with 13 investigational agent doses (IAD-2P through -8P) and another 16 aliquots of D_2O (DW-2M through -8P). They will also be furnished with further study diet instructions and begin the study diet thereafter and consume DW-2B at 13:00. At 13:00 on Day 2 they consume DW-2M followed at 21:00 by IAD-3 and DW-2P. They will continue to take investigational agent doses every 12 hours (*i.e.*, at 09:00 and 21:00) thereafter. On Days 3-4, participants continue consume D_2O aliquots three times daily, at 09:00 (DW 3A-4A), 13:00 (DW 3M-4M), and 21:00 (DW 3P-4P); they will collect a second saliva collection (SC-2) on the morning of Day 5. From Days 5 onward, D_2O aliquots are consumed only twice a day, at the same times as the corresponding IAD: "A"-labeled doses at 09:00 and "P"-labeled doses at 21:00. On Day 8, mirroring the procedure of Day 1, participants will be asked to fast except for discrete meals at 09:00, 13:00, and 18:00. Study Visit 3 (SV-3) will then take place on the morning of Day 9; fasting labs will be drawn (BD-3) and saliva collected (SC-3) at approximately 09:00 and IAD-9A + DW-9A will be administered immediately thereafter. Participants will then be provided with IAD-9P through -14p and DW-9P through -14P. They will continue to take doses of their assigned investigational agent every 12 hours, with IAD-9P and DW-9P taken as usual that day at 21:00. This pattern will repeat as shown in Figure 4.i again on Days 14-15: fasting except for three discrete meals on Day 14, followed by Study Visit 4 for morning blood draw (BD-4) and saliva collection (SC-4) on Day 15. CGM will be removed on Day 15 at SV-4. Study completion is defined as having reached the point of completing the SV-4 blood draw on Day 15.

We paused the study after three participants completed each of the three arms (*i.e.*, completion of 3 of the block-randomized cohorts of 3 participants each) to perform an unblinded interim analysis to determine the possibility of discontinuing one of the two diazoxide arms. The interim data were inconclusive (due to variability from the small sample size) and so we decided to continue the study without a protocol change.

4.3 – Rationale for research strategy

4.3.1. Rationale for study population

We propose to test our hypothesis in individuals with IR, which we define as prediabetic state (impaired fasting glucose and/or prediabetes, as defined by the American Diabetes Association [90]) and/or HOMA-IR score of ≥ 2.73 [91]. These individuals are expected to have garden-variety IR, marked by fasting hyperinsulinemia and excessive circulating lipogenic precursors. Note that earlier versions of this protocol sought only participants with prediabetic state. However, we now have expanded the inclusion criteria to include HOMA-IR ≥ 2.73 because many patients with significant IR are able to maintain euglycemia by dint of the very compensatory hyperinsulinemia that we intend this study to test. Inclusion only of participants with prediabetes, whose pancreatic insulin reserve has already begun to falter, therefore likely excludes some of the most hyperinsulinemic individuals – that is, the very people we most want to study. Although we would also like to study the question of "excess" insulin in non-IR volunteers as well, lowering insulin levels would not be expected to have the same physiologic significance as in participants with baseline hyperinsulinemia [3, 27] – particularly, again, as hyperinsulinemia itself may be a driver of IR [38, 44]. However, if this P&F study upholds our hypothesis, we may include a healthy control arm in further clinical studies. The fasting insulin cutoff of 13 $\mu IU\ mL^{-1}$ has been selected because this level of fasting hyperinsulinemia has been validated as reasonably sensitive and specific for IR in a similar population [92].

Although the main study will focus only on participants with a diagnosis of NAFLD, we will also include participants in this study who are at high risk of NAFLD (and meeting all other inclusion/exclusion criteria) according to the clinical judgment of the PI. We have decided also to include participants with IR but no formal NAFLD diagnosis because the primary endpoints in this study are fasting glucose and insulin levels rather than changes in lipid parameters. We expect the glycemic impact of diazoxide to be germane in these participants – especially as up to half of them may actually have undiagnosed NAFLD [93-96].

4.3.2. Pilot and feasibility study using diazoxide

Our overarching goal is to characterize the effect of hyperinsulinemia *per se* on hepatic *de novo* lipogenesis. As such, we ultimately aim to measure rates of DNL during overnight suppression of endogenous insulin secretion with diazoxide. However, because of inter-study variability in the effects of diazoxide on insulin and glucose levels [65, 70, 82, 83], we first need to define expectations for the effects diazoxide with IR-NAFLD in our hands. As aforementioned and outlined in Table 4.i below, diazoxide has indeed been used to efficaciously lower endogenous insulin levels in a number of human clinical studies for off-target indications, primarily weight loss and preservation of β-cell reserve.

Table 4.i. *Modern clinical studies of oral diazoxide for off-label indications in adults**

Study author (..., et al.)	Year	Diazoxide regimen					Ref.
		Total daily dose		Dose division		Duration [a]	
		Initial	*Max*	*Initial*	*Max*		
Alemzadeh [b]	1998	2 mpk [c,d]		0.67 mpk TID [e]		8 w	[31]
Björk (I) [f]	1996	4-6 mpk		Unspecified "divided doses"		3 mo	[75]
Björk (II) [f]	1998	300 mg		100 mg TID		7 d	[76]
Due [b,g]	2006	2 mpk [d]		0.67 mpk TID		8 w	[1]
Esterson [f]	2016	4-6 mpk		Once only			[83]
George [f]	2015	7 mpk		Once only			[77]
Guldstrand [b]	2002	300 mg		100 mg TID		5 d	[84]
Kadriu [f]	2018	300 mg	400 mg	150mg BID	200mg BID [j]	14 d	[97]
Kimonis [h, i]	2019	1.5 mpk	5.1 mpk	Once daily [i]	Once daily [i]	Up to 14w	[81]
Kishore [b]	2011	4 mpk		Once only			[82]
Loves (I) [b]	2018	200mg	700mg	100mg BID	200mg qAC [k] 100mg qHS [l]	6 mo	[74]
Loves (II) [b]	2018	200mg	900mg	100mg BID	300mg qAM [m] 200mg TID	Up to 1y	[73]
Nestler [b]	2012	300 mg		100 mg TID		10 d	[85]
Qvigstad [f]	2004	100 mg		100 mg qHS		9 w	[78]
Radtke (I) [f]	2007	100 mg		100 mg qHS		12 w	[79]
Radtke (II) [f]	2010	100 mg		100 mg qHS		6 mo	[98]
Raju [f]	2005	4 or 6 mpk		Once only			[65]
Ramanathan [f]	2011	6 mpk		Once only			[70]
Ratzmann [b]	1983	500 mg		Once daily		4 d	[86]
Schreuder	2005	150, 225, or 300 mg		50, 75, or 100mg TID		6 d	[72]
van Boekel [b]	2008	150mg	900mg	50mg TID	300mg TID	6 mo	[80]

* *N.B.* Short-term studies in comparable populations highlighted orange/yellow for single-dose studies and green for short courses
[a] Total duration of drug treatment, regardless of dose
[b] Study done in overweight or obese patients
[c] mpk = milligrams of diazoxide per kilogram body weight
[d] Max dose 200 mg d^{-1}
[e] TID = three times daily
[f] Study done in non-obese patients for other indications
[g] Highlighted because includes data post-1 week of treatment
[h] Study done in patients with Prader-Willi syndrome
[i] Study employed a controlled release formulation of diazoxide rather than usual oral suspension
[j] BID = twice daily
[k] qAC = at mealtimes
[l] qHS = at bedtime
[m] qAM = every morning

4.3.3. Rationale for diazoxide dosing

As diazoxide has not been used in clinical studies to study the impact of insulin on lipid metabolism, ideal dosing for this specific purpose is unknown. However, the doses we have selected (1-2 mg kg^{-1} per dose, for total daily doses of 2-4 mg kg^{-1}) are well within or below the dosing ranges both recommended by the FDA package insert (total daily dose [TDD] of 3-8 mg kg^{-1} d^{-1}) [99] and employed in other single-dose or short-course studies of diazoxide to blunt insulin secretion (see Tables 4.i, 4.ii) [1, 65, 70, 72, 77, 82-85]. Even in extended studies at quite high doses (*e.g.*, up to 700 mg TDD for 1 year) [73], diazoxide has been fairly well tolerated; safety considerations are further discussed in §12.3.

The insulinostatic effects of diazoxide in relevant populations (*cf.* highlighted studies in Table 4.i) is summarized in Table 4.ii. Two of the three studies employing diazoxide at doses of roughly 2 mg kg^{-1} d^{-1} for a short course reported

statistically significant decreases in fasting insulin levels, and in none of these three did fasting blood glucose levels rise significantly [1, 72, 85]. Because the volume of distribution of diazoxide is quite sensitive to body composition, obese participants achieve lower peak diazoxide concentrations than lean individuals at the same dose, hence the less-than-expected insulin response [85]. As such, a weeklong pharmacokinetic study estimated that obese men may require a total daily dose of 3.2-4.2 mg kg^{-1} in order to achieve effective insulin suppression [72]. Thus, overall, because our study population may exhibit a fairly wide span of body mass indices (from overweight 27 through obese 35 kg m^{-2}), it is necessary to evaluate the impact of both lower dose (1 mg kg^{-1} per dose, or 2 mg kg^{-1} TDD) and higher dose (2 mg kg^{-1} per dose, or 4 mg kg^{-1} TDD). We will ultimately select the diazoxide dose that best balances effects on insulin *vs.* glucose, as well as consideration of potential side effects. **Note that the FDA has granted an IND exemption to this diazoxide dosing scheme (IND #163465).** We conducted an unblinded interim analysis after accruing half of the project number of study completers (*i.e.*, 3 per arm) to assess the insulin-lowering effect of each dose of diazoxide. However, the data were inconclusive (due to variability from small sample size) and so we did not make any changes in the protocol.

Table 4.ii. *Effect of single-dose or short-course diazoxide on fasting insulin and glucose in obese/overweight people*

Lead author	Year	Diazoxide dosing scheme			Lead time [a]	Insulin levels [b]			Glucose levels [c]			Ref. [f]
		Regimen	*Total daily dose*	*Duration* [d]		*Pre*	*Post*	*ΔIns*	*Pre*	*Post*	*ΔGlc*	
Due	2008	Div. TID [e]	2 mg kg^{-1}	1 w	O/N [g]	26[h]	18[h, i]	-31%	109 [j]	112 [j, k]	+1%	[1]
Esterson	2016	4-6 mg kg^{-1}	n/a	Once	1 h	12.54	?	?	95.6	?	?	[83]
Kishore	2015	4 mg kg^{-1}	n/a	Once	3 h	11.36	7.41 [j]	-35%	92.9	96.8	+1%	[82]
Nestler	2012	100mg TID	300 mg	10 d	O/N	8.8	8.4[k]	-5%	84	92 [k]	+10%	[85]
Raju	2005	4 mg kg^{-1}	n/a	Once	4 h	5 [l]	3[k,m]	-40%	90[m]	96 [j,m]	+7%	[65]
		6 mg kg^{-1}	n/a	Once	4 h	5 [l]	3[k,m]	-40%	92[m]	99 [j,m]	+8%	
Schreuder	2005	100mg TID	300 mg [n]	6 d	O/N	16.3[m]	12.6[i,m]	-23%	106[m]	101 [m]	-5%	[72]

[a] Time between last dose and measurement of fasting insulin/glucose
[b] Insulin levels here are fasting and represented as μIU mL^{-1} [c] Glucose levels here are fasting and represented as mg dL^{-1}
[d] Duration of time diazoxide used prior to reported insulin/glucose levels; study may have continued on beyond this point (see total study durations in Table 1.i).
[e] Total daily dose in three divided doses
[f] Guldstrand, *et al.* (2002) [84] and Ratzmann, *et al.* (1983) [86] excluded from this table due to unavailability of full publication; Ramanthan, *et al.* (2011) [70] is excluded because only post-prandial insulin levels were reported
[g] Overnight fast since last dose [h] Converted as necessary from pmol L^{-1} [i] Change statistically significant
[j] Converted as necessary from mmol L^{-1} [k] Change not statistically significant
[l] Adduced from image analysis of graphs [m] "Pre" = placebo, "post" = diazoxide 100mg TID x 6 d
[n] Based on 114-kg average weight of obese participants, 300 mg TDD equates to a mean TDD of 2.6 mg kg^{-1}

4.3.4. Rationale for timing of procedures

The study timeline has been designed for two main reasons: (1) to evaluate the effects of two different diazoxide dosing schemes on insulin and glucose levels and (2) to simulate the optimal timing for a later lipid-kinetic study. Both of these purposes subserve the pilot aspect of this proposed study.

Dose time-effect relationship: This study will assess not only the effect of two different dosages of diazoxide on insulin and glucose levels, but also of two different dosing paradigms: a single-dose regimen followed immediately thereafter by a short-course, multi-dose regimen of either 3, 7, 10, or 13 days of regularly scheduled (*i.e.*, every 12 hours) dosing. Both such experimental designs have been shown successfully to reduce insulin levels with a minimal impact on glucose levels (see Tables 4.i, 4.ii). However, it is unclear which represents the optimal timing for studying the impact of insulin suppression on *de novo* lipogenesis, particularly in participants with IR-NAFLD. As our goal is to identify an optimal dosing scheme that balances investigative yield with participant risks and inconvenience, we may find that a single, larger dose of diazoxide suffices to adequately suppress fasting insulin levels and serum TG. If so, then we may not ultimately need to include a multi-dose course of diazoxide in the wider study. On the other hand, a durable impact on insulin and glucose and/or TG levels in this population may require achieving a steady state through a longer dosing period. In that case, expanding the study to include the 7- or 14-day course of diazoxide may be necessary.

Metabolic steady state: Our ultimate goal is to evaluate the impact of lowering insulin levels on hepatic *de novo* lipogenesis in NAFLD. This requires measuring the incorporation of stable isotope-labeled lipid precursors (*e.g.*, [1-$^{13}C_1$] sodium acetate

or D_2O) into VLDL-TG [100, 101] followed by compartmental modeling, for example *via* mass isotopomer distribution analysis (MIDA) [102]. Such lipid-kinetic analysis relies on multiple assumptions that can be relatively simplified by investigating a system at steady state [102, 103], yet our proposed system poses several challenges to achieving such stability. First, the fasting state, unlike a continuously fed state, is inherently not steady because of ongoing changes in fatty acid utilization [104]. Second, rates of DNL exhibit considerable intra- and inter-individual variability [4, 13, 55, 89]. Third, lowering insulin levels with a single dose of diazoxide further defies steady-state interpretation due to its pharmacodynamic (*i.e.*, non-uniform) impact on insulin secretion while drug levels themselves are not at steady state [72, 105].

We can mitigate these major analytic confounders in the wider study by performing a crossover study in which all participants act as their own controls by undergoing two-week treatment blocks with diazoxide and placebo (order of administration randomized) – hence our proposal to perform this pilot study "dress rehearsal" over two weeks' time. Indeed, measurement of DNL after diazoxide *vs.* placebo treatment will allow us to "normalize" each participant's response to their own baseline. This will help us to refine our statistical power by accounting for the heterogeneity attendant to the fasting state, rates of DNL, and, inevitably, circadian and behavioral factors among participants [4, 13, 88, 106]. Moreover, as diazoxide levels will have reached steady state after 3-4 days [72, 105], its impact on insulin levels will remain steadier during overnight assessment of DNL.

4.4 – Potential risks and benefits

4.4.1. Potential risks to participants: This study poses greater than minimal risk to participants.
See § 12 for full discussion of risks. See §§ 14, 15 for discussion of data and safety monitoring.

4.4.2. Potential benefits to participants
This study will not provide health benefits directly to participants. However, we do expect that the larger study that it will enable will deepen our understanding of IR, T2DM, and NAFLD pathophysiology. Such knowledge would likely contribute to the development of rational strategies for treating these common metabolic diseases. As such, we believe that these potential benefits to society outweighs the risk posed by study participation.

4.5 – Alternative research strategies

The study described herein presents greater than minimal risk to participants. However, there is no alternative protocol by which we can test our primary hypothesis with minimal risk. Potentially reasonable alternative studies are as follows.

4.5.1. Full pancreatic clamp
The most direct way to test our hypothesis is to directly vary insulin levels while maintaining constant, mild hyperglycemia. That is, some participants would be exposed to hyperinsulinemic hyperglycemia while others would be exposed to euinsulinemic hyperglycemia. This type of clamp-based approach has been used in rare instances to vary insulin levels independently of blood glucose [60-64], but to our knowledge this methodology never been applied to studies of triglyceride kinetics. However, because the pancreatic clamp is necessarily time limited, and is best performed during the day, it may not capture insulin effects on DNL that require longer periods and/or nocturnal circadian rhythm that this diazoxide protocol would allow us to detect.

Although an insulin clamp-based approach is attractive insofar as it can sustain insulin levels reliably and does not in itself pose risks associated with "drugs" like diazoxide, this methodology presents several concerns of its own. These include the higher risk of hypoglycemia with exogenous insulin infusion, discomfort (generally gastrointestinal) due to somatostatin receptor ligand infusion, and need for concomitant replacement with glucagon and growth hormone [59-64, 66, 107]. Somatostatin receptor ligands also can be difficult to tolerate due to adverse gastrointestinal side effects [108] and, like diazoxide, can also affect glucose metabolism independently of its effects on insulin secretion [109]. Thus, on the whole, the pancreatic clamp technique imposes greater than minimal risk, and arguably does not lower experimental risk *versus* the diazoxide treatment proposed herein.

4.5.2. Partial pancreatic clamp

We could consider using octreotide at doses lower than those required for full endocrine pancreatic suppression to manipulate endogenous insulin levels, akin to diazoxide, in lieu of the full pancreatic clamp. This would allow us to omit exogenous insulin and thereby reduce the risk of hypoglycemia. Octreotide has been applied to acute hyperglycemic clamp studies (*i.e.*, without exogenous insulin) [66, 110] as well as in longer weight-control trials based on its ability to lower insulin levels [68, 69]. However, to our knowledge, octreotide infusion has not been actively titrated to achieve specific levels of residual insulin secretion for metabolic studies. As such, we would need to perform further pilot studies in order to identify the optimal octreotide infusion rate to achieve goal insulin suppression. This approach still does not avoid potential adverse gastrointestinal side effects of somatostatin receptor ligands [108] and may still require partial replacement of glucagon and growth hormone [107]. Thus, this modified pancreatic clamp technique also imposes greater than minimal risk, and, again, does not appear to lower experimental risk *versus* the diazoxide treatment proposed herein.

4.5.3. Shorter diazoxide study

Finally, we could consider reducing the number of diazoxide doses by performing the study over a shorter period – perhaps even including a single-dose study. Indeed, we are attempting to assess the efficacy of shorter time courses in addition by building in testing following a single dose (*i.e.*, combining IAD-1 and -2 on the evening of Day 1) and shorter (7-day) diazoxide courses into the overall study design. If successful, we may be able to design the wider study over a similarly foreshortened time period. However, as explained in §4.3.4, obtaining data from a weeklong course is essential in order to be able to verifiably establish a pharmacokinetic steady state in which to test the effects of diazoxide on insulin, glucose, and lipid levels. In other words, we will need to proactively ensure that any negative results that might result from a short (single-dose or 7-day) study would not simply reflect a failure to attain a reliable drug steady state. This consideration is particularly salient in light of the insulinostatic variability posted in relevant clinical trials of diazoxide (see §4.3.3 and Table 4.ii).

If we are able to establish that a shorter (single-dose or 7-day) study is feasible, we can attempt to reformat the lipid-kinetic studies accordingly. However, the non-steady-state conditions that would result would considerably complicate the analysis and may result in false-negatives. Shorter studies of diazoxide may reduce but would not eliminate the risk of potential side effects. Because prolonged clinical trials of even higher doses of diazoxide have been conducted safely [73, 74, 80, 97], we believe that the benefits of obtaining the important pharmacodynamic data afforded by a longer study outweigh the marginal risk conferred by additional doses of diazoxide.

5. STUDY PARTICIPANTS

5.1 – Participants

Up to 72 participants (across all study groups) will be enrolled to achieve 18 evaluable participants.

5.2 – Inclusion criteria

a. Adults aged 18-70 years (using highly effective contraception if of childbearing potential, *cf.* §5.3.4)
b. Body mass index of 27-50 kg m^{-2}
c. Able to understand written and spoken English and/or Spanish
d. Able to have pre-randomization screening labs drawn and study protocol initiated within 30 days of eligibility determination
e. Diagnosed with, <u>or</u> clinically judged to be at high risk for, non-alcoholic fatty liver disease (NAFLD), also known as metabolic-associated fatty liver disease (MAFLD), by hepatologist or other qualified physician
f. Evidence of insulin resistance, represented by any or all of the following criteria:
    - Meeting either of the American Diabetes Association's definitions for prediabetes or IFG on screening labs:
        i. Prediabetes: Hemoglobin A_{1c} 5.7-6.4%
        ii. IFG: plasma glucose of 100-125 mg dL^{-1} after ≥ 8-h fast
    - Homeostasis Model of Insulin Resistance (HOMA-IR) score ≥ 2.73
g. Fasting hyperinsulinemia (fasting insulin level ≥ 13 μIU mL^{-1}) on screening labs

h. Written informed consent (in English or Spanish) and any locally required authorization (*e.g.*, Health Insurance Portability and Accountability Act) obtained from the participant prior to performing any protocol-related procedures, including screening evaluations.

