Study Protocol Title:
ABA - Abscisic Acid Effects on Glucose Homeostasis and Insulin Sensitivity

Study Sponsor:
Biotherapeutics Inc (BTI), a spinoff Company of Virginia Tech

Principal Investigator:
Principal investigator: Bret Goodpaster, PhD

List of Abbreviations:

AE	Adverse Event
ANCOVA	Analysis of Covariance
CFR	Code of Federal Regulations
CLIA	Clinical Laboratory Improvement Amendments
CMP	Clinical Monitoring Plan
COC	Certificate of Confidentiality
CONSORT	Consolidated Standards of Reporting Trials
CRF	Case Report Form
DCC	Data Coordinating Center
DHHS	Department of Health and Human Services
DSMB	Data Safety Monitoring Board
DRE	Disease-Related Event
EC	Ethics Committee
eCRF	Electronic Case Report Forms
FDA	Food and Drug Administration
FDAAA	Food and Drug Administration Amendments Act of 2007
FFR	Federal Financial Report
GCP	Good Clinical Practice
GLP	Good Laboratory Practices
GMP	Good Manufacturing Practices
GWAS	Genome-Wide Association Studies
HIPAA	Health Insurance Portability and Accountability Act
IB	Investigator's Brochure
ICH	International Conference on Harmonisation
ICMJE	International Committee of Medical Journal Editors
IDE	Investigational Device Exemption
IND	Investigational New Drug Application
IRB	Institutional Review Board
ISM	Independent Safety Monitor
ISO	International Organization for Standardization
ITT	Intention-To-Treat