5.3 – Exclusion criteria

1. Unable to provide informed consent in English or Spanish
2. Concerns arising at screening visit (any of the following):
   i. Documented weight loss of ≥ 5.0% of baseline within the previous 3 months
   ii. Abnormal blood pressure (including on treatment, if prescribed)
      - Systolic blood pressure < 95 mm Hg or > 160 mm Hg, *and/or*
      - Diastolic blood pressure < 65 mm Hg or > 100 mm Hg
   iii. Abnormal resting heart rate < 60 bpm or ≥ 100 bpm
      - Sinus brady- or tachycardia that has been appropriately evaluated and considered benign by the recruit's personal physician may be permitted at PI's discretion
   iv. Abnormal screening electrocardiogram (or if on file, performed within previous 90 d):
      - Non-sinus rhythm
      - Significant QT_c prolongation (≥ 480 ms)
      - New or previously unknown ischaemic changes that persist on repeat EKG:
         - ST elevations
         - T-wave inversions
   v. Laboratory evidence of diabetes mellitus:
      - Hemoglobin A_{1c} ≥ 6.5%, *and/or*
      - Fasting plasma glucose ≥ 126 mg dL^{-1}
   vi. Positive qualitative serum β-hCG (*i.e.*, pregnancy test) in women of childbearing potential
   vii. Liver function abnormalities
      - Transaminases (AST or ALT) > 3.0 x the upper limit of normal, *and/or*
      - Total bilirubin > 1.25 x the upper limit of normal
         - These exclusion criteria may be waived if the recruit's personal hepatologist/gastroenterologist approves an exception (communicated in writing)
   viii. Abnormal screening lipids: Triglycerides > 500 mg dL^{-1}, *and/or*
   ix. Abnormal screening serum electrolytes (any of the following)
      - Sodium, potassium, chloride, or bicarbonate levels that are considered clinically significant according to the clinical judgment of the PI (see §5.4).
      - Creatinine equating to estimated glomerular filtration rate < 60 mL min^{-1} 1.73 m^{-2}
   x. Platelet count below the lower limit of normal
   xi. White blood cell count below the lower limit of normal
   xii. Uric acid level above the upper limit of normal
   xiii. Glucose-6-phosphate dehydrogenase below the lower limit of normal
3. COVID-19 precautions
   i. Unwillingness to comply with masking requirements per NYP/CUIMC policy
   ii. Active, documented COVID-19 at any time after screening
4. Reproductive concerns
   i. Women of childbearing potential not using highly effective contraception, defined as:
      - Surgical sterilization (*e.g.*, bilateral tubal occlusion, bilateral oophorectomy and/or salpingectomy, hysterectomy)
      - Combined oral contraceptive pills taken daily, including during the study
      - Intrauterine device (levonorgestrel-eluting or copper) active at the time of the study
      - Medroxyprogesterone acetate (Depo-Provera®) injection active at the time of the study
      - Etonogestrel implants (*e.g.*, Implanon®, *etc.*) active at the time of the study

- Etonogestrel/ethinyl estradiol vaginal ring (*e.g.*, NuvaRing®) active at the time of the study
- Norelgestromin/ethinyl estradiol transdermal system (*e.g.*, Ortho-Evra®) active at the time of the study

ii. Women currently pregnant (tested by serum and/or urine β-hCG)
iii. Women currently breastfeeding

5. Concerns related to glucose metabolism
   i. History of having met any of the American Diabetes Association's definitions of diabetes mellitus (*i.e.*, overt diabetes):
      - Hemoglobin $A_{1c} \geq 6.5\%$
        - Or rapid rise in documented HbA_{1c} values causing clinical concern for evolving insulin deficiency
      - Plasma glucose ≥ 126 mg dL^{-1} after 8-h fast
      - Plasma glucose of ≥ 200 mg dL^{-1} at 2 h after ingestion of a 75-g glucose load
      - Random plasma glucose ≥ 200 mg dL^{-1} associated with typical hyperglycemic symptoms, diabetic ketoacidosis, or hyperglycemic-hyperosmolar state
   ii. History of gestational diabetes mellitus within the previous 5 years
   iii. Use of most antidiabetic medications within the 90 days prior to screening
      - Excluded: thiazolidinediones, sulfonylureas, meglitinides, DPP4 inhibitors, GLP-1 receptor agonists, SGLT2 inhibitors, amylin mimetics, acarbose, insulin
      - Metformin is acceptable provided that recruits meet all of the inclusion criteria at screening
   iv. Clinical concern for absolute insulin deficiency (*e.g.*, type 1 diabetes, pancreatic disease)
6. Concerns related to lipid metabolism
   i. Known diagnoses of familial hypercholesterolemia, familial combined hyperlipidemia, or familial hyperchylomicronemia
   ii. Use of certain lipid-lowering drugs within the 90 days prior to screening:
      - Statins or PCSK9 inhibitors for secondary prevention or treatment of familial hypercholesterolemia
        - Statins or PCSK9 inhibitors for primary prevention of ASCVD are acceptable
      - Fibrates (*e.g.*, fenofibrate, clofibrate, gemfibrozil)
      - High-dose niacin (>100 mg daily)
7. *Known, documented* history (*i.e.*, not to be newly screened/tested for study purposes), at the time of screening, of <u>any</u> of the following medical conditions:
   i. Pancreatic pathology, including but not limited to:
      - Pancreatic neoplasia, unless appropriately evaluated and considered benign and not producing hormones
      - Chronic pancreatitis
      - Acute pancreatitis within the previous 5 years
      - Autoimmune pancreatitis
      - Surgical removal of any portion of the pancreas
   ii. Cardiovascular disease *(N.B. uncomplicated hypertension is <u>not</u> exclusionary)*
      - Atherosclerotic cardiovascular disease
        - Stable or unstable angina
        - Myocardial infarction
        - Ischaemic or hemorrhagic stroke, or transient ischaemic attack
        - Peripheral arterial disease (claudication)
        - Use of dual antiplatelet therapy (aspirin + P_2Y_{12} inhibitor)
        - History of percutaneous coronary intervention
      - Heart rhythm abnormalities
      - Congestive heart failure of any New York Heart Association class
      - Symptomatic valvular heart disease (*e.g.*, aortic stenosis)
      - Pulmonary hypertension

iii. Chronic kidney disease, Stage 3 or higher (estimated glomerular filtration rate < 60 mL min^{-1} 1.73 m^{-2}), of any cause
iv. Chronic liver disease other than uncomplicated NAFLD, including but not limited to:
- Advanced liver fibrosis, as determined by non-invasive testing
- Cirrhosis of any etiology
- Autoimmune hepatitis or other rheumatologic disorder affecting the liver
- Biliopathy (*e.g.*, progressive sclerosing cholangitis, primary biliary cholangitis)
- Chronic liver infection (*e.g.*, viral hepatitis, parasitic infestation)
- Hepatocellular carcinoma
- Infiltrative disorders (*e.g.*, sarcoidosis, hemochromatosis, Wilson disease)

v. Gout
vi. Chronic viral illness *(N.B. diagnosis based only on medical history; we will <u>not</u> test for any of these viruses at any point in this study)*
- Hepatitis B virus (HBV), unless previously successfully eradicated with antiviral drugs that have been discontinued for at least 90 days prior to screening
- Hepatitis C virus (HCV) infection, unless previously successfully eradicated with antiviral drugs that have been discontinued for at least 90 d prior to screening
- Human immunodeficiency virus (HIV) infection

vii. Malabsorptive conditions
- Active inflammatory bowel disease (quiescent and off medication is acceptable)
- Celiac disease (in remission on gluten-free diet is acceptable)
- Surgical removal of a significant length of intestine

viii. Active seizure disorder (including controlled with antiepileptic drugs)
ix. Psychiatric diseases that…
- Are or have been decompensated within 1 year of screening, *and/or*
- Require use of antipsychotic drugs associated with significant weight gain/metabolic dysfunction (*e.g.*, clozapine, olanzapine), monoamine oxidase inhibitors, tricyclic antidepressants, or lithium

x. Glucose-6-phosphate dehydrogenase (G6PD) deficiency
- Due to presence of quinine in tonic water placebo

xi. Other endocrinopathies:
- Cushing syndrome (okay if considered in remission after treatment, provided that no exogenous corticosteroids are required)
- Adrenal insufficiency
- Primary aldosteronism

xii. Venous thromboembolic disease (deep vein thrombosis or pulmonary embolism) or any required use of therapeutic anticoagulation
xiii. Active malignancy, or hormonally active benign neoplasm, <u>except</u> allowances for:
- Non-melanoma skin cancer
- Differentiated thyroid cancer (AJCC Stage I only)

8. Clinical concern for increased risk of volume overload or hypotension (SBP <95 and/or DBP <65 mm Hg), including due to medications and/or heart/liver/kidney problems, as listed above
9. Use of certain medications currently or within 90 d prior to screening:
   i. Prescribed medications used for any of the indications in the preceding list of excluded conditions, or their use within 90 d prior to screening, <u>except</u> allowances for:
   - Statins for primary prevention of cardiovascular disease
   - Use of drugs prescribed for indications other than the exclusionary diagnoses/purposes listed above (*e.g.*, non-hydantoin antiepileptic drugs used for non-seizure indications, ACEi/ARB used for uncomplicated hypertension rather than for congestive heart failure, *etc.*)
     - Note, as above, that antidiabetic drugs except metformin within 90 d of screening <u>are</u> excluded

ii. Vasodilating drugs for any indication: hydralazine, nitrates, phosphodiesterase-5 inhibitors (*e.g.*, sildenafil, tadalafil), minoxidil (oral)
iii. Phenytoin or fosphenytoin for any indication
iv. Oral or parenteral corticosteroids (at greater than prednisone 5 mg daily, or equivalent) for more than 3 days within the previous 90 days; topical and inhaled formulations are permitted
v. Fludrocortisone

10. History of certain weight-loss (bariatric) surgeries, including:
    i. Roux-en-Y gastric bypass
    ii. Biliopancreatic diversion
    iii. Restrictive procedures (lap band, sleeve gastrectomy) performed within past 6 months
11. Clinical concern for alcohol overuse, including based on chart review and/or by participant's report of consuming more than 14 standard drinks per week for males or more than 7 standard drinks per week for females
12. Positive urine drug screen, except for:
    i. Lawfully prescribed medications
    ii. Marijuana/THC positivity, provided that the participant agrees not to use it during the same period that they will abstain from alcohol)
13. History of severe infection or ongoing febrile illness within 30 days of screening
14. Any other disease or condition or laboratory value that, in the opinion of the investigator, would place the participant at an unacceptable risk and/or interfere with the analysis of study data.
15. Known allergy/hypersensitivity to any component of the medicinal product formulations (including sulfa drugs), other biologics, IV infusion equipment, plastics, adhesive or silicone, history of infusion site reactions with IV administration of other medicines, or ongoing clinically important allergy/hypersensitivity as judged by the investigator.
16. Concurrent enrollment in another clinical study of any investigational drug therapy or use of any biologicals within 6 months prior to screening or within 5 half-lives of an investigational agent or biologic, whichever is longer.

5.4 – Follow-up of abnormal screening laboratory results

In the event of abnormal results, including those discovered incidentally, the PI or qualified physician designee will evaluate the clinical context. If the result is judged by the PI or qualified physician designee to be clinically insignificant (*i.e.*, would not be acted upon in a patient-care context), it may be noted but not require specific follow-up and not prevent the recruit from participating the in the study. For example, a serum chloride level in the basic metabolic panel of 1 or 2 mEq L^{-1} above or below the reference range in a patient-care context would almost certainly not trigger any specific response by the physician, and so also would not in the context of this research study. If the abnormal result is, according to the PI's clinical judgment, medically significant and plausibly true the PI or qualified physician designee will contact the enrollee to discuss the result, its meaning, and any further actions to be taken by the enrollee (including referral for urgent medical care, if necessary). If the PI determines that a laboratory value with potential clinical significance was likely spurious or transient, the PI may ask the enrollee to repeat the abnormal test and/or be fully re-screened later. For example, an elevated potassium level resulting from a grossly hemolyzed specimen should be repeated but will not automatically be exclusionary. If the lab result normalizes, and the clinical context remains stable, the enrollee may participate at the discretion of the PI.

5.5 – Inclusion of women and minorities

People of all genders, races, and ethnic groups are eligible for this trial. However, people of childbearing potential must use highly effective contraception in order to be included (see §5.3, above).

Table 5.i – *Accrual targets*

Accrual targets across all groups *(diazoxide and placebo)*		***Sex / gender***		
		Female	*Male*	*Total*
[redacted]	Hispanic or Latino	3	3	6

	Not Hispanic or Latino	6	6	12
	Ethnic category: total of all participants	9	9	18
Racial category	American Indian or Alaskan Native	0	0	0
	Asian	1	1	2
	Black or African-American	3	3	6
	Native Hawaiian or Pacific Islander	0	0	0
	White	5	5	10
	Racial category: total of all participants	9	9	18

The reason for this anticipated distribution of race/ethnicity among participants is that it generally mirrors the patient population from which participants will be recruited (*i.e.*, the Columbia Center for Fatty Liver Disease). However, these are only estimates and we will include any willing individual who satisfies all inclusion and exclusion criteria.

5.6 – Vulnerable populations

This study will not recruit children, prisoners, those with impaired decision-making capacity, or pregnant women. The study may enroll educationally or economically disadvantaged participants because a significant proportion of the patient population that we see at CUIMC has limited formal education and/or financial means. To exclude such individuals would mean excluding much of our own local community in Washington Heights and would jeopardize diverse inclusivity in recruiting. However, as previously explained, informed consent procedures will be undertaken at an 8th-grade level. The PI has considerable clinical experience in communicating complex information with educationally and/or financially disadvantaged individuals, both in English and Spanish.

5.7 – Participant recruitment

5.7.1. Direct referral from hepatology (NAFLD clinic)

Potential participants will be identified from the NAFLD clinic of Columbia University Medical Center by Dr. Julia Wattacheril (co-investigator), or other qualified specialist clinician, based on their past medical history. The recruiting clinician will provide information about the research and ask if they are interested in participating and being contacted by the study team (*i.e.*, oral consent for initial contact). Researchers other than the patient's treating physician will not contact the potential recruit unless it has been documented that they agree to discuss the study with the research team. Alternatively, the treating physician may provide information about the study along with contact information for the researchers, so the potential participants may contact the researchers if they are interested in joining the study. Once contact with the team has been established, recruits will undergo a screening procedure to determine eligibility under inclusion/exclusion criteria; this is described in detail in §§ 6.1, 7.1.

5.7.2. Direct referral from other providers

Additional potential participants, with or without known NAFLD, will be identified by physicians in the outpatient clinics of CUIMC and the Ambulatory Care Network (ACN) clinics of NewYork-Presbyterian Hospital on the basis of medical histories, physical examination findings, and laboratory results. The recruiting clinician will provide information about the research and ask if they are interested in participating and being contacted by the study team (i.e., oral consent for initial contact). Researchers other than the patient's treating physician will not contact the potential recruit unless they have given oral consent to discuss the study with the research team. Alternatively, the treating physician may provide information about the study along with contact information for the researchers, so the potential participants may contact the researchers if they are interested in joining the study. Once contact with the team has been established, recruits will undergo a screening procedure to determine eligibility under inclusion/exclusion criteria; this is described in detail in §§ 6.1, 7.1.

5.7.3. Recruitment using electronic medical record's research functionality (Epic Reports/TRAC)

We will use Epic, the electronic medical record (EMR), to assist with screening for potential patients to be assessed for eligibility by working with the Epic development team to create criteria to assist in screening ("Epic Reports" or TRAC functionality). A TRAC request will be made to provide a cohort of potential participants in Epic through the Reporting Workbench. The IRB-approved study description will be sent once to each patient via Connect, and they then can indicate in Connect if they are interested in receiving more information about the study or decline the invitation. If they indicate that they are interested in receiving more information, the study team may contact them by phone or email. Potential participants who do not respond in Connect will be sent the information by Qualtrics or Redcap. Reminders will be sent a maximum of two times. The use of Connect is to supplement other, currently IRB-approved recruitment methods. See also HIPAA Form C attached to this protocol submission.

The eligibility criteria or data that will be extracted in a TRAC report are:

- Patients who have answered "YES" to consent to be contacted for research
- Age (must be 18-70 years)
- Body mass index (must be 27-50 kg m^2)
- Presence of diagnoses of: acanthosis nigricans, hyperinsulinemia, impaired fasting glucose, impaired glucose tolerance, insulin resistance, overweight, obesity, polycystic ovary syndrome, prediabetes
- Absence of diagnoses of: diabetes mellitus, angina, atrial fibrillation, atrial flutter, bipolar disorder, chronic kidney disease (CKD) stage ≥ 3, cancer (current diagnosis only), cerebrovascular accident or stroke, cirrhosis or advanced liver fibrosis, congestive heart failure, coronary artery disease, deep venous thrombosis or pulmonary embolism, epilepsy, G6PD deficiency, gout, hepatitis B, hepatitis C, HIV/AIDS, myocardial infarction, peripheral arterial disease, pulmonary hypertension, schizophrenia or schizoaffective disorder, sulfa allergy, transplant, ventricular tachycardia
- Absence of the following medications:
    - Anticoagulants/antiplatelets: apixaban, argatroban, clopidogrel, dabigatran, dalteparin, edoxaban, enoxaparin, prasugrel, rivaroxaban, ticagrelor, warfarin
    - Antiepileptic drugs: phenytoin
    - Antipsychotics: clozapine, olanzapine
    - Beta blockers: atenolol, bisoprolol, carvedilol, labetalol, metoprolol, nebivolol
    - Corticosteroids: betamethasone, dexamethasone, fludrocortisone, hydrocortisone (oral or injected), methylprednisolone, prednisone, triamcinolone (injected)
    - DPP4 inhibitors: alogliptin, linagliptin, saxagliptin, sitagliptin
    - Fibrates: fenofibrate, gemfibrozil
    - GLP-1 receptor agonists: dulaglutide, exenatide, liraglutide, lixisenatide, semaglutide, tirzepatide
    - Insulin (any)
    - Lithium
    - Meglitinides: mitiglitinide, nateglinide, repaglinide
    - Monoamine oxidase inhibitors: isocarboxazid, phenelzine, selegiline, tranylcypromine
    - Niacin (high dose only)
    - Non-dihydropyridine calcium channel blockers: diltiazem, verapamil
    - Other diabetes medications: acarbose, amylin mimetics, pioglitazone
    - SGLT2 inhibitors: canagliflozin, dapagliflozin, empagliflozin, ertugliflozin, sotagliflozin
    - Sulfonylureas: glibenclamide, gliclazide, glimepiride, glipizide, glyburide
    - Urate-lowering therapies: allopurinol, febuxostat, rasburicase
    - Vasodilating drugs: hydralazine, isosorbide dinitrate, isosorbide mononitrate, nitroglycerin, sildenafil, tadalafil
- Laboratory values to search by:
    - Hemoglobin A_{1c}
        - < 5.7%
        - < 6.4%

  - Insulin level
    - < 10 μIU mL^{-1}
    - 10-12 μIU mL^{-1}
    - ≥13 μIU mL^{-1}
  - Triglyceride level
    - < 150 mg dL^{-1}
    - 150-400 mg dL^{-1}
    - < 400 mg dL^{-1}
  - Uric acid level
    - ≤ 8.6 mg dL^{-1} (*i.e.*, upper limit of normal for men)
    - ≤ 7.1 mg dL^{-1} (*i.e.*, upper limit of normal for women)
- Laboratory values to exclude:
  - Positive hepatitis B core antibody and/or surface antigen
  - Positive hepatitis C antibody
  - Positive HIV-1/-2 antigen and/or antibody

Note that any subset of the above TRAC criteria may also be applied, as not all of the above information will necessarily be available in the chart for all patients. For example, relatively few people have insulin levels recorded in their charts, but many of those who do not may still otherwise fit eligibility criteria for the study.

The following message will be sent to each patient who has indicated interest in receiving more information or decline the invitation:

Patient facing study name: *"Insulin lowering with diazoxide"*

You may be eligible for a research study conducted at the Columbia University Irving Medical Center.

We're seeking adult volunteers to help us study how the hormone insulin controls blood sugar in people at risk for type 2 diabetes. We'll ask participants to take an oral medication called diazoxide (at one of two doses) or a placebo (inert non-drug) and drink a particular type of water twice a day for two weeks while wearing a small continuous glucose (sugar) monitoring device on the back of the arm. Volunteers will come in for a brief screening visit and, if they agree and are eligible to participate in the study, will come in for four (4) brief morning study visits, with blood draws to look at blood levels of glucose (sugar), insulin, and certain fats.

Participants will be compensated up to a total of $700, plus reimbursement of certain travel expenses.

If you do not wish to be contacted in the future about any research studies, please contact the Columbia University Privacy Office at 212-305-7315 or by email at hipaa@cumc.columbia.edu.

We look forward to hearing from you soon.

Sincerely,
Joshua R. Cook, M.D., Ph.D.

The Qualtrics and Redcap survey templates for those who have given consent to be contacted for research:

Thank you for seeking healthcare at NewYork-Presbyterian Hospital/Columbia University Irving Medical Center.

At your visit with our healthcare provider(s) or through the CONNECT link, you agreed to be contacted to learn about participating in a research study.

Columbia University Irving Medical Center
AAAU2570 (PI: Cook) – [redacted]

Dr. Joshua Cook is conducting a research study to learn about the effects of lowering levels of the hormone insulin on blood sugar. You may be eligible to take part in this research study.

[Click here to learn about this research study]

If you do not wish to be contacted in the future about any research studies, please contact the Columbia University Privacy Office at 212-305-7315 or by email at hipaa@cumc.columbia.edu

If the recruit chooses to open the survey, they will be presented with the following information:

Patient facing study name: *"Insulin lowering with diazoxide"*

You may be eligible for a research study conducted at the Columbia University Irving Medical Center.

We're seeking adult volunteers to help us study how the hormone insulin controls blood sugar in people at risk for type 2 diabetes. We'll ask participants to take an oral medication called diazoxide (at one of two doses) or a placebo (inert non-drug) and drink a particular type of water twice a day for two weeks while wearing a small continuous glucose (sugar) monitoring device on the back of the arm. Volunteers will come in for a brief screening visit and, if they agree and are eligible to participate in the study, will come in for four (4) brief morning study visits, with blood draws to look at blood levels of glucose (sugar), insulin, and certain fats.

Participants will be compensated up to a total of $700, plus reimbursement of certain travel expenses.

If you want to learn more about this research study, a research team member will contact you. They will explain the study and answer any questions you may have. Finding out more about a study does NOT commit you to participate in it.

[Option 1: I am interested in learning more. Please contact me by email: _____]
[Option 2: I am interested in learning more. Please call my mobile phone or send a text message to: _____]
[Option 3: No thank you; I am not interested in this research study.]

5.7.4. Recruitment online via CUIMC RecruitMe and NIH ResearchMatch

We will also invite volunteers to self-refer for screening *via* the CUIMC RecruitMe and NIH ResearchMatch online portals. The study description will appear on the RecruitMe website as follows:

Study title: Effect of lowering insulin levels on blood sugar

We are conducting a research study on the effect of high insulin levels on the buildup of excessive liver fat ("fatty liver disease") in people at risk for type 2 diabetes. We are seeking adult volunteers, aged 18-70, who are overweight or obese with a body mass index of 27-50, and have been diagnosed with, or are at risk for, prediabetes. Participants will take either placebo (non-drug) or a medication called diazoxide, which lowers insulin levels, twice daily for two weeks, each time along with a drink of a particular type of water. We will perform blood tests at several time points during the study to determine the effect of diazoxide versus placebo on blood levels of insulin, glucose (sugar), and certain fats. Participants will attend four (4) outpatient visits plus a screening visit as part of the study and will be compensated up to a total stipend (salary) of $700 and reimbursement for travel expenses. If you are found ineligible to participate in this study, you may be asked to join similar, related studies that we are conducting.

The following yes-no questions will be asked as part of RecruitMe's "Do You Qualify?" survey:

- Is your body mass index between 27 and 50 kilograms per square meter?
- Have you ever been diagnosed with diabetes?
- Will you be able to attend a screening visit and then, over a period of 2 weeks, four (4) visits (each 1-2 hours) at 8:00 AM, including two on back-to-back days?
- Are you okay with having your blood drawn?
- Women under 51: do you use birth control (pills/implant/IUD/surgical), even if abstinent? (Men & women over 51: answer Yes)

- *(Note: we use the age 51 cutoff here because the character limit imposed by RecruitMe does not allow enough space to say, "women of reproductive potential." For women over 51 who have not yet gone through menopause, or for women under 51 who have gone through menopause, we will still clarify their reproductive/contraceptive status for all other recruits.)*

5.7.5. Recruitment flyers

We will post flyers at Columbia University locations on both the Medical Center and Morningside Campuses to advertise the study. A copy of the proposed flyer is attached in Documents. The copy of the flyer reads as follows:

Are you at risk of type 2 diabetes? We're seeking volunteers for a research study

We're evaluating the effects on blood sugar of an FDA-approved drug called diazoxide that lowers high insulin levels

Participation requirements:

- *Ages 18-70*
- *Pre-diabetes or at risk of diabetes (no personal history of diabetes)*
- *BMI 27-50 kg/m² (overweight/excess weight)*
- *27 doses of diazoxide + 32 drinks of heavy water to be taken at home over 14 days*
- *Initial screening visit followed by 4 study visits with blood draws*

Compensation: $700 for completing the study + travel reimbursement where applicable

For more information:
Ishwari Nagnur (212) 305-9336; imn2113@cumc.columbia.edu

5.7.6. Recruitment online via Craigslist

We will also invite volunteers to self-refer for screening *via* classified advertisement posted on Craigslist, an increasingly common recruiting practice in the human-subjects research community. The study description will appear in the "gigs" section of New York Craigslist (newyork.craigslist.org):

We are seeking volunteers for a research study at Columbia University Irving Medical Center (CUIMC) to determine the effect of lowering insulin levels on blood sugar.

Whom we're seeking: Adults with overweight or obesity (body mass index 27-50) and no history of diabetes

Duration of participation: One screening visit, then 4 outpatient visits of about an hour each over 2 weeks

What you have to do: Come to 4 study visits, take 27 doses of either diazoxide (medication that lowers levels of insulin) at one of two doses OR placebo (inert non-medication), drink 32 doses of heavy water, and follow dietary recommendations, all over 2 weeks

Compensation: $700 for those who complete the study, plus reimbursement for travel expenses within the NYC metro area

If you are potentially interested in volunteering to be in this research study, please follow the following link to the CUIMC RecruitMe website for more information on the study and how to enroll.

A link to the study's corresponding CUIMC RecruitMe page will then be posted.

5.7.7. Recruitment online via Reddit

We will also invite volunteers to self-refer for screening *via* creation of new posts on the online platform, Reddit. Reddit is a widely-used platform consisting of numerous communities ("subreddits") in which individuals can share information and engage in topic-based discussions. Reddit has been used successfully by other groups to recruit clinical-trial participants. The site offers a unique opportunity to reach a diverse population, including individuals who may not actively be engaged in traditional healthcare settings but are seeking information and support related to insulin resistance, prediabetes, and allied conditions. The anonymity and accessibility of Reddit may encourage participation from individuals who may otherwise be hesitant to participate in research studies. All content posted on Reddit must first be approved by the moderator of the relevant subreddit. If requested, we will share public-facing, IRB-approved study information (*e.g.*, the study's blank informed consent form) with subreddit moderators to authenticate our identity. No PHI/PII (or even any form of deidentified study data) will ever be disclosed or discussed on Reddit.

We will create a Reddit account to be used specifically and solely for the purpose of posting study-related information. Our posts will use a no-reply function and so we will not communicate directly with any individual users and we will not recruit people directly from Reddit. Rather, IRB-approved posts will direct potential recruits to the study's CUIMC RecruitMe page so they can signal their interest if they wish. We will also post only the following copy; because these discussion forums are meant to be used by laypeople who do already have some background knowledge about the topic, we will be a bit more detailed in our discussion of the study's background.

> *We are conducting research at Columbia University Irving Medical Center in New York City on the buildup of excessive liver fat ("fatty liver disease") in people with insulin resistance who are at risk of type 2 diabetes. We suspect that high insulin levels (hyperinsulinemia) that the body produces to compensate for the resistance to insulin's actions on blood sugar may still be able to drive excessive fat production and deposition in the liver. To test this idea, we plan to temporarily lower insulin levels in eligible participants by using a medication called diazoxide, which is approved by the U.S. Food & Drug Administration for treatment of certain high-insulin conditions that cause low blood sugar. In order to plan a larger study on how insulin lowering with diazoxide affects the liver's production of fats, we first are doing a small "pilot" study to test the effects of different doses and times of diazoxide administration on blood sugar, insulin levels, and certain other metabolic parameters.*
>
> *We are seeking adult volunteers, ages 18-70, who are overweight or obese with a body mass index of 27-50, and have been diagnosed with, or are at risk for, prediabetes. We are particularly eager to enroll volunteers who are known to have high fasting insulin levels, but this is not a requirement to be screened. (Insulin levels will also be measured at the screening visit.) Participants will take either placebo (non-drug) or a medication called diazoxide, which lowers insulin levels, twice daily for two weeks, each time along with a drink of a particular type of water. We will perform blood tests at several time points during the study to determine the effect of diazoxide versus placebo on blood levels of insulin, glucose (sugar), and certain fats. Participants will attend four (4) outpatient visits plus a screening visit as part of the study and will be compensated up to a total stipend (salary) of $700 and reimbursement for certain local travel expenses within the New York City metropolitan area. If found ineligible to participate in this study, enrollees may, with their consent, be asked to join similar, related studies that we are conducting.*
>
> *If you are potentially interested in volunteering to be in this research study, please follow the following link to the CUIMC RecruitMe website for more information on the study and how to enroll.*

5.7.8. Recruitment after enrollment in our other studies

This study is related to other studies we are conducting of the glycemic impact of lowering insulinization, IRB-AAAU3014 (Pancreatic Clamp P&F), AAAU9636 (Alpelisib I), AAAU9702 (Pancreatic Clamp), and AAAV2481 (Graded IST P&F). As a consequence, enrollees that fail screening for our other study(ies) but who gave consent for future contact may still fit the inclusion/exclusion criteria for this study. If so, we may invite those enrollees to come in for an abbreviated outpatient screening visit for this study. Similarly, we can perform abbreviated re-screening for enrollees in this study who previously failed screening but might now be eligible based on intervening changes in inclusion/exclusion criteria. In that case, we will

perform a separate, new informed consent process for this study (or, for re-screening, for the updated version of this study), and will need only to collect two additional labs: G6PD uric acid, and, where applicable, repeat β-hCG. That is, we will use all the other screening data that we will already recently have collected for the other study to satisfy the inclusion/exclusion criteria for this study. We may also identify potential participants from lab results obtained in IRB-AAAU9950 (Hyperinsulinemia Biobank). In that case, participants in AAAU9950 who have given permission for Future Contact may be invited to undergo a full informed consent process for this study followed by abbreviated screening procedures that carry over information obtained during the study visit for AAAU9950 (medical history information and some screening labs), while newly collecting necessary information not obtained during AAAU9950 (*e.g.*, EKG, certain lab tests).

Conversely, enrollees who first fail screening for this study but meet inclusion/exclusion criteria for IRB-AAAU3014/U9636/U9702/V2481 may be invited to an abbreviated screening visit for it (with a separate, new informed consent process). With the enrollee's permission (included as a condition in the Informed Consent Form), screening data from this study will then be applied to the other study in which they consent to participate.

Finally, we may also directly recruit people who have participated in one or more of our other studies if they have provided consent for future contact on the informed consent form of that (those) study(ies), provided that they do not undergo procedures for more than one of our studies concurrently.

5.7.9. Pre-consent process

Those who have signaled their potential interest in the study either through RecruitMe or the Epic CCR process may choose to undergo pre-consent pre-screening through either of two routes: (1) Qualtrics pre-screening survey or (2) by direct phone/email contact *via* HIPAA Form B. The latter option is available for those who feel less technologically comfortable. Recruits identified through CCR will be sent a message through Epic Connect, while those identified through RecruitMe will be sent an email (from @cumc.columbia.edu addresses only). The message will read as follows:

> *Hello,*
> *My name is Ishwari Nagnur, and I am a member of the research team for the "Diazoxide P&F" trial. Thank you for your interest in our study. In order to provide you with more information about the study, and to determine if you are potentially eligible to participate, we perform a screening process with two parts. The first part, called "pre-screening," involves asking you some questions about your contact information and medical history. This can be done in either of two ways:*
>
> 1. *You can fill out an online questionnaire ("Qualtrics"). Before answering the questions, you will be asked to review and electronically sign a "pre-consent" form that goes over some basics about the study and describes what the screening procedure entails. The information that you provide will be collected and stored confidentially until we are able to determine if you are eligible to come for an in-person screening visit, at which point your information will be deleted from the Qualtrics database. If you would like to proceed with the pre-consent form and Qualtrics survey, please navigate to the following link: [URL].*
> 2. *If you prefer not to take the online Qualtrics survey, you can speak directly with our study team by phone and/or email. We will ask you the same questions, but as you would not first electronically sign the pre-consent form, we would not record any information provided by phone and delete any information provided by email within one (1) business day. If you prefer this latter option, please reply to this message with your:*
>
> - *Full legal name*
> - *Sex/Gender*
> - *Ethnicity: Hispanic or Latino; Not Hispanic or Latino; Unknown*
>
> *Race: American Indian or Alaska Native; Asian; Black or African American; Native Hawaiian or Other Pacific Islander; White; Unknown*
>
> - *Date of birth*
> - *Current address*
> - *Phone number that is most reliable for you (and is it okay for us to send you text messages to coordinate?)*
> - *Emergency contact: name, relationship to you, and reliable phone number*
> - *BMI – You can use this calculator: https://www.nhlbi.nih.gov/health/educational/lose_wt/BMI/bmicalc.htm*

Based on your responses to the online or phone/email questionnaire, you may be invited to continue onto an in-person screening visit that will include collecting blood and urine for further testing and undergoing an electrocardiogram (heart rhythm check). If you have any questions, please feel free to respond to this message/email me at zds2120@cumc.columbia.edu or call (212) 305-9336.

All the best,
Ishwari

Qualtrics pre-consent. Qualtrics provides HIPAA-compliant online questionnaires that can be used for pre-screening potential recruits. Obtaining pre-screening information related to eligibility criteria will save people the time, effort, and money of coming in for a screening visit that they might automatically fail. In order to participate in the Qualtrics pre-screening process, participants first provide a "pre-consent" that allows collection of their health information. The pre-consent form is attached to the RASCAL protocol under Documents. Once recruits have provided their consent to proceed with the pre-screening process, they will be directed to a Qualtrics survey.

The Qualtrics survey questions/layout are also attached to the RASCAL protocol under Documents. Qualtrics allows the pre-screening process to terminate automatically if the recruit provides information that is incompatible with study eligibility. For recruits who pass the Qualtrics pre-screening, we will communicate directly by phone or email (sent from @cumc.columbia.edu addresses only) in order to coordinate the logistics of the screening visit and, if the person does not already have a record in CUIMC/NYP Epic, to obtain the information necessary to create one: full legal name, date of birth, phone number, address, emergency contact (name, relationship, reliable phone number), and, per CRR policy, race and ethnicity. All such pre-screening contact will be documented on the pre-screening log in the study binder.

HIPAA Form B-based pre-screening. For recruits who have expressed interest in the study (though any IRB-approved means) but who do not wish to perform the Qualtrics pre-screening process, including those who feel less comfortable with the use of technology, we can perform "manual" pre-screening by phone or email. These questions will include the use of contraception by women of reproductive potential, height/weight/body mass index, use of prescription medications, and questions about elements of their medical history that are directly pertinent and limited to the study's inclusion/exclusion criteria. Before asking any questions, we will ask recruits if they are in a place that they feel comfortable discussing their health and let them know that we will keep their answers strictly confidential. They will then be asked to provide verbal consent to proceed. Because this "manual" pre-screening will not include consent and authorization as for the Qualtrics survey, the "manual" pre-screening will be governed by HIPAA Form B (already IRB approved for this study), including disposal of any health information collected within one (1) business day.

As above, we will communicate with people who have expressed interest in the study (through any IRB-approved means) *via* phone or email (sent from @cumc.columbia.edu addresses only) in order to coordinate the logistics of the screening visit and, if the person does not already have a record in CUIMC/NYP Epic, to obtain the information necessary to create one: full legal name, date of birth, phone number, address, emergency contact (name, relationship, reliable phone number). Per the policy of the CRR, we will also inquire as to race and ethnicity. All such pre-screening contact will be documented on the pre-screening log in the study binder.

6. STUDY ONBOARDING AND WITHDRAWAL PROCEDURES

6.1 – CUIMC research participant onboarding

6.1.1. Informed consent procedures

Informed consent will be obtained from all recruits for the study in its entirety, either in English or Spanish, by authorized study staff before any study screening procedures begin. Informed consent will be obtained using the ACE method (Assessing readiness, Conferring information, and Ensuring understanding). Materials and language used will be at the eighth-grade level. For recruits who speak only Spanish, consent will be obtained either by a research staff member who is a fluent or native Spanish speaker or the CUIMC translation service will be used. All documents will also be provided as certified Spanish translations. Documentation of participation will be obtained from adult participants; minors are not eligible for the study, nor are individuals whose capacity to provide informed consent is questionable. The written Consent document is enclosed in the Attachments section of RASCAL.

6.1.2. Enrollment

A recruit is "enrolled" – that is, study participation begins – once written informed consent is obtained; they are at that point redesignated as an *enrollee*. Once informed consent is obtained, a study identification (SID) number will be assigned. Until the enrollee is verified to meet all the inclusion and exclusion criteria with screening, the suffix "-E" will be appended. Once the criteria are satisfied, the enrollee will be redesignated as a *participant*; the SID suffix "-E" will be dropped to leave only the number.

The numbers will be used to identify the enrollee/participant throughout study participation. A master log of all consented enrollees/participants will be maintained at the site and will document all screening failures (*i.e.*, participants who are consented but do not meet study eligibility criteria and/or are not randomized), including the reason(s) for screening failure. Enrollees who fail to meet the inclusion/exclusion criteria (*i.e.,* screening failures) will not be randomized or otherwise further participate in the study. However, enrollees who fail screening initially but become eligible to participate based on changes in inclusion/exclusion criteria under later versions of the protocol may be randomized for participation provided they (1) gave consent for future contact and (2) again provide informed consent under the most up-to-date version of the Informed Consent Form. In such cases, the enrollee will maintain their original SID number and file, but the eligibility redetermination and the re-consent process will be clearly documented in their study file.

If an enrollee fails screening because of a clinically significant finding, they will be notified and provided with a report that they can take to their physician. The investigators will follow up with such individuals to ensure that they have seen or have secured an appointment to see a physician regarding the clinically significant finding.

Enrollees who fail screening for this study but who do fulfill inclusion/exclusion criteria for participation in IRB-AAAU3014/U9636/U9702/V2481 may be invited to attend a screening visit for those studies instead. There, following a separate, new informed consent process, screening data collected for this study can additionally be used to make an eligibility determination for that study.

6.2 – Clinical trial design considerations

This will be a randomized, double-blinded, placebo-controlled clinical trial (pilot & feasibility study).

6.2.1. Randomization

Participants meeting all inclusion/exclusion criteria will be randomized to each of the three study groups (placebo, diazoxide 1 mg kg^{-1} ["DZ1"] diazoxide 2 mg kg^{-1} ["DZ2"]) at a ratio of 1:1:1, according to a computer-generated block-permuted schedule provided by an independent biostatistician. The biostatistician will email the schedule, or a link to generate the schedule, directly to the Research Pharmacy for consultation in making up the appropriate investigational agent preparation.

6.2. Blinding

Both investigators and participants will be blinded to the assigned treatment group. Routine unblinding will occur to investigators only after all of a participant's samples have been submitted for laboratory analysis. It should be noted, however, that investigators may get a sense of group allocation based on changes in blood glucose. However, due to interindividual variability in extent of insulin resistance and body mass index, it will not be possible to assuredly decode the randomization prior to unblinding. Unplanned unblinding of the study PI will occur only in the case of early withdrawal

and/or medical emergency (*e.g.*, severe hyperglycemia), which in most cases anyway will result in study termination. If they opt in, participants will be notified of their group assignment once all relevant data are collected and analyzed.

Again, described in §4.2, we conducted a pre-planned unblinding and interim analysis after accruing half of the project number of study completers (*i.e.*, 3 per arm) to assess the insulin-lowering effect *versus* tolerability of each dose of diazoxide with an eye to possibly eliminating one of the two diazoxide dosing arms. Those data were inconclusive due to variability from small sample sizes and so we did not make any changes to the protocol.

6.3 – Study sample policies

6.3.1. Biological samples obtained for the main study

Study data are protected by the use of a SID (study identification number), which is a number specific to the participant. Screening data obtained for IRB-AAAU3014/U9636/U9702/V2481, or AAAU9950 that is "double counted" toward this study after a screen-failure for either of the two others will be reassigned a new SID for this study, but a note (cross-reference) will be kept of the "old" SID in the biospecimen log. The investigator is in control of the information that is needed to connect a study sample to a participant. All investigators listed on the protocol may participate in the processing, storage, and/or assay of the samples.

A participant's consent to the use of the data does not have a specific expiration date, but the participant may withdraw consent at any time by notifying the investigator. If a patient withdraws or is removed from the study, stored samples (*i.e.*, prior to onset of events leading to study departure) deemed valid by the PI may still be included in analyses of the present study unless the participant withdraws their consent to use it. Withdrawal of consent will prompt immediate destruction of existing stored samples but does not require destroying results or study analyses that may have been generated using the participant's stored samples prior to withdrawal of consent.

Small quantities of the samples will be securely transferred to our collaborator, Dr. Marc Hellerstein, and his staff at the University of California Berkeley. This is necessary for determining D_2O enrichment and assessment of *de novo* lipogenesis. The transferred samples and/or data will be labeled with the SID, but neither Dr. Hellerstein nor his Berkeley team will have access to the key linking the SID to specific participants or PHI/PII. IRB-approved blanket material transfer (MTA) and data use (DUA) agreements between the CUIMC study team and Dr. Hellerstein's lab will be required to execute any transfer of specimens and/or data. The fully executed MTA/DUA is submitted in RASCAL. (Also see §14.7.)

6.3.2. Samples obtained for future research

Samples obtained for future research may be archived in the Insulin Resistance Biobank used for potential future analysis according to the participant's permission in the Consent Form. Samples obtained for future research will be labeled with a sample identification number but will not be labeled with personal identifiers such as a patient's name. A file linking this sample identification number with the SID will be kept in a secure place, with restricted access, by the study PI. If the participant withdraws consent for participating in the future research, this link will allow the PI to locate and destroy the participant's sample.

The coding of samples and results is to ensure that these research results are kept confidential by keeping the participant's identity and these results separate. If the participant consents to have their samples used for future research, this additional research may not start immediately and may start at any time during the storage period. The participant's sample(s) will be aliquoted and stored by the PI for at least seven (7) years.

6.3.3. Withdrawal of consent for future use of study samples

If the participant chooses not to allow their study samples to be used for future research, the samples will be destroyed by the PI once they are no longer required for the main study.

If consent is withdrawn after a sample has been taken, but before the participant's sample is stored for future research, the investigator will arrange to have it destroyed.

If consent is withdrawn after the participant's sample(s) have been stored for future research, the investigator will ensure that these sample(s) are destroyed unless the identification number has been removed and the participant can no longer be linked to any sample(s). However, if the participant's samples have already been used for research (*i.e.*, to generate data), the PI is not required to destroy the results of this research. In this case, only the remaining sample(s) will be destroyed.

6.4 – Study withdrawal

6.4.1 Study withdrawal policies
Participants are at any time free to withdraw from the study, without prejudice to any future treatment. if complete withdrawal of consent is established, then no further study procedures or data collection should take place.

The investigator may determine that an individual participant will not receive any further medicinal products if any of the following occur:

- Safety reasons as judged by the investigator where continued treatment may put the participant at undue risk; see §12.5 for further discussion of rules governing safety-related study interruptions.
- Any other adverse event that, in the opinion of the investigator, contraindicates further dosing
- Incorrectly randomized participant in whom inclusion/exclusion criteria violation would put the participant at undue risk
- Participant noncompliance that, in the opinion of the investigator, warrants treatment discontinuation for safety reasons or due to irreconcilable confounding of collected data
    - May include missed investigational agent doses, according to the PI's scientific discretion (*e.g.*, if dose missed early in the study then would have less of an impact on the data than a dose missed immediately before a study visit blood draw)
    - Includes any missed study visits. Due to the short duration of the study, there is not flexibility built in for missing study visits; we will review the participant's/enrollee's calendar prior to screening to ensure that no scheduling conflicts will arise during the study period

6.4.2. Replacement of participants
Participants who withdraw from the study will not count toward the accrual goal and may be replaced by the study PI to ensure that the data collected are sufficient to measure the study main outcomes. However, data collected prior to withdrawal may be included in study analyses, pending the discretion of the PI and no withdrawal of consent from the participant.

7. STUDY CALENDAR

7.1 – Screening visit (SV-0)

- *Consent:* Member of the study team will discuss and consent the enrollee for the study, following all registration procedures discussed above.
    - "Consent" includes a detailed description of all study procedures, answers any and all pertinent questions, and solicits informed consent for all study visit procedures.
- *Medical history:* Recruit's medical history to be discussed to the extent necessary to verify eligibility
    - All data will be captured on the appropriate CRF
- *Physical examination:* Targeted physical examination will be performed by the PI or qualified designee, including auscultation of heart/lungs, evaluation of clinical volume status, assessment of stigmata of chronic liver disease, and other pertinent items.
    - All data will be captured on the appropriate CRF.
- *Electrocardiogram:* 12-lead EKG will be performed by qualified technician and interpreted by the PI

- EKG requirement will be waived if result available from within the previous 90 d and no intervening clinical changes of concern have occurred
- All data will be captured on the appropriate CRF

- *Biometrics:* Measurements to be taken include vital signs (heart rate, blood pressure, respiratory rate, temperature), height, weight, and waist circumference.
  - Will use these data to calculate body mass index (BMI) and body surface area (BSA).
  - All data will be captured on the appropriate CRF.
- *Screening labs:* If the recruit has not consumed calories in the previous ≥ 8 hours, they can have screening fasting labs drawn and biometrics measured at the same time as the visit (see below). However, if the recruit is not fasting, or if the screening visit is scheduled such that blood draw cannot occur before 10:00, they will be asked to return as soon as possible for a phlebotomy-only visit to have morning fasting labs drawn (will be considered part of SV-0 for administrative purposes).
  - Screening labs can be drawn either by the study team at the IICTR CRR or at an NYP/CUIMC clinical phlebotomy site.
  - Screening labs to be drawn, and results necessary for inclusion, are as follows:
    - Plasma glucose
    - Serum insulin and *c*-peptide
    - Hemoglobin A_{1c}
    - Lipid profile (total cholesterol, LDL-C, HDL-C, triglycerides)
    - Liver function tests (total protein, albumin, total bilirubin, direct bilirubin, alanine aminotransferase, aspartate aminotransferase, total alkaline phosphatase)
    - Basic metabolic panel (sodium, potassium, chloride, bicarbonate, blood urea nitrogen, creatinine, calcium)
    - Uric acid
    - Glucose-6-phosphate dehydrogenase (*cf.* presence of quinine in tonic water placebo), unless prior result already on record
    - Complete blood count (CBC)
      - Only need this for platelet count to calculate baseline FIB-4 score
    - Serum or urine hCG (pregnancy test, where applicable)
    - Urine drug screen
      - Residual serum not consumed in above analyses for this and all subsequent study time points may be entered into Insulin Resistance Biobank (see §14.7).
  - Once the labs have resulted, the enrollee will be contacted to review the results. If lab results are not exclusionary (see §5.3.2), the study team will consider the enrollee to have passed screening and then will proceed on to Study Visit 1 (SV-1) as a participant (see §7.3).

7.2 – Screening Procedures

7.2.1. Informed consent

Prior to any screening procedures, a qualified member of the study team will discuss and consent the recruit for the study, following all registration procedures discussed above (see §6.1.1). Consenting includes a detailed description of all study procedures, answers all pertinent questions, and signature of an informed consent form (attached). Once informed consent is obtained, the recruit is designated as an *enrollee*. Note that enrollees who failed screening under prior versions of the protocol but would now be eligible for participation based on those screening data may repeat the consent process to update them to the most current version. We would only invite enrollees who originally gave consent for future contact to reenroll.

7.2.2. Medical history and physical examination

Medical history at screening will include history and current medical conditions, past or present cardiovascular, respiratory, gastrointestinal, renal, hepatic, neurologic, endocrine, lymphatic, hematologic, immunologic, dermatologic, psychiatric, or

genitourinary disorders; drug and surgical history; or any other diseases or disorders. A standard physical examination, including vital signs (see §7.2.3) will be conducted at every visit. Any focal deficit identified at baseline should be documented. Clinically significant abnormal findings will be recorded at baseline and follow up. For enrollees recently completing AAAU9950 or failing screening for AAAU2570 (*i.e.*, in previous protocol versions) or AAAU3014/U9636/U9702/V2481 medical history and physical exam will, with the consent of the enrollee, be co-opted for use in screening for this study. Thus, if there has been no clinical change during the short interval since screening for one of the other two studies, it will not be necessary to retake a full medical history and physical. Screening medical history and physical exam obtained for enrollees ultimately found ineligible to participate in this study, but who are eligible to participate in IRB-AAAU3014 or AAAU9636 will, with the enrollee/participant's permission, be applied for screening for the other study.

7.2.3. Vital signs

Vital sign measurements (blood pressure, heart rate, respiratory rate, and body temperature) will be obtained after the participant has rested in a seated position for at least 5 min. They will be collected at the screening visit (unless recently measured during screening for previous protocol versions of AAAU2570 or for AAAU3014/U9636/U9702/V2481) and at each Study Visit.

- Temperature will be taken orally according to standard operating procedure.
- Blood pressure and heart rate:
    - Calibrated and appropriately maintained automated devices will be used for measuring blood pressure and heart rate. The device cuff will be measured in the upper arm (*i.e.*, brachial artery), with the upper arm at heart level, for at least 30 s. We will use the same, properly sized cuff for each measure.
    - Participants must remain seated for the entire measurement. The same arm should be used, whenever possible, for each blood pressure assessment.

7.2.4. Biometrics

Height, body weight, and waist circumference will be measured at the screening visit (unless recently measured during screening for previous protocol versions of AAAU2570 or for AAAU3014/U9636/U9702/V2481, or AAAU9950) and at Study Visit 4. Body mass index and body surface area will be calculated on the basis of these data using the DuBois formula [111]:

$$\text{Body surface area } (m^2) = 0.007184 \times \text{weight (kg)}^{0.425} \times \text{height (cm)}^{0.725}$$

7.2.5. Electrocardiogram (EKG)

Twelve-lead EKG will be performed by qualified staff at the IICTR CRR or at another appropriate NYP/CUIMC clinical site using a standard device (*e.g.*, GE MAC 5500 or equivalent) that has been maintained and tested according to usual hospital standards. The reading will be taken after the recruit has rested comfortably for at least 10 min and has been asked about symptoms potentially concerning for angina. Obviously spurious or questionable results (*e.g.*, with movement artifact, incorrect lead placement, *etc.*) will be repeated. The screening EKG requirement is waived if a result is available in the EMR from within the previous 90 days, including if obtained during screening for previous protocol versions of AAAU2570 or for IRB-AAAU3014 and no concerning clinical changes have intervened.

The PI or qualified physician designate will review all EKG results within 48 hours. In the event of an abnormal EKG, the PI will discuss the overall clinical context with the recruit and may repeat the EKG as necessary. If the PI determines that the result is reliable and requires follow-up, he will notify the recruit's primary physician or other designated personal physician and. The impact of EKG on study eligibility will be made at the discretion of the PI in consideration of the benefit-to-risk ratio. EKG may be repeated during the course of the study on an as-needed basis, as judged by the PI.

7.2.6. Clinical laboratory tests

All screening laboratory tests (Blood Draw 0, BD-0) will be drawn either by study staff at the IICTR CRR or by NYP/CUIMC phlebotomy staff at CUIMC clinical phlebotomy sites (*e.g.*, HIP-1, VC-240, *etc.*). Blood will be collected by standard

venipuncture using evacuated collection tubes (BD™ Vacutainer® or equivalent) and sterile technique. Collection tubes will include serum-separator tubes (SST, gold-top tubes) for serum determinations, fluoridated tubes (NaF, gray-top tubes) for blood glucose measurements, and EDTA (lavender-top) tubes for hemoglobin A_{1c} determination on whole blood and for lipoprotein fractionation. See Table 7.v (in §7.5, below) for full schedule of labs.

All screening laboratory tests will be assayed at the CUIMC Center for Advanced Laboratory Medicine (or sent out by them to commercial reference laboratories, where necessary). Clinically significant (as determined by the investigator) abnormal laboratory results should be repeated as expediently as reasonable (see §5.4).

For enrollees recently completing AAAU9950 or failing screening for IRB-AAAU3014/U9636/U9702/V2481, or previous protocol versions of AAAU2570, screening lab data will, with the consent of the enrollee, be co-opted for use (or reuse) in screening for this study. Thus, if there has been no clinical change during the short interval since screening for one of the other two studies, it will be necessary only to draw G6PD and uric acid (*i.e.*, the two screening labs drawn for this but not the other studies). Screening labs obtained for enrollees ultimately found ineligible to participate in this study, but who are eligible to participate in IRB-AAAU3014/U9636/U9702/V2481 will, with the enrollee/participant's permission, be applied for screening for the other study.

7.2.7. HOMA-IR score

For participants not meeting the diagnosis of prediabetic state (impaired fasting glucose and/or prediabetic HbA_{1c}) on screening labs, we will calculate a Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) score based on the fasting plasma glucose and serum insulin levels [112], as follows:

$$\text{HOMA-IR score} = \text{fasting plasma glucose (mg dL}^{-1}) \times \text{fasting serum insulin (}\mu\text{IU mL}^{-1}) \div 405$$

Participants with a HOMA-IR score calculated as ≥ 2.73 will be considered to meet the inclusion criterion of insulin resistance (§5.2.f). This cutoff is based on data from NHANES, in which this specific cutoff identified the top tertile of HOMA-IR scores across a diverse United States patient population [91].

7.2.8. FIB-4 score

The FIB-4 score has been validated as a metric of NAFLD disease activity and predicts progression to cirrhosis [113]. We will calculate a FIB-4 score for all participants as a baseline characteristic but it will not be used as a study endpoint and therefore calculated only from data at the screening visit. FIB-4 is calculated based on age, transaminase levels (AST/ALT), and platelet count as follows:

$$\text{FIB-4 score} = \text{age (y)} \times \text{AST level (U L}^{-1}) \times [\ \text{platelet count (} \times 10^{9}\ \text{L}^{-1})\ ]^{-1} \times [\ \text{ALT level (U L}^{-1})\ ]^{1/2}$$

7.2.9. Determination and randomization

Following the screening visit, the study team will review the laboratory data and, coupled with the information obtained from history and physical at screening, an eligibility determination will be made according to the inclusion and exclusion criteria in §§5.2, 5.3. The enrollee will be deemed eligible for randomization if all inclusion and exclusion criteria are satisfied and will be notified of their eligibility determination as expediently as possible. For enrollees that were already screened for IRB-AAAU3014/U9636/U9702/V2481, or AAAU9950, we will render the eligibility determination pending the results of any lab results that we do not already have in hand from those studies. For enrollees in AAAU2570 previously determined to be ineligible but who become eligible after changes in inclusion/exclusion criteria, we will render the eligibility determination pending re-consent and discussion of any interval changes in health status. If an eligible enrollee wishes to continue in the study, they will then be redesignated as a participant. Randomization will occur (see §6.2.1) and study procedures will commence (see §§7.3, 7.4) within 30 d of the eligibility determination.

In the event of abnormal lab results, the PI will evaluate the clinical context. If the PI determines that the laboratory value was likely spurious or transient and without ongoing clinical significance, the recruit may be re-screened at a later time. If the lab result normalizes and the clinical context remains stable, the recruit may be enrolled at the discretion of the PI.

Enrollees who do not meet inclusion/exclusion criteria for this study, but who would be eligible to participate in our other insulinization-lowering pilot & feasibility studies (AAAU3014/U9636/U9702/V2481), may be invited to enroll (with separate, new informed consent) in that study instead. In that case, screening data obtained for this study would, with the enrollee/participant's permission, be counted as screening data for one of those other studies.

7.3 – Study Visits *(see also schematic Figure 4.i and summary in Table 7.i, below)*

Within 30 d of eligibility determination, participants will be randomized (see §6.2.1) to one of three study groups: placebo, diazoxide 1 mg kg^{-1} (DZ1 group), or diazoxide 2 mg kg^{-1} (DZ2 group), and study procedures will commence as outlined below. Note that the procedures listed in the following schedule are explained in further detail in §7.3. Because of the complexity of the study, participants will be provided with easy-to-read instruction sheets and schedule (see attachment) to aid in compliance.

Study Day	Time of day	Study procedures
Day -1	21:00	• Begin overnight fast (except water)
Day 1	09:00	**Study Visit 1 (SV-1)** 1. Present to Outpatient Clinical Research Unit (OCRU); pertinent history and physical (H&P) 2. Check weight and waist circumference 3. Undergo **Blood Draw 1 (BD-1)** 4. Download USDA MyPlate mobile app and instruct on its use for dietary guidance 5. Undergo **Saliva Collection 1 (SC-1)** 6. Apply FreeStyle Libre Pro continuous glucose monitor (CGM) on upper arm (while commercially available) 7. Provide **Investigational Agent Doses** 1P (x 2) **(IAD-1P)** with instructions for home use 8. Administer **Deuterated Water Dose 1A (DW-1A)** Provide **Deuterated Water Doses 1M/P (DW-1M/P)**, with instructions for home use.
	13:00	• Consume lunch, based on dietary guidance, by 13:30 • Ingest **Deuterated Water Dose 1M (DW-1M)**
	18:00	• Consume dinner, based on dietary guidance, by 18:30 • Thereafter, begin overnight fast (except water)
	21:00	• Ingest **Investigational Agent Doses 1P** (x 2) **(IAD-1P)** • Ingest **Deuterated Water Dose 1P (DW-1P)**
Day 2	09:00	**Study Visit 2 (SV-2)** 1. Present to OCRU; perform pertinent H&P 2. Review diet and recheck weight and waist circumference 3. Undergo **Blood Draw 2 (BD-2)** 4. Collect empty containers from **IAD-1P** and **DW-1M/-1P** 5. Provide **Investigational Agent Doses 2P-8P (IAD 2P-8P)** with instructions for home use 6. Administer **Deuterated Water Dose 2A (DW-2A)** 7. Provide **Deuterated Water Doses 2P-8P (DW 2P-8P)**, with instructions for home use. 8. May begin to eat *ad libitum*, following dietary guidance, from this point between 07:00 and 21:00

	13:00	• Ingest **Deuterated Water Dose 2B (DW-2M)**
	21:00	• Ingest **Investigational Agent Dose 3 (IAD-2P)** • Ingest **Deuterated Water Dose 2C (DW-2P)** • Fast overnight (21:00-07:00) except water
Day 3	09:00	• Ingest **Investigational Agent Dose 4 (IAD-3A)** • Ingest **Deuterated Water Dose 2 (DW-3A)** • Consume study diet *ad libitum* between 07:00-21:00
	13:00	• Ingest **Deuterated Water Dose 3M (DW-3M)**
	21:00	• Ingest **Investigational Agent Dose 3P (IAD-3P)** • Ingest **Deuterated Water Dose 3P (DW-3P)** • Fast overnight (21:00-07:00) except water
Day 4	09:00	• Ingest **Investigational Agent Dose 4A (IAD-4A)** • Ingest **Deuterated Water Dose 4A (DW-4A)** • Consume study diet *ad libitum* between 07:00-21:00
	13:00	• Ingest **Deuterated Water Dose 4M (DW-4M)**
	21:00	• Ingest **Investigational Agent Dose 4P (IAD-4P)** • Ingest **Deuterated Water Dose 4P (DW-4P)**
Day 5	09:00	• Undergo **Saliva Collection 2 (SC-2)**, save to bring to **SV-3** • Ingest **Investigational Agent Dose 5A (IAD-5A)** • Ingest **Deuterated Water Dose 5A (DW-5A)** • Consume study diet *ad libitum* between 07:00-21:00
	21:00	• Ingest **Investigational Agent Dose 5P (IAD-5P)** • Ingest **Deuterated Water Dose 5P (DW-5P)** • Fast overnight (21:00-07:00) except water
Day 6	09:00	• Ingest **Investigational Agent Dose 10 (IAD-6A)** • Ingest **Deuterated Water Dose 6A (DW-6A)** • Consume study diet *ad libitum* between 07:00-21:00
	21:00	• Ingest **Investigational Agent Dose 6P (IAD-6P)** • Ingest **Deuterated Water Dose 6P (DW-6P)** • Fast overnight (21:00-07:00) except water
Day 7	09:00	• Ingest **Investigational Agent Dose 12 (IAD-7A)** • Ingest **Deuterated Water Dose 7A (DW-7A)** • Consume study diet *ad libitum* between 07:00-21:00
	21:00	• Ingest **Investigational Agent Dose 13 (IAD-7P)** • Ingest **Deuterated Water Dose 7B (DW-7P)** • Fast overnight (21:00-07:00) except water
Day 8	09:00	• Ingest **Investigational Agent Dose 14 (IAD-8A)** • Ingest **Deuterated Water Dose 8A (DW-8A)** • Consume breakfast, based on dietary guidance, by 09:30
	13:00	• Consume lunch, based on dietary guidance, by 13:30
	18:00	• Consume dinner, based on dietary guidance, by 18:30; fast overnight except water
	21:00	• Ingest **Investigational Agent Dose 15 (IAD-8P)** • Ingest **Deuterated Water Dose 5P (DW-8P)**

Day 9	09:00	**Study Visit 3 (SV-3)** 1. Present to OCRU; perform pertinent H&P 2. Review diet and recheck weight and waist circumference 3. Undergo **Blood Draw 3 (BD-3)** 4. Undergo **Saliva Collection 3 (SC-3)** 5. Collect empty containers from **IAD-2P** through **-8P** and **DW-2P** through **-8P** 6. Administer **Investigational Agent Dose 9A (IAD-9A)** 7. Provide **Investigational Agent Doses 9P-14P (IAD 9P-14P)** with instructions for home use 8. Administer **Deuterated Water Dose 9A (DW-9A)** 9. Provide **Deuterated Water Doses 9P-14P (DW 9P-14P)**, with instructions for home use 10. May resume eating *ad libitum*, following dietary guidance, between 07:00 and 21:00
	21:00	• Ingest **Investigational Agent Dose 9P (IAD-9P)** • Ingest **Deuterated Water Dose 9P (DW-9P)** • Fast overnight (21:00-07:00) except water
Day 10	09:00	• Ingest **Investigational Agent Dose 10A (IAD-10A)** • Ingest **Deuterated Water Dose 10A (DW-10A)** • Consume study diet *ad libitum* between 07:00-21:00
	21:00	• Ingest **Investigational Agent Dose 10P (IAD-10P)** • Ingest **Deuterated Water Dose 10P (DW-10P)** • Fast overnight (21:00-07:00) except water
Day 11	09:00	• Ingest **Investigational Agent Dose 11A (IAD-11A)** • Ingest **Deuterated Water Dose 11A (DW-11A)** • Consume study diet *ad libitum* between 07:00-21:00
	21:00	• Ingest **Investigational Agent Dose 21 (IAD-11P)** • Ingest **Deuterated Water Dose 11P (DW-11P)** • Fast overnight (21:00-07:00) except water
Day 12	09:00	• Ingest **Investigational Agent Dose 12A (IAD-12Aa)** • Ingest **Deuterated Water Dose 12A (DW-12A)** • Consume study diet *ad libitum* between 07:00-21:00
	21:00	• Ingest **Investigational Agent Dose 12P (IAD-12P)** • Ingest **Deuterated Water Dose 12P (DW-12P)** • Fast overnight (21:00-07:00) except water
Day 13	09:00	• Ingest **Investigational Agent Dose 13A (IAD-13A)** • Ingest **Deuterated Water Dose 13A (DW-13A)** • Consume study diet *ad libitum* between 07:00-21:00
	21:00	• Ingest **Investigational Agent Dose 13P (IAD-13P)** • Ingest **Deuterated Water Dose 13P (DW-13P)** • Fast overnight (21:00-07:00) except water
Day 14	09:00	• Ingest **Investigational Agent Dose 14A (IAD-14A)** • Ingest **Deuterated Water Dose 10A (DW-14A)** • Consume breakfast, based on dietary guidance by 09:30
	13:00	• Consume lunch, based on dietary guidance, by 13:30
	18:00	• Consume dinner, based on dietary guidance, by 18:30; fast overnight except water

	21:00	• Ingest **Investigational Agent Dose 14P (IAD-14P)** • Ingest **Deuterated Water Dose 14P (DW-14P)**
Day 15	09:00	**Study Visit 4 (SV-4)** 1. Present to OCRU; perform pertinent H&P 2. Review diet and recheck weight and waist circumference 3. Undergo **Blood Draw 4 (BD-4)** 4. Undergo **Saliva Collection 4 (SC-4)** 5. Collect empty containers from **IAD-9P** through **-14P** and **DW-9P** through **-14P** 6. Remove FreeStyle Libre Pro continuous glucose monitor • **Study Completion** is defined as reaching this point

Table 7.ia. *Summary of Study Procedures: Study Days 1-8*

Study procedures	Screening	Study Day																	
		-1	1		2		3		4		5		6		7		8	
		PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM
Informed consent	●																	
Study Visit (SV-)	●		1		2													
History & physical	●		●		●													
Biometrics	●		●		●													
Electrocardiogram	●																	
Diet counseling			●		●													
Continuous glucose monitor			●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Blood draw (BD-)	0		1		2						3							
Saliva collection (SC-)			1								2							
Investigational Agent dose (IAD-)				0+1		2P	3A	3P	4A	4P	5A	5P	6A	6P	7A	7P	8A	8P
D_2O aliquot (DW-)			1A1M	1P	2A 2M	2P	3A 3M	3P	4A 4M	4P	5A	5P	6A	6P	7A	7P	8A	8P

Table 7.ib. *Summary of Study Procedures: Study Days 9-15*

Study procedures	Study Day												
	9		10		11		12		13		14		15
	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM
Informed consent													
Study Visit (SV-)	3												4
History & physical	●												●
Biometrics	●												●
Electrocardiogram													
Diet counseling	●												●
Continuous glucose monitoring	●	●	●	●	●	●	●	●	●	●	●	●	●
Blood draw (BD-)	3												4
Saliva collection (SC-)	3												4
Investigational Agent dose (IAD-)	9A	9P	10A	10P	11A	11P	12A	12P	13A	13P	14A	14P	
D_2O aliquot (DW-)	9A	9P	10A	10P	11A	11P	12A	12P	13A	13P	14A	14P	

7.4 – Explanation of study procedures

This section discusses participant-facing procedures. Laboratory methods are discussed in §8 and pharmacy procedures/investigational agents in §§10-11.

7.4.1. Study site

Treatment will be administered on an outpatient basis at the Columbia University Irving Institute for Clinical and Translational Research (IICTR) Clinical Research Resource's (CRR) Outpatient Clinical Research Unit (OCRU). The study PI will be the attending physician of record for all study visits and will comply with all IICTR CRR policies.

Prior to presentation of participants to the OCRU, all study staff (nurses, lab techs, other phlebotomists, and any research assistants who later join the team) will be trained in the study protocol by the PI or qualified, authorized co-investigator. They will review and discuss the requirements of the clinical study protocol and related documents with the investigational staff and will also train them in any study-specific procedures and system(s) utilized. The PI will ensure that appropriate training relevant to the study is given to all of these staff members, and that any new information relevant to the performance of this study is forwarded to the staff involved. The PI will maintain a record of all individuals involved in the study (*i.e.*, medical, nursing, and other staff).

7.4.2. Phlebotomy

Blood will be collected *via* standard venipuncture using evacuated collection tubes (BD™ Vacutainer® or equivalent) by registered nurses or qualified phlebotomists employing sterile technique. Collection tubes will include serum-separator tubes (SST, gold-top tubes) for serum determinations, fluoridated tubes (NaF, gray-top tubes) for blood glucose measurements, and EDTA (lavender-top) tubes for hemoglobin A_{1c} determination on whole blood plus plasma lipoprotein fractionation (for VLDL). See Table 7.v (in §7.4, below) for full schedule of labs.

7.4.3. Study diet

Participants will follow a diet plan that is designed to maintain their baseline (screening) weight throughout the study and that will mimic and accommodate the design of the wider study. Participants will be advised not to consume alcohol during, or for 48 h before and after, the study period.

<u>*Fasting:*</u> Fasting means that participants may not consume any food or drink except for water and/or necessary medications. Participants will be asked to fast overnight, beginning either at 18:30 following consumption of mixed meals on Days 1, 8, and 14, or at 21:00 on the other days. Participants will also be asked to fast between discrete meals (breakfast, 09:00-09:30; lunch, 13:00-13:30; dinner, 18:00-18:30) on "discrete meal days" (Days 1, 8, and 14). Oral intake may resume after at least 10 h of overnight fasting.

<u>*Diet:*</u> Although there is not a standardized study diet, participants will be advised to follow a roughly weight-maintaining diet during the study, designed to provide each participant's daily energy requirements. We will base estimated daily caloric requirements using the U.S. Department of Agriculture's MyPlate widget, which provides caloric goals for weight maintenance based on age, sex, height, weight, and activity level.

During Study Visit 1, participants will be instructed to download and follow the United States Department of Agriculture's MyPlate mobile app. The app provides general dietetic guidance based individuals' daily calorie goals (*i.e.*, as calculated based on the MyPlate widget). Participants will be required to follow the app's recommendations for the "discrete meal days" (Days 1, 8, 14) and strongly advised to follow the app's recommendations on other days as well. Water, black coffee, plain tea, and calorie-free drinks may be consumed at will, but participants will be strongly advised not to consume any other beverages. They will be cautioned especially against consumption of juices, sodas, or other sugary beverages due to risk of hyperglycemia. Sugary snacks such as ice cream, candies, *etc.* will also be discouraged. Again, alcohol consumption is prohibited during the study period. We will discuss dietary adherence and recheck weight and waist circumference at each Study Visit, although we will not require participants to formally document or otherwise prove that they are strictly following dietary guidance.

7.4.4. Investigational agent administration

Investigational agents (*i.e.*, diazoxide 1 mg kg $^{-1}$, diazoxide 2 mg kg $^{-1}$, or placebo) will be dispensed by the Research Pharmacy pre-portioned in single-dose, single-use oral syringes whose contents are obscured by labeling covering the entire surface of the syringe barrel. Handling, preparation, and dosing of investigational agents are discussed in detail in §10. Participants will empty the investigational agent dose (IAD) contained within each single-use oral syringe directly into the mouth, all at once, and may wash it down with water (preferably the associated co-occurring D_2O aliquot as scheduled, see §7.4.6). All further IAD are labeled as Study Day/Time, where "A" denotes AM and "P" denotes PM. Beginning with IAD-2P (*i.e.*, Day 2, PM) on Day 2 at 21:00 (t = 24 h), doses will be taken every 12 hours. Note that on Study Day 1, the first two IAD (both labeled for participants as "1P") are taken together at 21:00 (t = 0 h). Participants will be instructed not to manipulate the labeling covering the syringe barrel or otherwise to visually inspect the investigational agent; they will also be instructed to return all empty syringes at the next study visit. Study personnel will inspect the integrity of the labeling when the empty containers are returned to ensure that unblinding has not occurred. A summary of the dosing scheme is provided in Table 7.iv.

Table 7.iv. *Investigational agent dose (IAD) scheme*

IAD		IAD syringe picked up		IAD admin-istration time		IAD syringe returned		IAD #		IAD syringe picked up		IAD admin-istration time		IAD syringe returned	
#	Label	Study Visit	Study Day	Study Day	Time of day	Study Visit	Study Day	#	Label	Study Visit	Study Day	Study Day	Time of day	Study Visit	Study Day
1	IAD-1P [a,b]	SV-1	Day 1	Day 1	21:00 [a]	SV-2	Day 2	16	IAD-9A [c]	SV-3	Day 9	Day 9	09:00	SV-3	Day 9
2	IAD-1P [a,b]	SV-1	Day 1	Day 1	21:00	SV-2	Day 2	17	IAD-9P	SV-3	Day 9	Day 9	21:00	SV-4	Day 15
3	IAD-2P	SV-2	Day 2	Day 2	21:00	SV-3	Day 9	18	IAD-10A	SV-3	Day 9	Day 10	09:00	SV-4	Day 15
4	IAD-3A	SV-2	Day 2	Day 3	09:00	SV-3	Day 9	19	IAD-10P	SV-3	Day 9	Day 10	21:00	SV-4	Day 15
5	IAD-3P	SV-2	Day 2	Day 3	21:00	SV-3	Day 9	20	IAD-11A	SV-3	Day 9	Day 11	09:00	SV-4	Day 15
6	IAD-4A	SV-2	Day 2	Day 4	09:00	SV-3	Day 9	21	IAD-11P	SV-3	Day 9	Day 11	21:00	SV-4	Day 15
7	IAD-4P	SV-2	Day 2	Day 4	21:00	SV-3	Day 9	22	IAD-12A	SV-3	Day 9	Day 12	09:00	SV-4	Day 15
8	IAD-5A	SV-2	Day 2	Day 5	09:00	SV-3	Day 9	23	IAD-12P	SV-3	Day 9	Day 12	21:00	SV-4	Day 15
9	IAD-5P	SV-2	Day 2	Day 5	21:00	SV-3	Day 9	24	IAD-13A	SV-3	Day 9	Day 13	09:00	SV-4	Day 15
10	IAD-6A	SV-2	Day 2	Day 6	09:00	SV-3	Day 9	25	IAD-13P	SV-3	Day 9	Day 13	21:00	SV-4	Day 15
11	IAD-6P	SV-2	Day 2	Day 6	21:00	SV-3	Day 9	26	IAD-14A	SV-3	Day 9	Day 14	09:00	SV-4	Day 15
12	IAD-7A	SV-2	Day 2	Day 7	09:00	SV-3	Day 9	27	IAD-14P	SV-3	Day 9	Day 14	21:00	SV-4	Day 15
13	IAD-7P	SV-2	Day 2	Day 7	21:00	SV-3	Day 9	[a] Both doses taken together on the evening of Day 1							
14	IAD-8A	SV-2	Day 2	Day 8	09:00	SV-3	Day 9	[b] The first two IAD will both be labeled for participants as "1P" for ease							
15	IAD-8P	SV-2	Day 2	Day 8	21:00	SV-3	Day 9	[c] IAD-9A administered at Study Visit 3							

7.4.5. Continuous glucose monitoring (CGM)

We will perform continuous glucose monitoring (CGM) using a FreeStyle Libre Pro (Abbott) intermittently scanned glucose monitor, which is approved by the FDA for glycemic monitoring in patients with diabetes. The device automatically records interstitial fluid glucose levels, which are directly proportional to blood glucose levels, at 15-min intervals. It does not require calibration or cross-checking with capillary glucose measurements (see attached product manual). The device includes two components:

- *Sensor:* The small, disc-shaped non-invasive CGM sensor is worn unobtrusively on the back of the upper arm. Placement is done with a custom-made device and is nearly painless. Once placed, the device samples interstitial fluid using a flexible microfilament that is 5 mm long and < 0.4 mm thick. It can be worn for up to 2 weeks in patients with diabetes, but will be applied for ~24 h to study participants. The device is water resistant up to 3 feet for 30 min and thus can be worn while showering or bathing. All participants will be fitted with brand new devices.
- *Reader:* A single reader device is used to activate and scan glucose data from all FreeStyle Libre Pro Sensors. The reader will remain with the study team but will not be used during study procedures.

This device is widely used for *professional CGM*, a modality in which patients are blinded from the CGM reading so that clinicians can observe their native glycemic trends without feedback behavioral modification. The device will be placed by the PI or their authorized designee at SV-1 and will be worn continuously until removal at SV-4. Study participants will be provided with an information sheet published by the manufacturer (Abbott) containing relevant information (see Attachments). Study participants will be blinded to the CGM readings as per usual professional CGM protocol. However,

investigators will also remain blinded to each participant's CGM readings until they reach study completion unless premature unblinding is medically necessary (see §12.5). The CGM tracing is downloaded at each study visit in order to ensure that the CGM sensor is functioning and as a means of preserving data in case the sensor comes off before the next study visit. However, the study team will ensure only that the data have been collected and will not look through the CGM data until each participant's study completion.

As of February 2025, we have been notified that Abbott is no longer manufacturing the FreeStyle Libre Pro CGM. As such, we will not be able to obtain CGM readings from the final three participants to go through the study (not enrolled yet). As there have not been any instances of clinically significant hyperglycemia in any of the 14 subjects who had completed the study at the time of the notification, we do not believe that this poses any real safety risk, particularly as the data remain by default effectively blinded until study's end anyway. We do not think it is advisable to switch to a different brand of CGM (*e.g.*, Dexcom) because the data would not be directly comparable to the Libre Pro. Unblinded CGM, such as Libre 2 or Libre 3, would not be feasible for the study either. The Libre 2 requires the patient to scan every 8 hours in order to preserve data, which would unblind them to the readings. The Libre 3 has a bothersome alarm for hypoglycemia that cannot be silenced; as these patients are not taking exogenous insulin, any hypoglycemia they experience will be endogenous and physiologic. Thus, we feel that the best approach would be to omit CGM data for the last participants who are unable to receive the Libre Pro. As CGM is only a secondary endpoint, it does not imperil overall data analysis.

7.4.6. Deuterated water enrichment

We will enrich participants' total body water pools to up to ~1.5% with deuterium oxide to enable measurement of *de novo* lipogenesis. To do this, participants will consume 50-mL aliquots of 70% D_2O (*i.e.*, 30% H_2O) every 12 hours, timed for convenience alongside investigational agent doses. Aliquots will be portioned by the study team in single-use containers labeled with the aliquot (DW-) number, using the same Study Day/Time code employed for IAD; from Days 1-4, these will also include a third "M" dose, for "midday." Handling, preparation, and dosing of D_2O are discussed in detail in §11. Participants will drink the D_2O aliquots fully within 5-10 minutes and save the empty containers to return at the following study visit. A summary of the dosing scheme is provided in Table 7.v. Compliance with D_2O intake will be ascertained by accounting for the empty returned containers and by monitoring the D_2O enrichment of plasma and saliva (obtained using sublingual salivette sponges) at each study visit.

Table 7.v. *Deuterated water dosing (DW-) scheme*

DW- #		DW container picked up		DW admin-istration time		DW container returned		DW- #		DW container picked up		DW admin-istration time		DW container returned	
#	*Label*	*Study Visit*	*Study Day*	*Study Day*	*Time of day*	*Study Visit*	*Study Day*	#	*Label*	*Study Visit*	*Study Day*	*Study Day*	*Time of day*	*Study Visit*	*Study Day*
1	DW-1A [a]	SV-1	Day 1	Day 1	09:00	SV-1	Day 1	21	DW-9A [a]	SV-3	Day 9	Day 9	09:00	SV-3	Day 9
2	DW-1M	SV-1	Day 1	Day 1	13:00	SV-2	Day 2	22	DW-9P	SV-3	Day 9	Day 9	21:00	SV-4	Day 15
3	DW-1P	SV-1	Day 2	Day 1	21:00	SV-2	Day 2	23	DW-10A	SV-3	Day 9	Day 10	09:00	SV-4	Day 15
4	DW-2A [a]	SV-2	Day 2	Day 2	21:00	SV-3	Day 9	24	DW-10P	SV-3	Day 9	Day 10	21:00	SV-4	Day 15
5	DW-2M	SV-2	Day 2	Day 2	13:00	SV-3	Day 9	25	DW-11A	SV-3	Day 9	Day 11	09:00	SV-4	Day 15
6	DW-2P	SV-2	Day 2	Day 2	21:00	SV-3	Day 9	26	DW-11P	SV-3	Day 9	Day 11	21:00	SV-4	Day 15
7	DW-3A	SV-2	Day 2	Day 3	09:00	SV-3	Day 9	27	DW-12A	SV-3	Day 9	Day 12	09:00	SV-4	Day 15
8	DW-3M	SV-2	Day 2	Day 3	13:00	SV-3	Day 9	28	DW-12P	SV-3	Day 9	Day 12	21:00	SV-4	Day 15
9	DW-3P	SV-2	Day 2	Day 3	21:00	SV-3	Day 9	29	DW-13A	SV-3	Day 9	Day 13	09:00	SV-4	Day 15
10	DW-4A	SV-2	Day 2	Day 4	09:00	SV-3	Day 9	30	DW-13P	SV-3	Day 9	Day 13	21:00	SV-4	Day 15
11	DW-4M	SV-2	Day 2	Day 4	13:00	SV-3	Day 9	31	DW-14A	SV-3	Day 9	Day 14	09:00	SV-4	Day 15
12	DW-4P	SV-2	Day 2	Day 4	21:00	SV-3	Day 9	32	DW-14P	SV-3	Day 9	Day 14	21:00	SV-4	Day 15
13	DW-5A	SV-2	Day 2	Day 5	09:00	SV-3	Day 9	[a] D_2O aliquot administered at the indicated study visit							
14	DW-5P	SV-2	Day 2	Day 5	21:00	SV-3	Day 9								
15	DW-6A	SV-2	Day 2	Day 6	09:00	SV-3	Day 9								
16	DW-6P	SV-2	Day 2	Day 6	21:00	SV-3	Day 9								
17	DW-7A	SV-2	Day 2	Day 7	09:00	SV-3	Day 9								
18	DW-7P	SV-2	Day 2	Day 7	21:00	SV-3	Day 9								
19	DW-8A	SV-2	Day 2	Day 8	09:00	SV-3	Day 9								
20	DW-8P	SV-2	Day 2	Day 8	21:00	SV-3	Day 9								

7.5 – Schedule of blood draws

Each blue dot indicates that the test at the head of its row is drawn at the time listed at the head of its column. Laboratory details of blood draws discussed further in §8.1.

Table 7.v. *Schedule of blood draws (all fasting)*

Tube type	Lab test	Screening	Study Visit			
			SV-1	SV-2	SV-3	SV-4
NaF	Glucose	●	●	●	●	●
SST [a]	Insulin	●	●	●	●	●
	c-peptide	●	●	●	●	●
	Apolipoprotein B		●	●	●	●
	Triglycerides	●	●	●	●	●
	Bile acid profile		●	●	●	●
	Adiponectin [g]		●	●	●	●
	IGFBP-1 [g,h]		●	●	●	●
	Total cholesterol	●				
	HDL-cholesterol	●				
	LDL-cholesterol	●				
	BMP [b,d]	●				
	LFT [c,d]	●				
	β-hCG [e]	●				
	Uric acid	●				
EDTA	Hemoglobin A_{1c}	●				
	Complete blood count	●	●	●	●	●
	Free fatty acids		●	●	●	●
	VLDL-TG		●	●	●	●
	VLDL-ApoB		●	●	●	●
	D_2O enrichment		●	●	●	●
	De novo lipogenesis				●	●
	Diazoxide level [g]		●	●	●	●
Li-hep	Glucose-6-phosphate dehydrogenase	●				
Saliva	D_2O enrichment [f]		●	●	●	●
Urine	Drug screen	●				

[a] Serum separator tube
[b] Basic metabolic panel (see § 8.1)
[c] Liver function tests (see § 8.1)
[d] Will also be repeated as needed at later time points should clinical concern arise
[e] Human chorionic gonadotropin, β subunit (only for women of childbearing potential)
[f] Saliva collection 2 (SC-2) done at home on Study Day 5 and brought back to SV-3
[g] May be performed in either EDTA-plasma or serum
[h] Insulin-like growth factor binding protein-1

8. MEASUREMENT OF EFFECT

8.1 – Blood tests

All treatment effects will be assessed by blood tests, drawn according to the schedule in Table 7.v. For this purpose, the following blood sample tubes will be drawn at intervals over the course of the screening and study visits. If not used immediately, residual material will be stored frozen within a biospecimen bank at the IICTR Biomarkers Core Laboratory or in a secure freezer unit in our research laboratory, as necessary based on assay (see table below). Measures of the primary and secondary endpoints will be discussed in subsequent sections.

Blood draw at the screening visit will be done by standard venipuncture into evacuated collection tubes (BD Vacutainer® or equivalent). For enrollees invited to screen after failing screening for IRB-AAAU768, AAAU3014/U9636/U9702/V2481, only G6PD, uric acid, and repeat β-hCG (where applicable) will need to be drawn. Those invited to screen after participating in AAAU9950 will also require BMP, LFT, and CBC. Assays requiring serum will be drawn in serum separator tubes (SST, gold top), those requiring plasma into fluoridated tubes (gray top) or EDTA (lavender top) tubes, and those requiring whole blood into EDTA (lavender top) tubes. Blood samples will be processed as expediently as possible, according to the specifications for each individual assay (see §§ 8.2-8.4). If the quantity of a blood sample is not sufficient for performing all assigned assays (see Tables 8.i, 8.ii), lab tests will be triaged according to hierarchy of endpoints (*i.e.*, primary endpoints prioritized over secondary endpoints, secondary endpoints over exploratory endpoints).

Table 8.i. *Volumes and types of blood tubes needed for each draw*

Tube type	Screening	Study Visit			
		SV-1	SV-2	SV-3	SV-4
SST, 5 cc (gold top)	10 [a]	10	10	10	10
NaF K_2EDTA, 2 cc (gray top)	2	2	2	2	2
EDTA, 4 cc (lavender top)	4	14	14	14	14
Li-heparin, 4cc (green top)	4				
Total blood volume at time point (cc)	20 [b,c]	26	26	26	26
Blood volume, running total (cc)	20	46	72	98	124

[a] All volumes entered in the above table are in cc of whole blood
[b] As only uric acid, G6PD, and repeat β-hCG (where applicable) will need to be drawn for recruits failing screening for IRB-AAAU3014/U9636/U9702/V2481, we will collect only 5 cc in SST and 4 cc in Li-heparin tubes, for total of 9 cc screening blood volume. As a result, the running total blood volume will be reduced by 11 cc.
[c] Recruits previously participating in AAAU9950 will also require BMP, LFT, and CBC, and so will require at least 3 cc to be drawn in EDTA, for a total of 12 cc blood volume, reducing the running total by 8 cc.

Plan for blood draws (see summary in Tables 8.i & 8.ii):

- At screening visit *(fasting ≥ 8 h)*
  - SST (gold top), ~15 cc whole blood total
    - Insulin
    - C-peptide
    - Basic metabolic panel: sodium, potassium, chloride, bicarbonate, blood urea nitrogen, creatinine, calcium
    - Liver function tests: total protein, albumin, bilirubin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase
    - Lipid profile: total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides
    - Uric acid
    - β-hCG (only for people of childbearing potential)
  - NaF (gray top), ~4 cc whole blood
    - Glucose (plasma)
  - EDTA (lavender top), ~4 cc whole blood
    - Hemoglobin A_{1c}

- Complete blood count
- Lithium-heparin (green top), ~4 cc whole blood
  - Glucose-6-phosphate dehydrogenase, unless prior result already on record
- *Note:* Urine will also be collected for drug screening using McKesson Drug Abuse Test Kit (point-of-care-assay). Results will be used only for the purpose of this study and will not be entered in the EMR or other health record.
- *Note:* With the exception of G6PD, uric acid, and repeat β-hCG (where applicable), these labs will not be redrawn for enrollees/participants invited to screen for this study after failing screening for IRB-AAAU3014/U9636/U9702/V2481. Those previously participating in AAAU9950 will also require collection of BMP, LFT, and CBC. None will need to be redrawn for re-enrollees previously screen-failed for AAAU2570.

- At Study Visits 1, 2, 3, 4 *(fasting ≥ 12 h)*
  - SST (gold top), ~15 cc whole blood total at each time point
    - Insulin
    - *c*-peptide
    - Total apolipoprotein B
    - Triglycerides
    - Bile acid profile
      - Bile acid intermediary metabolites:
        - 7α-hydroxy-4-cholesten-3-one (7-HCO)
        - 7α,12α-dihydroxy-4-cholesten-3-one (7,12-diHCO)
      - 12α-hydroxylated bile acids (12-HBA):
        - Cholic acid (CA)
        - Deoxcyholic acid (DCA)
        - Glycocholic acid (G-CA)
        - Glycodeoxycholic acid (G-DCA)
        - Taurocholic acid (T-CA)
        - Taurodeoxycholic acid (T-DCA)
      - Non-12α-hydroxylated bile acids (non-12-HBA):
        - Glycochenodeoxycholic acid (G-CDCA)
        - Glycoursodeoxycholic acid (G-UDCA)
        - Hyodeoxycholic acid (HDCA)
        - Lithocholic acid (LCA)
        - Taurochenodeoxycholic acid (T-CDCA)
        - Taurolithocholic acid (T-LCA)
        - Tauroursodeoxycholic acid (T-UDCA)
        - Ursodeoxycholic acid (UDCA)
        - (Muricholic acids, which are not produced in humans)
    - Adiponectin (may be performed in serum or plasma)
    - Insulin-like growth factor binding protein-1 (IGFBP-1) (may be performed in serum or plasma)
    - Diazoxide level (may be performed in serum or plasma)
  - NaF (gray top), ~2 cc whole blood
    - Glucose (plasma)
  - EDTA (lavender top), ~1 cc whole blood total at each time point
    - Complete blood count
    - VLDL-triglyceride
    - VLDL-apolipoprotein B
    - D_2O enrichment
    - *De novo* lipogenesis (in VLDL-TG and/or total plasma TG)
    - Free fatty acids

- Diazoxide level (may be performed in serum or plasma)
- *Note:* Saliva will be collected for detection of D_2O enrichment in body water

Table 8.ii. *Summary of lab collection requirements and processing sites*

Tube type →		SST	NaF	EDTA	Li-heparin
Adiponectin (serum or plasma)		£ + €		£ + €	
Basic metabolic panel (serum)		$			
Bile acid profile (serum)		£			
C-peptide (serum)		$			
Complete blood count (whole blood)				$	
Diazoxide (serum or plasma)		£		£	
Free fatty acids (serum)					£ + €
Glucose (plasma)			$		
Glucose-6-phosphate dehydrogenase					$
Hemoglobin A_{1c}				$	
β-hCG (serum or urine)		$			
Insulin (serum)		$			
Insulin-like growth factor binding protein-1 (serum or plasma)		£ + €		£ + €	
Liver function tests (serum)		$			
Uric acid (serum)		$			
Serum lipids/ lipoproteins (unfractionated)	Apolipoprotein B	£ + €			
	Triglycerides	$			
	Total cholesterol	$			
	HDL-cholesterol	$			
	LDL-cholesterol [a]	$			
Plasma lipids	VLDL-triglycerides			£ + €	
	VLDL-apolipoprotein B			£ + €	
	D_2O enrichment [b]			€	
	De novo lipogenesis [b]			€	
Site of sample processing:		$ = CUIMC Center for Advanced Lab Medicine £ = IICTR Biomarkers Core Lab € = Research lab			

[a] These values are calculated based on other lab assays (*i.e.*, not measured directly)
[b] DNL and D_2O enrichment may be assessed in fractionated VLDL and/or in total plasma fatty acids

8.2 – Measurement of Primary Endpoint

8.2.1. Primary endpoint

The primary endpoint of the study is the overnight change in insulin, *c*-peptide, and glucose levels at each time point following administration of diazoxide (at 1 mg kg^{-1} or 2 mg kg^{-1}) *versus* placebo.

8.2.2. Laboratory assessment of insulin, c-peptide, and glucose levels

- Insulin:
  - Collected in SST (gold-top tube)
  - Sent out to ARUP Laboratories by CUIMC Center for Advanced Laboratory Medicine, assayed by quantitative chemiluminescent assay
  - Insulin is the principal hormonal cue for entry of glucose into key metabolic cell types as well as coordinating lipid metabolism between the fasted and fed states. Resistance to the effects of insulin is a *sine qua non* of type 2 diabetes, as progressive failure of compensatory insulin over-secretion by the pancreas results in overt diabetes mellitus [114]. Insulin resistance is also an important contributor to the development of non-alcoholic fatty liver disease [3].

- Fasting insulin levels will be ≥ 13 μIU mL^{-1} per inclusion criteria [115]. We expect that diazoxide treatment will lower insulin levels by 25-50% relative to placebo while maximally active (*e.g.*, after reaching steady state) [82].

- C-peptide:
  - Collected in SST (gold-top tube)
  - Sent out to ARUP Laboratories by CUIMC Center for Advanced Laboratory Medicine, assayed by quantitative chemiluminescent assay
  - C-peptide is an inactive fragment of the proinsulin polypeptide that is excised in the processing of the active hormone. C-peptide circulates at levels proportional to total insulin, but has a longer half-life and thus provides more information on integrated insulin levels over time [116].
  - We expect that *c*-peptide levels will largely parallel insulin levels, but given its longer plasma half-life, we expect that it will accumulate relative to insulin and changes in insulin levels will show a delayed reflection in c-peptide levels.
- Plasma glucose:
  - Collected in fluoridated tube (gray-top tube)
  - Assayed at CUIMC Center for Advanced Laboratory Medicine by standard methods
  - Glucose is the principal carbohydrate energy source used by the cells of the body. Chronic elevations of fasting blood glucose are diagnostic of prediabetic dysglycemia and diabetes mellitus as follows, and contribute to the micro- and macrovascular complications thereof [117]:
    - Prediabetes: hemoglobin A_{1c} 5.7-6.4%
    - Impaired fasting glucose: FPG 100-125 mg/dL on ≥ 2 occasions
    - Diabetes mellitus: FPG ≥126 mg/dL or hemoglobin A_{1c} ≥ 6.5% on ≥ 2 occasions
  - We expect that fasting glucose levels will fall either in the euglycemic or impaired fasting glucose (100-125 mg dL^{-1}) ranges at baseline, and that diazoxide will elicit a dose-dependent rise in fasting glucose levels. However, the increase in blood glucose will likely be mild or moderate, and not directly proportionate to the fall in insulin levels [1, 65, 72, 82].
- Homeostasis model assessment of insulin resistance (HOMA-IR) index
  - HOMA-IR is a mathematical index calculated to provide a rough quantification of the degree of IR: (fasting plasma glucose x fasting plasma insulin) ÷ 405 [112].
  - Normal HOMA-IR scores fall in the range of 0.7-2. For the purposes of this study, "insulin resistance" will be considered as a HOMA-IR score of ≥ 2.73 [91].
  - We expect that HOMA-IR levels will be either unchanged by diazoxide treatment, due to opposing effects on insulin *vs.* glucose levels, or will slightly decrease, if blunting insulin levels does not lead to a fully proportionate rise in blood glucose [1, 65, 72, 82, 112].

8.3 – Measurement of Secondary Endpoints

8.3.1. Secondary endpoints

To investigate the impact of diazoxide on lipid/lipoprotein metabolism, including:

1. Total circulating lipid/lipoprotein levels:
   a. Serum triglyceride (TG) levels
   b. Serum free fatty acid (FFA) levels
   c. Serum apolipoprotein B (apoB) levels

8.3.2. Laboratory assessment of serum biomarkers

Blood samples for plasma/serum lipids will be collected in the morning, under fasting conditions (at least a 12-hour fast) at each blood draw (see Table 7.v). Alcohol consumption within 48 h and intense physical exercise within 24 h preceding blood sampling will be discouraged. The lipid parameters to be investigated specifically include:

- Triglycerides (TG, serum)

- Collected in SST (gold-top tube)
- Assayed at CUIMC Center for Advanced Laboratory Medicine using Cobas Integra 400 Plus analyzer and Roche Diagnostic Systems reagents
- Triglycerides (TG) are the body's major storage form of fat and is transported between tissues primarily within triglyceride-rich lipoproteins such as chylomicrons and very low-density lipoproteins (VLDL). Excessive buildup of triglycerides in the liver result in hepatic steatosis, which can progress to non-alcoholic fatty liver disease and non-alcoholic steatohepatitis.
- Fasting TG levels are expected to be high-normal or slightly high relative to the reference range in IR [118]. We expect that diazoxide treatment will have opposing effects on TG levels, by decreasing their *de novo* synthesis while also derepressing their hepatic secretion in VLDL, possibly also assisted by a rise in fasting FFA [55, 119, 120]. This, overall, may result in no difference in serum TG levels early in the time course of the study (*e.g.*, blood draws 2 ± 3), but that it will increasingly become reflected such that total TG levels will be significantly lower with diazoxide treatment than with placebo [73, 74, 80, 81].

- Free fatty acids (FFA, serum), also known as non-esterified fatty acids (NEFA)
  - Collected in EDTA (lavender top) tube
  - Assayed in the IICTR Biomarkers Core Facility, or in our research lab by colorimetric enzymatic assay (Wako NEFA HR-2, Fujifilm).
    - Note that this is not a CLIA-approved test. Results are for research purposes only and will not be returned to participants.
  - Free fatty acids (FFA), also known as non-esterified fatty acids (NEFA), are individual (single chain) fatty acids that have not been esterified with glycerol to produce mono, di-, or triglycerides. Free fatty acid levels, particularly in the fasting state, are a reliable marker of insulin action, as insulin potently blunts FFA production by adipocyte lipolysis [121].
  - We expect FFA levels to either to slightly rise or to remain the same with diazoxide treatment, as residual insulin secretion is expected to suppress lipolysis [27, 74].
- Apolipoprotein B (ApoB), total (serum)
  - Collected in SST (gold-top tube)
    - Note that apolipoprotein B will also be separately measured in fractionated VLDL, see §8.3.3.
  - Assayed in the IICTR Biomarkers Core Facility by ELISA, or in our research lab by ELISA (Mabtech ELISA Pro: Human ApoB) [122]
    - Note that this is not a CLIA-approved test. Results are for research purposes only and will not be returned to participants.
  - Apolipoprotein B forms the backbone upon which the lipids of VLDL, chylomicrons, and LDL are loaded to generate the lipoprotein holoparticles [118, 123]. There is one ApoB molecule per individual lipoprotein, and thus is directly proportional to the circulating lipoprotein burden [118, 123, 124]. ApoB is produced in two isoforms: full length (ApoB100) and truncated (ApoB48). ApoB48 is produced only by enterocytes and is therefore specific to chylomicrons [118, 124]. ApoB100 is predominantly produced by the liver, and thus is mainly relegated to hepatic VLDL, although small quantities of ApoB100-containing TRLs are produced by enterocytes as well [118, 125].
  - We expect total serum ApoB levels largely to parallel serum TG. However, a change in the ratio of TG – or particularly of newly synthesized TG (*i.e.*, DNL) – to ApoB suggests differences in TRL size and/or composition that may merit further investigation.

8.4 – Measurement of Exploratory Endpoints

8.4.1. Exploratory endpoints

To explore the effects of decreasing insulin levels in the setting of fasting or constant hyperglycemia on various metabolic parameters, including:

d. Continuous glucose monitoring (CGM) profile
e. *De novo* lipogenesis in VLDL-TG and/or total plasma TG (also requires measurement of D_2O enrichment in total body water)
f. Triglyceride levels in VLDL (VLDL-TG)
g. Apolipoprotein B-100 in VLDL (VLDL-ApoB)
h. Plasma levels of bile acids (BA) and their metabolic intermediates, by LC-MS/MS
i. Plasma or serum levels and complex distribution of adiponectin, by ELISA and/or Western blot
j. Plasma or serum levels of insulin-like growth factor binding protein-1 (IGFBP-1), by ELISA

8.4.2. Continuous glucose monitoring

- The FreeStyle Libre Pro continuous glucose monitor (CGM) samples interstitial fluid for glucose levels every 15 min for up to 2 weeks.
- CGM readings/tracing be accessed from device using reader device to download. The system produces a standardized report that includes individual daily profiles and summaries of daily patterns for the 2-week recording period (only ~24 h for this study).
- The data can be summarized as the mean glucose with standard deviation during the overnight period (or subintervals thereof), the integrated glucose (area under the curve) for the overnight period (or subintervals thereof), or percent of time spent within particular glucose ranges [126].
- We expect that participants will exhibit higher glucose levels during diazoxide treatment than with placebo, particularly after meals [65, 70].

8.4.3. De novo lipogenesis

De novo lipogenesis will be assessed as the incorporation of deuterium (from orally ingested D_2O) into fatty acids (particularly palmitate) esterified within VLDL-TG and/or total plasma TG. Blood samples will be drawn into EDTA tubes, as above, at the time of each study visit's blood draw and plasma will be separated. Further processing of the plasma to prepare for gas chromatography-mass spectrometry (GC-MS) will be performed either by us in our research lab or by the lab of our collaborator, Dr. Marc Hellerstein at University of California Berkeley. (Again, the samples assayed at Berkeley would be coded and not accompanied by any information that could be used to identify any research participant. See §6.3.1 for discussion of the logistics of this specimen sharing.)

Plasma TG may be assessed directly and/or specifically from VLDL via ultracentrifuge fractionation. VLDL-TG and/or total plasma TG fatty acids will be transesterified to fatty acid methyl esters to prepare for GC-MS. Mass spectrometry for glucose tracer enrichment will be performed in the lab of Dr. Marc Hellerstein at the University of California Berkeley. Dr. Hellerstein will also provide guidance in the analysis of the GC-MS data, particularly including Mass Isotopomer Distribution Analysis (MIDA). Interpretation of DNL data also requires establishing the enrichment of the total body water pool with D_2O. This is ascertained also based on samples of plasma as well as of saliva, the latter collected alongside blood draws via sublingual sponge (salivette). We expect that lowering of insulin levels with diazoxide will also decrease DNL (*i.e.*, incorporation of deuterium into TG palmitate). Although this study is likely not sufficiently powered to detect a statistically significant difference from placebo, this preliminary data will lay the groundwork for the future main study.

8.4.4. Lipid/lipoprotein laboratory analyses

Blood for lipoprotein fractionation and lipid/lipoprotein kinetic analyses will be collected in EDTA-containing lavender-top tubes. Plasma will be ultracentrifuged using a density gradient to isolate the VLDL fraction [127]; we will store the remainder (containing other lipoprotein fractions) in the freezer for future use per §14.7. The quantity (in mg dL^{-1}) of TG and apoB will be measured in VLDL, by colorimetric assay and liquid chromatography-tandem mass spectrometry (LC-MS/MS), respectively. ApoB may also be detected by Western blot.

Expected results of lipoprotein/lipid analyses:

- Triglycerides in fractionated plasma lipoproteins

- Triglyceride synthesis is expected to be high with hyperinsulinemia, but this stimulus for increased VLDL-TG may be offset by residual impairment of VLDL-TG secretion. However, as insulin's suppression of VLDL secretion is blunted in IR, the magnitude of the change in total VLDL-TG may be small [14, 119, 120] or even flat [128, 129] in the placebo group during fasting.
- Because lowering insulin levels may exert opposing effects on hepatic TG metabolism, diazoxide treatment may also result in no net change in VLDL-TG *versus* placebo at earlier time points, but is expected to be lower with diazoxide treatment by the end of the study. [55, 119, 120].

- Apolipoprotein B in fractionated plasma lipoproteins
  - We expect that diazoxide treatment will reduce insulin's inhibition of VLDL-apoB secretion. VLDL-apoB secretion may therefore trend higher than in the diazoxide-treated groups [14, 119, 120].

8.4.5. Laboratory assessment of circulating biomarkers

- Bile acid profile:
  - Collected in SST (gold-top tube)
  - Assayed at IICTR Biomarkers Core Facility by LC-MS/MS
    - Note that this is not a CLIA-approved test. Results are for research purposes only and will not be returned to participants.
  - Bile acids are amphipathic derivatives of cholesterol that are produced in the liver and stored in the gallbladder. Bile acids aid in the emulsification of lipids and lipid-soluble ingested nutrients in the intestine, thereby facilitating their absorption [130, 131]. In addition to their role in lipid digestion, bile acids are active signaling molecules that help to coordinate enterohepatic metabolism by stimulating secretion of other signaling molecules [130, 132].
  - Includes the following bile acid species:
    - Bile acid intermediary metabolites
      - 7α-hydroxy-4-cholesten-3-one (7-HCO)
      - 7α,12α-dihydroxy-4-cholesten-3-one (7,12-diHCO)
    - 12α-hydroxylated bile acids (12-HBA):
      - Cholic acid (CA)
      - Deoxcyholic acid (DCA)
      - Glycocholic acid (G-CA)
      - Glycodeoxycholic acid (G-DCA)
      - Taurocholic acid (T-CA)
      - Taurodeoxycholic acid (T-DCA)
    - Non-12α-hydroxylated bile acids (non-12-HBA):
      - Glycochenodeoxycholic acid (G-CDCA)
      - Glycoursodeoxycholic acid (G-UDCA)
      - Hyodeoxycholic acid (HDCA)
      - Lithocholic acid (LCA)
      - Taurochenodeoxycholic acid (T-CDCA)
      - Taurolithocholic acid (T-LCA)
      - Tauroursodeoxycholic acid (T-UDCA)
      - Ursodeoxycholic acid (UDCA)
      - (Muricholic acids, which are not produced in humans)
  - Insulin, acting through inhibition of FoxO transcription factors, regulates the expression of bile-acid synthetic enzymes. This notably includes CYP8B1, which catalyzes the 12α-hydroxylation of 7-HCO *en route* to the synthesis of cholic acid and all other 12α-hydroxylated BA (12-HBA) species [133]. In the setting of insulin resistance, 12-HBA levels rise well out of proportion to non-12-HBA and thus acts as a readout of liver-specific insulin action [115, 132-134]. We therefore expect dysglycemic participants to have an elevated ratio of 12-HBA to non-12-HBA. As BA synthesis lies downstream of FoxO [132], the key exponent

of the glucose arm of the insulin signaling pathway [135], we expect that reduction of compensatory hyperinsulinemia will further augment excess 12-HBA synthesis. This will serve as a non-glucose metric by which we can assess the glucose arm of the pathway, thereby complementing TG measures of the lipid arm.

- Adiponectin (serum or plasma)
  - Collected in SST (gold-top tube) or EDTA (lavender-top tube) for serum or plasma, respectively
  - Assayed at IICTR Biomarkers Core Facility *or* in our research lab by ELISA and/or non-denaturing Western blot
    - Note that this is not a CLIA-approved test. Results are for research purposes only and will not be returned to participants.
  - Adiponectin is an abundant serum protein produced by adipocytes that can oligomerize into complexes of varying molecular weights (*i.e.*, high molecular weight complexes, low molecular weight complexes, monomeric). Although its true biologic function remains controversial, it has been considered an insulin-sensitizing signaling molecule [136]. Despite the uncertainty as to its biologic role, adiponectin is very well established as a biomarker of insulin resistance: its levels – and particularly levels of high-molecular weight complexes – correlate inversely with IR *except* in the case of patients with mutations in the gene for or antibodies to the insulin receptor, in which case adiponectin levels are paradoxically high [136]. Adiponectin levels and complex distribution should provide an index of degree of adipose tissue IR in dysglycemic *vs.* control participants that does not reflect hyperglycemia *per se.*
- Insulin-like growth factor binding protein-1 (IGFBP-1, serum or plasma)
  - Collected in SST (gold-top tube) or EDTA (lavender-top tube) for serum or plasma, respectively
  - Assayed at IICTR Biomarkers Core Facility *or* in our research lab by ELISA
    - Note that this is not a CLIA-approved test. Results are for research purposes only and will not be returned to participants.
  - Insulin-like growth factor binding protein-1 (IGFBP-1) is a liver-derived protein that binds to and modulates the half-life of circulating insulin-like growth factors (IGF). IGFBP-1 levels are regulated by nutrient status: its levels are raised by glucagon and cortisol and decreased by insulin [145]. Insulin inhibits the expression of the IGFBP-1 gene through its inhibition of FoxO transcription factors [146, 147], thus IGBFP-1 mRNA levels rise in hepatic IR [21, 148]. As such, IGFBP-1 can be used as a glucose-independent biomarker of IR that is relatively specific to the liver [145, 149].
- Diazoxide level (serum or plasma)
  - Collected in EDTA (lavender-top) or SST (gold-top) tube for plasma or serum, respectively
  - Assayed at IICTR Biomarkers Core Facility, which will also develop the assay based on a sample of pure diazoxide obtained from a reputable chemical manufacturer.
    - Note that this is not a CLIA-approved test. Results are for research purposes only and will not be returned to participants.
  - Diazoxide will be measured as a quality-control measure and to get a better sense of drug absorption and dose-response relationship in the fasting serum/plasma of those who received it, with placebo-treated serum/plasma as a negative control.

9. STATISTICAL CONSIDERATIONS

9.1 – General statistical considerations

All analyses will be performed as diazoxide 1 mg kg^{-1} group *vs.* diazoxide 2 mg kg^{-1} group *vs.* placebo (control) group. Comparative data will be analyzed for each individual time point. *Post-hoc* analyses may include stratification of the data by different sub-intervals within the study and/or by age, sex, or other participant characteristics. We may also re-analyze

data *post hoc* that consolidates both diazoxide dose groups into one for comparison to placebo, or based on whether they meet criteria for prediabetes or not.

Descriptive statistics for various variables will be summarized. Data will be presented as mean ± standard error in the mean for normally distributed variables and as median ± interquartile range for non-normally distributed variables. Graphical tools such as time-course curves, histograms, and boxplots will be generated where applicable using commercially available software (*e.g.*, Microsoft Excel, GraphPad Prism). Data will be analyzed by mixed-effects model (to overcome missing-data limitation of repeated measures ANOVA) followed by Tukey's multiple comparisons test or, where applicable, by simple linear regression Multivariate and partial regression analyses will be performed where needed by standard methods. All analyses will be two sided with a significance threshold of $p < 0.05$.

9.2 – Size and accrual rate

For this pilot and feasibility study, we plan to enroll up to 24 participants per group in order to obtain 6 evaluable participants per group, for a total of 18 participants. Based on diazoxide-induced changes in insulin levels in a similar population [1], by obtaining complete study data on 6 participants per group, we could detect a diazoxide-induced change of ~25% in fasting insulin levels at $\alpha = 0.05$, $\beta = 0.80$. This is the magnitude of reduction in hyperinsulinemia that we aim to induce for later studies, and this degree of change in insulin levels has been shown to correlate with significantly lower rates of hepatic *de novo* lipogenesis [2-4]. However, we will justify expansion of the study if a smaller yet clear trend emerges that may be underpowered to reach statistical significance. In case of attrition or incomplete data, replacement participants will be recruited on an as-needed basis. Participants with IR-NAFLD will be recruited by their hepatologist at CUIMC. The estimated accrual rate is 2-3 patients per month, until at least 72 total participants have been enrolled. The fatty liver clinic at CUIMC operates full time and has a panel of approximately 900 patients. Therefore, an accrual of 18 patients is feasible.

10. INVESTIGATIONAL STUDY PRODUCTS

10.1 – Investigational study product regulatory policies

Investigational study products will be used only under the supervision of the PI or their qualified, designated co-investigator. The PI is a licensed, actively registered physician in New York State carrying medical liability insurance and is fully credentialed by CUIMC and NYP. (See attached *Curriculum Vitae*.)

All participants will be informed that the drug product being used is investigational. Informed consent of the participant must be obtained prior to the use of an investigational drug. Per NYP policy, investigational study products will be reviewed by, stored in, dispensed by, and destroyed by the Research Pharmacy (RP), in consultation with the PI. The RP will have full oversight of investigational drugs, will maintain drug accountability records, and will provide training to designated pharmacy areas that may have to dispense investigational drugs outside of RP pharmacy hours. The PI or designated co-investigator will ensure that all drug information is readily available in the medical record:

- Name of the study protocol and any protocol number designated by the IRB
- Names of co-investigator(s)/authorized prescriber(s)
- Name of investigational drug and any synonyms
- Preparations (dosage form and strength) available from the pharmacy
- All pertinent data concerning the storage and preparation of the investigational drug
- Method of administration
- Expected therapeutic effect, potential long- and short-term adverse effects, and monitoring of response(s)
- Any other important information relevant to the investigational product

Table 10.i. *Investigational study products*

Investigational Agent	Manufacturer		Dosing regimen

		Study group	Dose number	Dose name	Study Days taken	Dosing interval
Diazoxide (50 mg mL^{-1} oral suspension)	e5 Pharma [a,b]	Diazoxide 1 mg kg^{-1}	1+2	1P (x 2)	Day 1	Simultaneous
			3-27	2P-14P	Days 2-14	Every 12 hours
		Diazoxide 2 mg kg^{-1}	1+2	1P (x 2)	Day 1	Simultaneous
			3-27	2P-14P	Days 2-14	Every 12 hours
Placebo [c] (drug-free vehicle)	CUIMC RP [d]	Placebo (40 µL kg^{-1})	1+2	1P (x 2)	Day 1	Simultaneous
			3-27	2P-14P	Days 2-14	Every 12 hours

[a] Drug suspended in vehicle of sorbitol, mint flavor, sodium benzoate, methylparaben, poloxamer 188, propylparaben, and water
[b] Verified as IND-exempt by FDA (#163465)
[c] Comprises flat diet tonic water (Canada Dry®) with added peppermint extract (McCormick®) and thickened with xanthan gum (Carrington Farms®)
[d] CUIMC Research Pharmacy

10.2 – Investigational product handling

Diazoxide oral suspension will be requisitioned from the manufacturer or supplier and placebo will be prepared in house (see §10.4.2). Bottles will be stored, per manufacturer's instructions, in the dark at room temperature (min. 15ºC, max 30ºC) within the RP. Bottles may be reused for dispensing doses to multiple participants, provided that the contents have not reached the expiry date marked by the manufacturer. Per NYP policy, investigational study products will be reviewed by, stored in, dispensed by, and destroyed by the CUIMC RP, or designated pharmacy, in consultation with the PI. The RP or designated pharmacy will label all investigational drugs with the following information:

- A statement that identifies the drug as for investigational use only
- Protocol name and/or IRB number
- Name of the drug
- Dosage form and strength
- Directions for the use of the drug

10.3 – Investigational product inspection

Each bottle used for dose preparation should be inspected. Diazoxide appears as a white- to light brown-colored, mint-flavored suspension and placebo as a colorless, mint-flavored viscous solution. Diazoxide oral suspension is packaged in 30-mL resealable bottles. If there are any defects noted with the investigational product, including passed expiry date, the bottle will be discarded and the investigator should be notified immediately.

10.4 – Dose preparation steps

10.4.1. Preparation of diazoxide

Diazoxide will be purchased pre-formulated by the manufacturer, containing the drug suspended in a solution containing sorbitol, mint flavor, sodium benzoate, methylparaben, poloxamer 188, propylparaben, and water [99]. Diazoxide will be packaged for dispensing as individual doses of 1 mg kg^{-1} or 2 mg kg^{-1} by the Research Pharmacy (or designated pharmacy) as below (see §10.4.3).

10.4.2. Preparation of placebo

Because of the distinctive taste of diazoxide oral suspension and unavailability of drug-free vehicle to use as placebo, the Research Pharmacy will produce a suitable placebo that reproduces the unique minty bitterness of the original. The placebo's formulation will be based closely on a recipe developed at the National Institutes of Health and successfully used in similar human-subjects research [82, 83]. The placebo solution consists of 0.1 mL of McCormick® pure peppermint extract added *via* sterile syringe into 1 L of flat Canada Dry® diet tonic water. The 1-L mixture is then thickened by adding 1.1 g of calorie-free xanthan gum (Carrington Farms® or equivalent) and mixing well for a 0.11% (w/v) solution. This viscosity

resembles that of sorbitol-thickened solutions [137] such as diazoxide oral suspension, but the quantity of xanthan gum may be modified as needed to best match the viscosity of the active drug suspension.

10.4.3. Preparation of investigational agent doses

In order to maintain blinding, an equivalent volume of diazoxide at either dose, or of placebo, must be prepared by the Research Pharmacy (or designated pharmacy) to the nearest 0.1 mg, as outlined in Table 10.ii. Each and every dose will be aliquoted into a separate pharmaceutical-grade single-use oral syringe. Because we do not have pharmacologic data to prove that diazoxide would be stable if mixed with placebo, we will have to dispense different volumes to the two groups. However, we will seek to maintain blinding by completely masking the contents of each syringe with labeling covering the entire surface of its barrel.

Table 10.ii. *Preparation of investigational study products*

Product	Diazoxide dose (group)	Diazoxide concentration		Preparation of individual dose		Total volume of dose
		Initial (as supplied)	*Final (as dosed)*	*Volume of active drug*	*Volume of placebo to add*	
Diazoxide	1 mg kg^{-1}	50 mg mL^{-1}	25 mg mL^{-1}	20 µL kg^{-1}	0 µL kg^{-1}	20 µL kg^{-1}
	2 mg kg^{-1}	50 mg mL^{-1}	50 mg mL^{-1}	40 µL kg^{-1}	0 µL kg^{-1}	40 µL kg^{-1}
Placebo	0 mg kg^{-1}	n/a	n/a	0 µL kg^{-1}	40 µL kg^{-1}	40 µL kg^{-1}

Per NYP policy, investigational products will be prepared by the Research Pharmacy or other designated pharmacy; the pharmacy will be provided with a copy of the investigational drug protocol to use as a source document for drug information. As aforementioned, the Research Pharmacy is responsible for preparing and dispensing each and every IAD according to the pre-ordained 1:1:1 randomization scheme furnished by a disinterested third party. For each participant, the RP will prepare individual vessels containing single doses (IAD) and will be labeled with the following information prior to dispensing:

- Identification of "AAAU2570 Investigational Agent"
- Investigational agent dose (IAD) number (*e.g.*, Dose Number 1, 2, *etc.*)
- Date and time at which the dose is to be taken
- Directions for use of the drug
- Participant's name
- Participant's date of birth
- Participant's medical record number
- Date and time dispensed
- Expiration time or date
- Dispenser's initials

10.5 – Dispensing doses of investigational product

Per NYP policy, investigational products will be dispensed by the Research Pharmacy, or designated pharmacy, following placement of an electronic order signed by an authorized prescriber identified in the approved IRB protocol. All dispensing procedures must be approved by the Research Pharmacy or designated pharmacy before study initiation. Investigational agent doses will be administered and/or provided for home administration only if it has been dispensed by the RP or designated pharmacy within the institution. The RP may dispense one or multiple doses of IAD for a particular participant for short-term (up to overnight) storage by the PI/study team prior to being handed over to the participant. This storage will be undertaken according to RP and manufacturer instructions. The RP will be aware of any storage of IAD by the study team, and the study team will not do so without the permission of the RP. The PI or a designated, qualified research team member will be present at each study visit to oversee the handover of the doses to the participant, to review them with participants and any relevant clinical staff, and to answer any pertinent questions the participant may have.

At the appointed time for each dose, the participant will consume the entire dispensed dose of diazoxide or placebo oral suspension within 30 s and may then take sips of water as necessary to wash it down. The participant may not

manipulate the label covering the surface of each syringe or otherwise visually inspect the investigational agent. Participants must return all used IAD syringes according to the schedules outlined in Tables 7.iv and 10.iii. As previously detailed, the RP will prepare IAD for dispensing according to the following schedule (Table 10.iii).

Table 10.iii. *Schedule of IAD provision and recovery*

Study Visit	Study Day	Investigational Agent Dose (IAD)													
		0	1	2P	3A	3P	4A	4P	5A	5P	6A	6P	7A	7P	8A	8P
SV-1	1	●	●													
SV-2	2	○	○	●	●	●	●	●	●	●	●	●	●	●	●	●
SV-3	9			○	○	○	○	○	○	○	○	○	○	○	○	○

Study Visit	Study Day	Investigational Agent Dose (IAD)												IAD handling:
		9A	9P	10A	10P	11A	11P	12A	12P	13A	13P	14A	14P	
SV-3	9	◐	●	●	●	●	●	●	●	●	●	●	●	● Container given to participant
SV-4	15		○	○	○	○	○	○	○	○	○	○	○	○ Container recovered ◐ Admin-istered at SV

11. NON-INVESTIGATIONAL MEDICINAL STUDY PRODUCTS

11.1 – Deuterated water/deuterium oxide ($^2H_2O/D_2O$), 70% (in H_2O)

11.1.1. Sourcing, handling and inspection

Deuterium oxide (70%, in H_2O) will be purchased from Sigma-Aldrich (#613428) or Cambridge Isotope Laboratories (#DLM-4-70-1000), either in bulk (for cost reasons) or as individual 50-mL aliquots within sterile screw-top bottles. D_2O will be stored, per manufacturer's instructions, at room temperature in a dry and well-ventilated place. The Research Pharmacy has confirmed that they do not need to dispense the D_2O (see correspondence in Documents); the PI will instead maintain records of dispensation and accountability. Each container used for dose preparation should be inspected. The appearance of D_2O is indistinguishable from tap water. If there are any defects noted with the product, including passed expiry date, the bottle will be discarded and the investigator should be notified immediately.

11.1.2. Dose preparation, distribution, and administration

When purchased in bulk, D_2O will be portioned by the study team by decanting 55.5-g (*i.e.*, 50-mL) aliquots into new, clean, food-safe screw-top containers atop a tared electronic balance. Individual D_2O aliquots, whether pre-portioned or aliquoted by the study team, will each be labeled with a unique dose number and will be distributed to participants according to the following schedule (Table 10.iv). Note that certain doses (DW-1A, 2A, 9A) will be consumed at Study Visits 1, 2, and 3, respectively. Participants will drink 50 mL of 70% D_2O three times a day, at least 3 hours apart (*e.g.*, morning, afternoon, evening, total of 150 ml per day), for the first four days. They will then drink 50 mL of 70% D_2O twice a day, 12 hours apart (*e.g.*, morning and evening, total of 100 ml per day), thereafter. Aliquots should be consumed within 2-3 minutes apiece. This labeling protocol has been shown in >500 human subjects in Dr. Hellerstein's studies to produce a steady rise to plateau body water enrichments of 1.0-2.0% D_2O.

Table 10.iv. *Schedule of D_2O provision and recovery*

	Study	D_2O dose/aliquot (DW-)

Study Visit	Day	1A	1M	1P	2A	2M	2P	3A	3M	3P	4A	4M	4P	5A	5P	6A	6P
SV-1	1	◐	●	●													
SV-2	2		○	○	◐	●	●	●	●	●	●	●	●	●	●	●	●
SV-3	9					○	○	○	○	○	○	○	○	○	○	○	○

Study Visit	Study Day	D_2O dose/aliquot (DW-)															
		7A	7P	8A	8P	9A	9P	10A	10P	11A	11P	12A	12P	13A	13P	14A	14P
SV-2	2	●	●	●	●												
SV-3	9	○	○	○	○	◐	●	●	●	●	●	●	●	●	●	●	●
SV-4	15						○	○	○	○	○	○	○	○	○	○	○

D_2O handling: ● Container given to participant ○ Container recovered ◐ Administered at SV

11.1.3. Safety

There are no known medical risks of heavy water (D_2O) intake at the doses used in this study. D_2O is not radioactive and has been extensively tested in animals and humans. We have selected the same product and protocol safely used by our collaborator, Dr. Hellerstein, in over 3000 study patients (see Letter of Collaboration). D_2O has shown no potentially serious adverse effects until it reaches an enrichment of above 15-20% of total body water [138]. Since the dose we administer is sufficient only to enrich total body water to 1.0-2.0% (an order of magnitude below the threshold for concern) over 2 weeks, reaching a high enough enrichment to cause symptoms is not a concern. Rarely, individuals (<3%) may experience temporary light-headedness, dizziness, or vertigo when they take the first doses of D_2O. These symptoms are self-limited and typically resolve within 2-3 hours. Participants will be instructed on the intake of D_2O to ensure a slow rise in initial body water enrichment and thereby avoid these symptoms. However, we may discontinue further D_2O doses for participants that develop recurrent or intractable symptoms that, in the PI's judgment, are likely related to D_2O consumption. As D_2O serves an exploratory endpoint only, participants may otherwise continue the study if they're willing and able to continue taking their investigational agent doses faithfully.

12. SAFETY AND ADVERSE EVENTS

12.1 – Adverse Events and Unanticipated Problems

12.1.1. Definition of adverse events (AE)

An *adverse event* (AE) is any symptom, sign, illness or experience that develops or worsens in severity during the course of the study. Intercurrent illnesses or injuries should be regarded as adverse events. Abnormal results of diagnostic procedures are considered to be adverse events if the abnormality:

- results in study withdrawal
- is associated with a serious adverse event
- is associated with clinical signs or symptoms
- leads to additional treatment or to further diagnostic tests
- is considered by the investigator to be of clinical significance

An AE can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporarily associated with the use of a medicinal product, whether or not it is considered related to the medicinal product. An AE includes, but is not limited to, any clinically significant worsening of a participant's pre-existing condition.

AEs may be treatment-emergent (*i.e.*, occurring after initial receipt of investigational product) or non-treatment-emergent. A non-treatment-emergent AE is any new sign, symptom, disease, or other untoward medical event that begins after written informed consent has been obtained but before the participant has received investigational product. Elective treatment, surgery, or pre-planned treatment (*i.e.*, scheduled prior to the participant's enrollment in the study) for a documented pre-existing condition that did not worsen from baseline is not considered an AE. An untoward medical event occurring during a pre-scheduled elective procedure or routinely scheduled treatment should be recorded as an AE or serious AE.

12.1.2. Definition of serious adverse events (SAE)

AE are classified as serious or non-serious. A *serious adverse event* (SAE) is any AE that:

- Results in death
- Is immediately life threatening
- Requires inpatient hospitalization
- Results in persistent or significant disability/incapacity
- Is a congenital anomaly/birth defect in offspring of the participant
- Is an important medical event that may jeopardize the participant or may require medical intervention to prevent one of the outcomes listed above

Medical or scientific judgment should be exercised in deciding whether expedited reporting is appropriate in this situation. All adverse events that do not meet any of the criteria for serious AE should be regarded as *non-serious adverse events*.

12.1.3. Unanticipated problems

Any AE will be assessed for whether or not it was an unanticipated problem. An *Unanticipated Problem (UP)* is any incident, experience or outcome involving risks to participants or others in any human-subjects research that meets all of the following criteria:

3. Unexpected (in terms of nature, severity, or frequency) given:
    a. The research procedures that are described in the protocol-related documents, including the IRB-approved research protocol and informed consent document; and
    b. The characteristics of the subject population being studied
4. Definitely, probably, or possibly related to participation in the research study
5. Suggests that the research places subjects or others at a greater risk of harm than was previously known or recognized

Reports of all events (including follow-up information) that meet the definition of an unanticipated problem posing risk to subjects or others must be submitted to the IRB within one week (5 business days) following the occurrence of the unanticipated problem or the investigator's acquiring knowledge of the unanticipated problem in accordance with IRB policy. Additionally, the investigator will submit a summary of all unanticipated problems that occurred since the beginning of the study at the time of continuing review.

12.1.4. Recording and reporting of adverse events

At each contact with the participant, the investigator must seek information on adverse events by specific questioning and, as appropriate, by examination. AE will be recorded immediately on the source document and in the AE module of the CRF using a recognized medical term or diagnosis that accurately reflects the event as well as the date on which the PI or research staff became aware of the event. All clearly related signs, symptoms, and abnormal diagnostic results should be

recorded in the source document, grouped as possible under a single diagnosis. AE/SAE that meet the criteria of an unanticipated problems will be reported to the IRB within 1 week (5 business days) of when the study team becomes aware.

All AE occurring during the study period must be recorded. The clinical course of each event should be followed until resolution, stabilization, or until it has been determined that the study treatment or participation is not the cause. SAE that are still ongoing at the end of the study period must be followed up to determine the final outcome. Any SAE that occurs after the study period and is considered to be possibly related to the study treatment or study participation should be recorded and reported immediately.

Abnormal test results: An abnormal laboratory finding (including on electrocardiogram) that requires medical intervention by the investigator, or a finding judged by the investigator as medically significant should be reported as an AE. If clinical sequelae are associated with a laboratory abnormality, the diagnosis or medical condition should be reported (*e.g.*, renal failure, hematuria) rather than the laboratory abnormality (*e.g.*, elevated creatinine or increased urinary erythrocyte count, respectively). Abnormal laboratory values that are not, in the investigator's opinion, medically significant and do not require intervention should not be recorded as an AE.

Pre-existing condition: A pre-existing condition is one that is present at the start of the study. A pre-existing condition should be recorded as an AE if the frequency, intensity, or character of the condition worsens during the study period.

Hospitalization, prolonged hospitalization, or surgery: Any adverse event that results in hospitalization or prolonged hospitalization should be documented and reported as a serious adverse event unless specifically instructed otherwise in this protocol. Any condition responsible for surgery should be documented as an adverse event if the condition meets the criteria for and adverse event.

12.1.5. Time period for collection of adverse events
AE will be collected from the time of the dispensing of Investigational Agent Doses 1 and 2 on Study Visit 1 through the completion of the study protocol (*i.e.*, following blood draw at SV-4). Any AE that are unresolved at the end of the study protocol will be followed up by the investigator for as long as medically indicated, including for treatment, as necessary, but without further recording in the CRF.

12.1.6. Medical monitoring
It is the responsibility of the PI to oversee the safety of the study at their site. This safety monitoring will include careful assessment and appropriate reporting of AE as noted above, as well as implementation of the Data and Safety Monitoring Plan. Medical monitoring will include a regular assessment of the number and type of SAE.

12.2 – Safety matters related to basic study procedures

12.2.1. Phlebotomy
Aside from temporary mild pain, we do not expect any adverse events to arise in response to the peripheral venipuncture. Drawing blood poses small risks of bleeding and infection. However, we will mitigate this risk by using sterile technique, exclusively by authorized, trained professionals.

12.2.2. Continuous glucose monitoring
The FreeStyle Libre Pro continuous glucose monitor is exceptionally safe and well tolerated. Participants may feel a minor pinch upon application of the device and a brief tearing sensation with device removal. Irritation of the skin (*e.g.*, redness, itching) is possible but uncommon with this widely used device. We will use antiseptic technique when placing and removing the device. Participants will be provided with an information sheet published by the manufacturer that includes

safety information, including the need to remove the device prior to any X-ray, CT scan, MRI, or diathermy treatment. (See Attachments.)

12.3 – Safety matters related to diazoxide

Potential adverse reactions related to use of diazoxide include hyperglycemia, hypotension, fluid retention, gout flare, hypertrichosis, and gastrointestinal upset [99]. Given the age of the drug (first FDA approval in 1973), pre-modern clinical trials did not define the frequencies of specific adverse events, and no such data are listed in the FDA package insert [99]. However, data from single-dose or short-course clinical trials offer a sense of what we might expect [1, 65, 70, 72, 77, 82, 85].

12.3.1. Hyperglycemia

Hyperglycemia is an expected, on-target effect of diazoxide treatment due to suppression of glucose-stimulated insulin secretion. However, it tends to be mild, particularly during fasting and with administration of single or few doses [1, 65, 70, 72, 77, 82, 85]. For example, in healthy volunteers, a dose of diazoxide 6 mg kg^{-1} raised peak blood glucose during a mixed-meal tolerance test only by about 10 mg dL^{-1} above the placebo group while reducing peak insulin secretion by roughly 20% [70]. Clinical trial data support the glycemic safety of diazoxide in obesity as well. Obese men treated with a total daily diazoxide dose of 2 mg kg^{-1} for one week showed a rise in mean fasting blood glucose from 109 mg dL^{-1} at baseline to 112 mg dL^{-1}, while fasting insulin levels fell on average by 32% [1]. Exact peak blood glucose values during OGTT were not reported, but the integrated glucose area under the curve was not significantly changed from baseline after diazoxide treatment [1]. In a similar weeklong study of obese men, diazoxide at a total daily dose of 300 mg (mean: 2.6 mg kg^{-1} d^{-1}) lowered fasting insulin levels by 23% *versus* placebo while not at all affecting fasting or peak postprandial glucose [72]. Diazoxide treatment even at relatively high doses for prolonged periods has also been documented as safe from a glycemic perspective. For example, uptitration of diazoxide in obese men (up to 900 mg d^{-1}, mean dose achieved 422 mg d^{-1} for ~3.4 mg kg^{-1} d^{-1}) for 6 months increased fasting glucose levels increased on average by 12.6 mg dL^{-1} to 113.5 mg dL^{-1}; mean peak postprandial glucose levels rose by about 27 mg dL^{-1} to 171 mg dL^{-1} [74].

Taken together, these data do not suggest a high risk of dangerous hyperglycemia during a short course of diazoxide 2-4 mg kg^{-1} d^{-1}. Nevertheless, we will take multiple precautions against the development of clinically significant hyperglycemia. First, we will exclude participants with a history of diabetes mellitus or using medications that impact on blood glucose levels (see §5.3.5). Second, we will monitor blood glucose levels at 12 h after the first two doses, and then after 7 d of taking regularly scheduled (*i.e.*, every 12 h) diazoxide. Third, participants will be provided with an isocaloric, nutritionally balanced study diet low in simple carbohydrates. Finally, the PI, a board-certified endocrinologist, will be reachable for consultation at all times.

We will educate participants on the risk, recognition, and management of severe hyperglycemia. If they do develop symptoms potentially consistent with severe hyperglycemia (*e.g.*, polyuria, polydipsia, blurred vision), they should contact a member of the study team immediately. The PI or other qualified study personnel will be reachable around the clock to discuss concerns and to provide advice on corrective actions. If symptoms are untenable, the participant will be advised to seek emergency medical attention. Should symptomatic hyperglycemia be verified to occur, the participant's study participation will be terminated and all further doses of investigational agent will be surrendered. We will also terminate study participation if any blood glucose level ≥ 250 mg dL^{-1} is documented.

12.3.2. Ketoacidosis (theoretical risk)

Because diazoxide reduces insulin secretion and raises blood glucose levels, it theoretically increases the risk of diabetic ketoacidosis. However, the FDA package insert recommends regular monitoring of the urine for sugar and ketones only in patients receiving prolonged treatment with the drug [99]. Indeed, ketoacidosis was not reported in clinical studies administering single doses or short courses of diazoxide [1, 65, 70, 72, 77, 82, 85] or even in clinical trials in which insulin-resistant patients were treated with high doses of diazoxide for extended periods [73, 74, 80]. Moreover, diazoxide has been used safely in extended clinical trials of patients with early type 1 diabetes for induction of "β-cell rest" without excess episodes of diabetic ketoacidosis [75, 76, 98].

Nonetheless, we will further minimize this risk by excluding participants with a history of diabetes mellitus or taking SGLT2 inhibitors for any indication, those with known pancreatic structural pathology, and those with clinical suspicion of impaired β-cell reserve. Should concern for ketoacidosis arise (*e.g.*, symptoms of severe hyperglycemia plus prominent gastrointestinal distress), the participant will be advised to notify the study team at once and to seek emergency medical attention.

12.3.3. Hypotension

Diazoxide has vasodilatory properties, although the FDA package insert notes that "the hypotensive effect of diazoxide on blood pressure is usually not marked with the oral preparation", tending only to manifest with intravenous use [99]. In two studies administering single doses of oral diazoxide to healthy volunteers, diastolic blood pressures were slightly lower ($|\Delta DBP| < 5$ mmHg) and heart rates slightly higher ($|\Delta HR| < 10$ bpm) than with placebo [65, 70]; there were no between-group differences in these vital signs in two other single-dose studies [77, 82]. There was no apparent effect on systolic blood pressures in any of the four [65, 70, 77, 82]. Multi-dose studies also did not report any significant hypotensive effect of oral diazoxide. For instance, a 6-day trial of diazoxide at 300 mg d^{-1} (mean: 2.6 mg kg^{-1} d^{-1}) in obese men had no effect on systolic or diastolic BP [72]. Likewise, a more extended obesity trial found no significant difference in systolic or diastolic BP after 8 weeks of treatment at 2 mg kg^{-1} d^{-1} [1].

Nevertheless, to avoid the risk of hypotension, we will exclude patients with screening systolic BP < 95 mm Hg (with or without antihypertensive treatment), diastolic BP < 65 mm Hg (with or without antihypertensive treatment), and/or using concomitant treatment with beta blockers or vasodilators. We will educate participants on the risk, recognition, and management of hypotension and instruct that they notify the study team if they develop concerning symptoms. We will check vital signs, assess clinical volume status, and interview the participant about adverse effects at each study visit. If the participant reports any symptoms concerning for hypotension (*e.g.*, lightheadedness [especially orthostatic], dizziness, malaise), we will obtain orthostatic vital signs. If orthostasis is confirmed (*i.e.*, $\Delta SBP \geq 20$ mmHg, and/or $\Delta DBP \geq 10$ mmHg, and/or $\Delta HR \geq 20$ bpm at 3 min after rising from a recumbent position), we will remove the participant from the study and offer treatment at the discretion of the PI (board certified in internal medicine) in consultation with any relevant following physicians.

12.3.4. Fluid retention

Peripheral edema has been a relatively common side effect of diazoxide treatment in obesity trials due to sodium and water retention. Acute volume overload was not reported in clinical studies administering single doses of diazoxide [65, 70, 77, 82]. However, in comparable studies reporting adverse effects occurring within 2 weeks of diazoxide initiation at 2 mg kg^{-1} d^{-1}, the incidence of peripheral edema was 43% (placebo subtracted) [1] or 33% [31], although it was not reported by participants in another such study [72]. Diazoxide-associated edema is reversible with dose reduction or discontinuation [31, 73, 74, 80] and has not been associated with elevated *N*-terminal pro-brain natriuretic peptide (NT-proBNP) [73]. None of these studies reported pulmonary edema or serious clinical sequelae of volume overload [1, 31, 72-74, 80].

In order to decrease the risk of fluid retention, we will exclude participants with clinical volume overload or with predisposing conditions including congestive heart failure, chronic kidney disease (Stage 2 or worse), or chronic liver disease beyond uncomplicated NAFLD, even if presently compensated. We will assess clinical volume status on physical exam at Study Visits and discuss any volume-related concerns that participants have. If peripheral edema occurs, we will reassure the participant that it is expected to abate after drug discontinuation. However, if the edema is intolerable, we will advise the participant to withdraw from the study. If clinical concern arises for pulmonary edema (*e.g.*, reported new-onset shortness of breath or cough), we will perform a thorough clinical evaluation. If pulmonary edema is confirmed, we will remove the participant from the study and offer treatment at the discretion of the PI in consultation with any relevant following physicians.

12.3.5. Gout flares

Diazoxide can decrease excretion of uric acid and thereby increase the risk of gout flares [99, 139], although no such events were reported in clinical studies administering single doses or short courses of diazoxide [1, 65, 70, 72, 77, 82, 85]. Participants with known gout or elevated uric acid on screening labs are excluded from the study to minimize this risk.

However, should participants develop signs or symptoms concerning for a new gout attack, we will pause their study participation while checking uric acid levels. If uric acid is found to be elevated, the participant will be withdrawn from the study and referred to their personal physician for further evaluation and management.

12.3.6. Hypertrichosis

Diazoxide has been noted to cultivate lanugo hypertrichosis that can be cosmetically distressing but that recedes upon drug cessation [99]. However, this was not reported in clinical studies administering single doses or short courses of diazoxide [31, 65, 70, 77, 82]. The absence of hypertrichosis with short-term diazoxide treatment appears also to include female participants [1, 31], although some diazoxide obesity trials were limited to men [73, 74, 80].

12.3.7. Gastrointestinal symptoms

Gastrointestinal symptoms may occur with use of diazoxide and include nausea, vomiting, abdominal pain, diarrhea, dyspepsia, dysgeusia, or loss of appetite [99]. One of 12 participants in one single-dose study developed an episode of nausea and vomiting with diazoxide [77]. No such symptoms were mentioned in three other single-dose studies [65, 70, 82]. Even in protracted clinical trials of high-dose diazoxide, GI symptoms developed in relatively few participants and were reversible upon dose reduction or drug discontinuation [31, 73]. If participants report any such symptoms, they will be evaluated by the PI or other qualified study personnel and offered supportive care at the discretion of the PI. If symptoms are intolerable despite such measures, the participant will be advised to withdraw from the study.

12.3.8. Thrombocytopenia

Thrombocytopenia has been reported with diazoxide use. The incidence of thrombocytopenia is not specified on the package insert, but a retrospective cohort study of 295 children treated with diazoxide over an 11-year period documented the occurrence of thrombocytopenia in 14 (4.7%), in seven of whom the lab abnormality was first detected while taking diazoxide, in four within 4 weeks of discontinuing the drug, and in the remaining three, over four weeks after stopping diazoxide. Mean time to resolution was 4 days [140]. Data on the incidence and characteristics of diazoxide-associated thrombocytopenia specifically in adults are sparse, appearing at the case-report level [141, 142]. Thrombocytopenia was not reported nor was the platelet count routinely tracked in the clinical trials of diazoxide in adults listed in Table 4.i.

We will exclude participants with low platelet count at screening and will check a complete blood count at each study visit. If participants develop signs or symptoms concerning for low platelets, such as petechial rash or purpura, epistaxis, new or increased rectal bleeding, we will advise the participant to hold further investigational agent doses pending expedient evaluation and blood count check by the PI or other qualified physician (*e.g.*, the participant's personal physician). If the platelet count returns at < 100,000, either on routine check after starting diazoxide or when checked in response to a clinical concern, the participant will be withdrawn from the study. If the platelet count returns normal and there are no other medical reasons to withdraw, the participant may continue in the study if they wish. If abnormal, platelet counts will be rechecked at least weekly until normalization. (Of course, in the unexpected event of a major bleed, the participant will be directed to seek emergency medical attention.)

12.3.9. Leukopenia

Leukopenia has been reported with diazoxide use. The package insert specifically discusses only neutropenia; it does not specify the incidence, but does mention that it is "transient, not associated with increased susceptibility to infection, and ordinarily does not require discontinuation of the drug." In the aforementioned retrospective cohort analysis of diazoxide in children, neutropenia was diagnosed in 46/295 (15.6%), of whom 29 (9.8% overall) occurred during diazoxide treatment, 13 (4.4% overall) within 28 days after discontinuing diazoxide, and in 4 (1.4% overall) was at least 28 days after discontinuation. Mean absolute neutrophil count at detection was 940 μL^{-1} and mean time to resolution was 10 days (even though diazoxide was not stopped in everyone). Data on the incidence and characteristics of diazoxide-associated leukopenia in adults are sparse, appearing at the case-report level [142, 143]. There were no reports of leukopenia published in the diazoxide adult clinical trials listed in Table 4.i.

We will exclude participants with low white blood cell count at screening and will check a complete blood count at each study visit. If participants develop signs or symptoms concerning for leukopenia, such as fever or infection, we will

advise the participant to hold further investigational agent doses pending expedient evaluation and blood count check by the PI or other qualified physician (*e.g.*, the participant's personal physician). If the leukocyte count returns newly low, either on routine check after starting diazoxide or when checked in response to a clinical concern, the participant will be withdrawn from the study. If the blood count returns normal and there are no other medical reasons to withdraw, the participant may continue in the study if they wish. If abnormal, blood counts will be rechecked at least weekly until normalization.

12.3.8. Sulfa allergy
Diazoxide contains a sulfa moiety and thus participants known to be allergic to sulfa-containing drugs are excluded from the study.

12.4 – Safety matters related to investigational placebo

Investigational placebo consists entirely of supermarket ingredients: flat diet tonic water flavor-enhanced with peppermint extract and thickened with xanthan gum. Other than the theoretical potential of hypersensitivity reactions, we are unaware of any potential safety concerns for peppermint extract and xanthan gum. Diet tonic water, as manufactured by Canada Dry®, contains carbonated water, citric acid, sodium benzoate (preservative), saccharin, quinine, and natural flavors. The characteristic bitterness of tonic water – hence its utility as a placebo for the bitter-tasting diazoxide – is due to quinine. Quinine, the *sine qua non* of tonic water, is FDA-approved for malaria treatment. Although a variety of adverse reactions have been attributed to quinine, we would be concerned primarily with:

- Hemolysis in people with glucose-6-phosphate dehydrogenase deficiency
    - We will minimize this risk by excluding participants with known G6PD deficiency (§5.3.7) or abnormal G6PD result on screening labs (§5.3.9)
- Heart rhythm abnormalities, including QT_c prolongation
    - We will minimize this risk by excluding participants with known heart rhythm abnormalities (§5.3.7) and/or taking antiarrhythmic medications (§5.3.9)
    - We will also perform a screening EKG (§7.2.5) and exclude those with concerning findings, including $QT_c \geq 480$ ms (§5.3.7)
- Cutaneous eruption
- Hypersensitivity reaction

We consider these risks highly unlikely, as the quinine content of tonic water is minimal; Canada Dry® tonic water has been found to contain quinine at 61.3 mg L^{-1} [144]. As we will administer the placebo at 40 μL kg^{-1}, a 100-kg individual will receive 4 mL per dose. This equates to 245 μg of quinine per placebo dose, or less than $1/1000^{th}$ (< 0.1%) of the typical 324-mg starting malaria-treatment dose. During the 27-dose course of the study, cumulative quinine consumption in the same participant would amount to around 6.6 mg, still approximately $1/50^{th}$ (2%) of a single quinine treatment dose, over 14 days. Sale of the beverage is unrestricted and its labeling (see attachment) contains no health warnings, FDA-mandated or otherwise.

12.5 – Safety-related study interruption

12.5.1. – Pause of study procedures
Clinical trial procedures, including administration of investigational agents, will be <u>paused</u> *for individual participants* pending further investigation if one or more of the following events occur:

- Occurrence of a serious adverse event deemed to be possibly related to the study drugs or unanticipated problem
- Signs or symptoms concerning for severe hyperglycemia, including ketoacidosis
- Signs or symptoms concerning for clinically significant volume depletion or high risk for its development (*e.g.*, due to intractable vomiting)
- Signs or symptoms concerning for volume overload beyond isolated peripheral edema
- Signs or symptoms concerning for new onset of gout

- Signs or symptoms concerning for thrombocytopenia or neutropenia
- Signs or symptoms concerning for infection
- Other signs, symptoms, or events that, in the medical judgment of the PI, are sufficiently concerning to merit further investigation

Investigation and management of these events will be undertaken according to the medical judgment of the PI.

12.5.2. – Stop (early termination) of study procedures

Clinical trial procedures, including administration of investigational agents, will be stopped (terminated) early *for individual participants* if one or more of the following events occur:

- Hospitalization for any cause
- Hypersensitivity reaction, including urticaria or other rash, mucosal edema, respiratory distress, or other concerning signs/symptoms
- Blood glucose level ≥ 250 mg dL^{1} and/or ketoacidosis detected
- Hypotension
    - Systolic BP < 90 mm Hg and/or diastolic BP < 60 mm Hg
    - Orthostatic change in systolic BP of ≥ 20 mm Hg and/or diastolic BP of ≥ 10 mm Hg and/or rise in heart rate of ≥ 30 bpm with 2-5 min of upright postural change
- Platelet count < 100,000 μL^{1}, with or without signs/symptoms
- White blood cell count and/or absolute neutrophil count below the lower limit of normal, with or without signs/symptoms
- Confirmation of a new diagnosis of gout or uric acid level newly above the upper limit of normal
- Clinical evidence of pulmonary edema or other significant serous effusion
- Diagnosis of COVID-19 or, at the medical discretion of the PI, other serious infection
- Other signs, symptoms, or events that, in the medical judgment of the PI, are sufficiently concerning to merit immediate cessation of study procedures

Investigation and management of these events will be undertaken according to the medical judgment of the PI. The decision to resume *versus* terminate study procedures will be made on a case-by-case basis at the discretion of the PI with reference to the parameters above and in accordance with all relevant safety reporting/monitoring regulations.

12.6 – Unanticipated risks or adverse events

The study team will discuss study risks with recruits during the informed consent process. However, there may be as-yet unknown or unanticipated adverse effects of this study. The study team will continually monitor for these effects and will report anything meeting the definition of unanticipated problems in §12.1.3 to the IRB and DSM within 1 week (5 business days) of study team awareness. All unanticipated, non-serious AE and the study team's response to the non-serious AE will be included in a report at the time of annual continuing IRB review. The study team may consider amending the protocol as needed to ensure patient safety. Changes in the procedures of the study, as well as any changes in the risks and/or benefits, will be presented to and discussed with participants upon approval from the IRB for implementation of such revisions, and any IRB-revised written consent will be signed, as appropriate.

13. PROTECTION OF HUMAN PARTICIPANTS

This study is to be conducted according to U.S. and international standards of Good Clinical Practice, applicable government regulations, and institutional research policies and procedures.

This protocol and any amendments will be submitted to a properly constituted independent ethics committee (EC) or institutional review board (IRB), in agreement with local legal prescriptions, for formal approval of study conduct. The decision of the EC/IRB concerning the conduct of the study will be made in writing to the PI and a copy of this decision will

be provided to the PI before commencement of this study. The investigator will retain a list of EC/IRB members and their affiliates.

All participants for this study will be provided a consent form describing the study and providing sufficient information for recruits to make an informed decision about their participation in this study. This consent form will be submitted with the protocol for review and approval by the EC/IRB for this study. The formal consent of a participant, using the EC/IRB-approved consent form, must be obtained before that participant is submitted to any study procedure. This consent form must be signed by the participant and the investigator-designated research professional obtaining the consent.

14. DATA COLLECTION, MAINTENANCE, AND REPORTING

14.1 – Data Handling

14.1.1. Data collection

Data for this study will be collected and stored both on paper and electronically. The IICTR has an electronic clinical trials and data management system (CTMS) that will be used for data collection. CTMS has full auditing capabilities, which is web-based and housed on a server in a fully HIPAA-compliant server room with restricted access and video monitoring. All users must log in with their own application user ID and password. Users off campus must first access the Virtual Private Network (VPN) with their assigned campus user ID and password and then use their application credentials. Users are only able to see study information if they are indicated as authorized study personnel in our electronic IRB system. Users are limited to access based on the role assigned in their corresponding protocol. Participant data is entered directly into the system, which, in the case of Columbia participants, confirms the correct identity of patients *via* an interface with the electronic medical patient index. Staff with the appropriate IRB-defined roles scan run reports within the system for reporting purposes. Data and other study-related files may be shared among authorized study personnel through the use of a CUIMC IT/RSAM-approved systems that are accessible only by password-protected computers connected to the CUIMC network (RSAM system ID entered under Privacy & Data Security).

Case Report Forms (CRF) for the study will be collected on paper and numerical elements will be transcribed and maintained electronically. Data and other study-related files may be shared among authorized study personnel through the use of a P: drive or Columbia University-licensed Microsoft Office 365 applications that are accessible only by password-protected computers connected to the CUIMC ethernet or Mercury WiFi (system IDs entered under Privacy & Data Security).

14.1.2. Data management

The study team will be responsible and accountable for the data management of this study according to the following:

- All study data will be collected on paper CRFs that will be transcribed into electronic form by authorized study personnel
- Baseline characteristics will be entered by study personnel into a local electronic file developed by the study team
- These files are maintained by the PI or delegated research staff and query as needed
- The PI and co-investigators will ensure that data are recorded on the CRFs and specified in the study protocol, in accordance with the instructions provided
- The PI and co-investigators will ensure the accuracy, completeness, and timeliness of the data recorded
- The PI will sign the completed CRFs

It is the investigators' responsibility to ensure that all clinical and laboratory data entered on the corresponding CRFs are complete, accurate, and authentic.

14.1.2. NIH-compliant Data Management and Sharing plan

Although this is not an NIH-funded study, as a matter of best practice, we wish to abide by NIH's Data Management and Sharing Policy (DMSP). In compliance with the DMSP, study data will eventually be submitted to an NIH- and Columbia

CTO-endorsed online data repository such as Dryad prior to competitive grant renewal or journal publication of study results. Such study data may include generic characteristics of participants (e.g., age, sex, race/ethnicity, height/weight/BMI, vital signs, baseline lab results), study results (raw and/or analyzed data) obtained, and information on biospecimen inventory for sharing according to the practices described in this protocol. All study data submitted to the repository will be coded or deidentified. Nobody except IRB-authorized study personnel will have access to the code key and so it will not be possible for anyone else to link participant-level study data to a specific person. No sensitive data will be transmitted outside of IRB-authorized study personnel. No genetic testing will be performed on these samples and thus no genetic/genomic data will be generated.

14.2 – Data and Safety Monitoring

14.2.1. Data and Safety Monitoring Plan

Data and safety monitoring will be carried out by the PI continually and annually (and as needed) by a designated physician data and safety monitor (DSM) not otherwise involved with the study. Adjudication of serious adverse events that are possibly related to the study drugs and/or events that require pausing or terminating the intervention will be performed by Dr. Judith Korner, Professor of Medicine at Columbia University Irving Medical Center. Data and safety monitoring activities for the study will continue until all participants have completed their participation and, as needed, until a sufficient amount of time has passed beyond which any study-related AE are unlikely.

During the study, the study PI and co-investigators will be responsible for the following:

- Confirm that the investigational team is adhering to the protocol, that data are being accurately and expeditiously recorded in the CRFs, and that biological samples are handled in accordance with the laboratory manual
- Verify that the research pharmacy maintains drug accountability
- Perform source data verification, including verification of informed consent of participating participants
- Ensure that withdrawal of informed consent to the use of a participant's biological samples is reported, that relevant biological samples are identified and destroyed accordingly, and that these actions are documented and reported back to the participant
- As above, a physician not involved in the research project will serve as data and safety monitor (DSM): Dr. Judith Korner, Professor of Medicine at Columbia University Irving Medical Center

In addition, reports summarizing study progress (including recruitment, interim data analysis and data quality, adverse events, and protocol changes) will be submitted to the IRB annually plus as needed/requested.

14.2.2. Audits and inspections

The PI will permit study-related monitoring, audits, and inspections by the EC/IRB, government regulatory bodies, and university compliance and quality assurance groups of all study-related documents (*e.g.*, source documents, regulatory documents, data collection instruments, study data, *etc.*). The investigator will ensure the capability for inspections of applicable study-related facilities (*e.g.*, pharmacy, diagnostic laboratory, *etc.*). Participation as an investigator in this study implies acceptance of potential inspection by government regulatory authorities and applicable university compliance and quality assurance offices.

14.3 – Confidentiality

Information about study participants will be kept confidential and managed according to the requirements of the Health Insurance Portability and Accountability Act (HIPAA) of 1996. Those regulations require a signed participant authorization informing the participant of the following:

- What protected health information (PHI) will be collected from participants in this study
- Who will have access to that information and why
- Who will use or disclose that information

- The rights of a research participant to revoke their authorization for use of their PHI

In the event that a participant revokes authorization to collect or use PHI, the investigator, by regulation, retains the ability to use all information collected prior to the revocation of participant authorization. Participant binders will be maintained within the PI's locked office within a limited-access area of the Black Building; only the investigator and authorized study staff will have access to the files.

14.4 – Source Documents

Source data is all information, original records of clinical findings, observations, or other activities in a clinical trial necessary for the reconstruction and evaluation of the trial. Source data are contained in source documents. Examples of these original documents and data records include: hospital records; clinical and office charts; laboratory notes; memoranda; participants' diaries or evaluation checklists; pharmacy dispensing records; recorded data from automated instruments; copies or transcriptions certified after verification of accuracy and completeness; microfiches; photographic negatives; microfilm or magnetic media, radiographs, participant files; and records kept at the pharmacy, at the laboratories, and at medico-technical departments involved in the clinical trial.

14.5 – Case Report Forms

The study case report form (CRF) is the primary data collection instrument for the study. CRF will be recorded on paper for inclusion into the study binder and will also be transcribed electronically where applicable. All data requested on the CRF must be recorded. All missing data must be explained. If a space on the CRF is left blank because the procedure was not done or the question was not asked, write "N/D". If the item is not applicable to the individual case, write "N/A". All entries must be printed legibly in black or blue ink. If any entry error has been made, the error should be corrected by drawing a single strike across the incorrect entry, without erasure or obliteration, and entering the correct data above it. All such changes must be initialed and dated. For clarification of illegible or uncertain entries, print the clarification above the item, then initial and date. Once the paper CRF are completed, the relevant information will be entered into the electronic study binder by authorized study personnel (*e.g.*, PI or clinical research coordinator). The PI must sign off on CRF once they are entered and verified. Only study personnel listed on the IRB protocol may be granted access to CRF or electronic study data by the PI. All study personnel entering data on or accessing CRF and/or electronic study binder must have up-to-date training on all relevant topics, including CTMS, GCP, HIPAA, *etc.*

14.6 – Records Retention

Records relating to a specific research activity, including research records collected by investigators, must be maintained for at least the longest period that accounts for all applicable policies and regulations. This minimum retention period applies whether or not any participants were enrolled in the study. Clinical records, including consent forms that document clinical intervention or clinical/diagnostic research-related procedures must be retained in medical records by the institution for at least seven (7) years, per CUIMC and NYP policy, in turn based on New York State law.

14.7 – Sample and data use by collaborators on this study

14.7.1. Nature and purpose of collaboration

Because our ultimate primary endpoint in the main study (*i.e.*, the future study that this P&F study will support) will be change in DNL with insulin lowering, this P&F study provides a valuable opportunity to derive some exploratory preliminary data and to acclimate the study team to the use of stable-isotope tracers. Although the PI (Dr. Cook) is readily able to administer the stable isotope tracer needed to measure DNL (*i.e.*, oral D_2O), the onerous sample preparation for GC-MS and the analytical complexity of deriving DNL from it do not fall within the PI's area of expertise. We have therefore forged a collaboration with Dr. Marc Hellerstein, Professor of Nutrition and Toxicology at the University of California Berkeley, who is one of the world's foremost experts on lipid kinetics and developed the canonical Mass Isotopomer

Distribution Analysis (MIDA) method of quantifying DNL. Dr. Hellerstein and his team have provided considerable technical guidance on the incorporation of heavy water labeling into this protocol. More importantly, the Hellerstein lab will perform the DNL-related aspects of specimen handling and data analysis that fall outside Dr. Cook's expertise. That is, we will send samples of plasma and saliva obtained during these study procedures to the Hellerstein lab, which will in turn prepare for and run the GC-MS on those samples and analyze the resulting data in order to assess DNL. We expect that the collaboration begun with this study will continue on for the subsequent, main study of DNL with insulin lowering.

All participant-facing study procedures will take place at CUIMC; no study participants will be seen at any institution outside of CUIMC. As noted in his Letter of Collaboration, because no participants are seen at his institution, and all samples and data he receives are coded or deidentified (see below), Dr. Hellerstein will rely on the CUIMC IRB for approval of his analytic services. This is the established approach that Dr. Hellerstein uses in his many extramural collaborations and is endorsed by the University of California Berkeley Committee for the Protection of Human Subjects.

14.7.2. Specifications of sample use

Only coded samples will be shared with our collaborators in the Hellerstein lab. Samples and data shared between the CUIMC study team and collaborators at Berkeley are directly related to – and are defined endpoints of – this study. They are therefore considered neither to be future uses nor to have been drawn from the Insulin Resistance Biobank (see §14.8). Samples to be transferred to the Hellerstein lab will be drawn, processed, handled, and stored prior to transfer along with all other samples for this study. Each sample will be transferred frozen in cryovials labeled with the associated study ID numbers, sample type (*i.e.*, plasma or saliva), date of collection, and study time point. Each specimen aliquot will also be assigned a tracking number that is appended to the sample ID number for inventory purposes. No PHI/PII will ever be shared outside of IRB-approved CUIMC study personnel or authorized university or governmental regulatory agencies. The key linking the sample's corresponding study ID number to PHI will remain securely stored within CUIMC IT/RSAM-approved systems within CUIMC and/or locked in the PI's office, as outlined elsewhere in this protocol. Only IRB-authorized CUIMC study personnel as described in this protocol will ever have access to the key and its associated PHI. An electronic log of all samples ("Biospecimen Log") – also containing *no* PHI/PII – will be maintained by the PI detailing the inventory of the specimens to be transferred (*i.e.*, one entry per specimen) as well as:

- Coded sample ID, including specimen's individual tracking number (*no PHI*)
- Specimen type: plasma or saliva
- Sample tracking number (see above)
- Date of collection
- Study time point
- Date of transfer from CUIMC
- Date of receipt at Berkeley

Specimen aliquots will be shipped to the Hellerstein lab at Berkeley *via* courier service in sealed, insulated containers (*e.g.*, polystyrene) containing dry ice. An IRB-approved blanket material transfer agreement (MTA) and data use agreement (DUA) between Dr. Hellerstein and the study team will be required to execute any transfer of specimens or data. The specimens will be used only to perform prespecified assays by mass spectrometry: D_2O enrichment of body fluids (blood, saliva) and any assays necessary to quantify DNL. Other lipid-kinetic parameters, such as VLDL-TG secretion or ApoB kinetics, may also be performed. These other possible endpoints will be considered as future uses and so would only be performed on samples where consent has been given for future use. The samples will never be used for genetic testing.

14.7.3. Specifications of data use

All transfer/use of data with the Hellerstein lab will take place only once covered by an IRB-approved blanket Data Use Agreement (DUA). Transferred samples will be furnished alongside a slip of paper containing the same information listed on the cryovials labels; the slip will be sealed in an envelope addressed to the rightful recipient. The PI and IRB-authorized study personnel may also communicate electronically *via* institutional email addresses (*e.g.*, @cumc.columbia.edu, @berkeley.edu) with Dr. Hellerstein and his staff. Such electronic communication may include correspondence regarding

study samples, their associated data, data analysis, and other topics relevant to this study. Data will be supplied only on a need-to-know basis and in the smallest necessary quantity, as judged by the PI. Again, no PHI/PII, including the key to sample codes, will ever be shared outside of IRB-authorized CUIMC study personnel. All of the data/results to be generated in Dr. Hellerstein's lab are they are purely for research purposes and will not be returned to participants.

14.8 – Sample retention for Insulin Resistance Biobank (Future Use of Biospecimens)

14.8.1 – Purpose of repository

Lowering of insulin levels with diazoxide provides unique and valuable insights into human physiology and metabolism that extend beyond the specific objectives of this study and are not otherwise readily obtainable. As such, these samples are expected to be of value in testing additional hypotheses in the future, including by independent research groups. As such, we propose to add to our Insulin Resistance Biobank (repository) in which we store aliquots of residual, coded study specimens for such future analyses. This Insulin Resistance Biobank was approved by the IRB in Protocol #AAAU0504, but that study has since been closed and the Biobank, now renamed the Hyperinsulinemia Biobank, will be governed by AAAU9950. Note that no specimens were entered into the Biobank from AAAU0504 because of poor recruiting. Biospecimens from AAAU2570 that were stored in the Biobank under AAAU0504 will remain physically in the exact same location under AAAU9950 and the specimen records transferred over. We plan to maintain this repository indefinitely and plan to supplement it with additional specimens from other IRB-approved mechanistic studies of insulin resistance to be carried out in the future. The consent form for study participants includes language specifically outlining the purpose and general methods of specimen biobanking, as well as the measures taken to prevent breach of confidentiality. Participants may elect not to allow their coded biospecimens to be shared with non-study personnel, in which case this prohibition will be clearly noted in the electronic biospecimen log (see below). However, participants are required to allow the PI and co-PIs of this study to perform additional, relevant analyses of their samples for at least seven (7) years. This ongoing access to the study team may be needed to perform additional analyses later requested by peer reviewers for publication of the study(ies) generated by this protocol.

14.8.2 – Repository specifications

Specimens in the repository will be labeled with the participant ID number, sample type (*i.e.*, blood tube type in which it was collected), date of collection, and study time point. Each specimen aliquot will also be assigned a tracking number that is appended to the sample ID number for inventory purposes (*e.g.*, XXXX-01, -02, -03, *etc.*). No protected health information will be included with these specimens at the repository site. The key linking the participant ID number to PHI will remain securely stored within CTMS and/or locked in the PI's office, and physically separated from the repository, as outlined elsewhere in this protocol. Only authorized study personnel as described in this protocol will ever have access to the key and its associated PHI. Although personnel authorized in AAAU9950) will have access to biospecimens and associated data, any such individuals will not have access to the key linking participant ID numbers to PHI for this study unless they are also authorized personnel on this study. An electronic log of all samples ("Biobank Log") – also containing *no* protected health information – will be maintained by the PI detailing the inventory of specimens (*i.e.*, one entry per specimen) as well as:

- Coded sample ID, including specimen's individual tracking number (*no PHI*)
- Biospecimens' physical layout/location within the freezers (see below)
- Any prohibition on sharing of specimens based on patients' consent forms
- Blood tube type from which aliquot was taken
- Date of collection
- Study time point
- Age, sex, and biometric data (*i.e.*, vital signs, weight, height, BMI, and waist circumference) of the participant from whom the sample was drawn
- Results of lab tests already performed on each specimen

Columbia University Irving Medical Center
AAAU2570 (PI: Cook) – [redacted]
The biospecimen repository will be located in -80ºC freezers within the lab space of the PI (Dr. Cook), or of Dr. Rebecca Haeusler (co-investigator under AAAU0504), with temporary storage as needed for assays within the IICTR Biomarkers Core Laboratory. Each of these sites is guarded by CUIMC security services and access to the freezers is restricted to unauthorized personnel. Residual blood samples will be aliquoted in our research lab, using sterile technique, from original collection vials into small tubes (*e.g.*, Eppendorf Quality™ microcentrifuge tubes, #0222363204, or equivalent); each aliquot will contain blood only from a single collection vial type and participant. The tubes containing aliquots will be labeled as above and stored, along with other samples from the same participant, in freezer-safe boxes (*e.g.*, Fisherbrand™ 03-395-455) capable of storing up to 81 specimens apiece. Each freezer box will be clearly labeled with the study name and protocol number, contact information for the PI, and sample type(s) contained. These boxes will be stored at -80ºC on dedicated storage racks. Only Dr. Cook (PI) will have direct access to the repository, but other study co-investigators may handle biospecimens as needed once furnished by Dr. Cook.

14.8.3 – Sharing of de-identified biospecimens or data from repository
Re-coded (see below) specimens and/or associated data will be available for sharing with other qualified researchers, including those at CUIMC and externally, as deemed appropriate by the study PI. Requests for material and/or data must be made formally in writing (email is acceptable) to the PI. All requests must include a scientific justification for their use and evidence of legitimacy – that is, whether the requesting researcher is affiliated with a reputable institution, has the physical plant necessary for appropriate specimen handling and storage, and has a respectable reputation.

All formal requests for material must be routed through the PI, Dr. Cook. Scientifically justified requests must be narrowly tailored to utilize the minimum possible amount of material or data. The PI will consider all such requests on a case-by-case basis, based on the scientific merit of the proposed use and the legitimacy of the individual(s) making the request (*i.e.*, appropriate institutional setting, ability to properly handle specimens, no known history of scientific misconduct, *etc.*). We will not approve requests for biospecimens or data to be used for clinical investigation of a medical device or for genetic testing.

When granting a request for sharing material, authorized study personnel will provide small specimen sub-aliquots that are labeled only with (i) a de-identifying new subaliquot code (*i.e.*, not the same as the study ID number), (ii) the sample type (*i.e.*, blood tube type in which it was collected), and (iii) study time point. Samples will be furnished alongside a slip of paper containing the same information that will be sealed in an envelope addressed to the rightful recipient; sample information data will not be transmitted electronically to investigators external to CUIMC. Data (*e.g.*, results of lab tests, biometrics) will be supplied only on a need-to-know basis and in the smallest necessary quantity, as judged by the PI. No PHI will ever be communicated to personnel not authorized to take part in this study, and, again, samples are coded even within the repository itself. Given the two-step removal of such specimens and data from PHI (*i.e.*, coded participant ID number with key sequestered separately within CTMS *and* unique de-identifying subaliquot code), breaches of confidentiality are highly unlikely.

Specimen subaliquots will be shipped to the institutional address of requesters *via* courier service in sealed, insulated containers (*e.g.*, polystyrene) containing dry ice. A material transfer agreement (MTA) between the requesting researcher/institution and the study team will be required to execute any transfer of specimens. Similarly, a Data Use Agreement (DUA) between the requesting researcher/institution and the study team will be required to execute any transfer of data. In either case, any proposed material and/or data transfer, and the accompanying finalized MTA/DUA, will be submitted to the IRB for approval prior to the transfer of any data or materials. An electronic record of all such requests, and specimens disbursed, will be kept by the study team and not shared with any non-study personnel.

15. STUDY FINANCES

15.1 – Conflict of interest

Any investigator who has a conflict of interest with this study (*e.g.*, patent ownership, royalties, or financial gain greater than the minimum allowable by their institution, *etc.*) must have the conflict reviewed by the Columbia University Conflict of Interest Committee with a Committee-sanctioned conflict management plan that has been reviewed and approved prior to participation in this study. All CUIMC investigators will follow the University conflict-of-interest policy.

15.2 – Participant compensation: stipends and reimbursement

15.2.1. Compensation

Participants will be provided financial compensation for participation. Stipends will be paid on a *pro rata* graduated scale for each Study Visit attended; attending SV-1 and -2 pay $50 per visit and attending SV-3 and -4 pay $100 per visit. Participants will also be paid study completion bonuses of $250 for taking all investigational agent doses and $150 for drinking all D_2O aliquots. If any investigational agent doses or D_2O aliquots are missed, bonuses will be proportionally downwardly prorated for participants permitted to remain in the study through SV-4. Participants withdrawn before SV-4 will not be eligible for the bonuses. The maximum stipend to be paid per participant is $700 and compensation will be paid as a single lump sum after the end of a participant's time in the study (whether completion or withdrawal). As missed study visits necessitates withdrawal from the study, participants will not be eligible to receive payment for attempted attendance of further study visits. Table 15.i, below, summarizes the compensation scheme on a per-visit basis for each participant.

15.2.2. Travel reimbursements

We will also provide travel-expense reimbursement for public transportation fares, tolls, or parking for participants coming to CUIMC specifically for study visits. We will *not* provide additional transportation compensation for participants otherwise coming to CUIMC at that time to attend a regularly scheduled medical appointment. Compensation for subway or bus fare will be on the current rate set by the Metropolitan Transportation Authority or for Port Authority Trans-Hudson (PATH) (currently $2.90 per ride, for $5.80 per round trip). If participants furnish a receipt for round-trip travel *via* the Long Island Rail Road, the Metro-North Railroad, or New Jersey Transit, we will provide commensurate reimbursement. Reimbursement for tolls will be paid based on contemporaneous passenger car toll rates set by the Port Authority of New York & New Jersey for Hudson River crossings (currently $16.00 one way only); by the Metropolitan Transportation Authority for East River crossings (currently $10.17 in both directions, includes the Verrazano-Narrows Bridge) or the Henry Hudson Bridge (currently $7.50 in both directions). Reimbursements for toll crossings will be paid based on the shortest route from the participant's home address, as calculated by Google Maps. Participants must provide either a copy of the bill mailed to them for toll-by-mail or a copy of their electronic toll collection statement that displays the relevant study-visit travel in order to be reimbursed for tolls. (All other personal information besides the name on the bill or statement can be redacted as the participant desires.) Compensation for up to 3 h of parking will be paid upon furnishing of receipt. Receipts can be authenticated by sending images to the principal investigator or other designated study personnel by email or SMS.

Table 15.i. *Study compensation and travel reimbursement scheme*

Study participant compensation scheme			
Stipend	Study Visit stipend	SV-1, -2 ($ per SV)	$ 50.00
		SV-3, -4 ($ per SV)	$ 100.00
	Bonus for taking all 27 IADs [h]		$ 250.00
	Bonus for drinking all 32 D_2O aliquots [h]		$ 150.00
Transportation reimbursement (amount)	Car tolls [a,b,c]	PANYNJ crossings [d]	$ 16.00
		Tappan Zee Bridge	$ 5.75
		MTA toll crossings [e]	$ 20.34
		Henry Hudson Bridge	$ 15.00
	Parking (with receipt only) [a,f]		$ x 3 h

	MTA subway/bus fare or PATH (round trip) [a,g]	$ 5.80
	Commuter rail fare (with receipt only) [a,g]	$ x round trip

a. Paid on a per-visit basis, only to participants coming to CUIMC exclusively for study procedures; not paid if otherwise attending regularly scheduled medical appointment
b. Tolls charged by MTA Bridges & Tunnels are bidirectional, and thus calculated as round-trip tolls for relevant crossings; does not include toll-free crossings (Brooklyn Bridge, Manhattan Bridge, Williamsburg Bridge). Participants must provide a redacted copy of their toll-by-mail bill or a their electronic toll collection statement that highlights the relevant trip(s).
c. Subject to change depending on contemporaneous passenger car toll rate charged by the relevant public authority
d. Crossings managed by the Port Authority of New York & New Jersey: George Washington Bridge, Lincoln Tunnel, Holland Tunnel, Bayonne Bridge, Goethals Bridge, Outerbridge Crossing
e. Includes Queens-Midtown Tunnel, Throgs Neck Bridge, Triboro Bridge, Verrazano-Narrows Bridge, Whitestone Bridge; excludes Henry Hudson Bridge, as above
f. Subject to change depending on contemporaneous hourly rate charged as verified on receipt
g. Subject to change depending on contemporaneous individual passenger fare charged by the relevant public authority
h. Bonuses will be downwardly prorated for doses missed, if the participant is allowed to continue in the study through SV-4.

15.2.3. Method of payment

Study stipends/compensation will be issued either by direct deposit through Columbia Finance's Payment Works system or as university-issued check(s), in either case to be issued after study completion. Participants may choose the option they prefer, but, in either case, payments may require up to 6-8 weeks for processing. Payments in excess of $600 per year are tax reportable. All participants will be therefore be asked to complete a standard IRS Form W9, including social security number, in order to be registered as vendors with the university for this purpose.

Travel reimbursements will be paid *via* the Columbia Pay Card system by loading onto an unregistered (anonymous) U.S. Bank-issued Visa®-branded debit card using Columbia Finance online infrastructure. The debit card can be used like any other debit card, wherever Visa® cards are accepted. The Pay Card used for travel reimbursement will be distributed to participants along with a packet of Columbia- and U.S. Bank-provided information about its use (see Documents). The instructions include a step-by-step guide that recipients will follow to activate and use their cards. The anonymous Pay Card will be loaded with the total travel reimbursement (i.e., based on receipts and statements provided) after the completion of the study.

16. PUBLICATION PLAN

Neither the complete nor partial results of the study carried out under this protocol, nor any of the information provided by the sponsor for the purposes of performing the study, will be published or passed on to any third party without the consent of the study sponsor. Any investigator involved with this study is obligated to provide the sponsor with complete results and all data derived from the study.

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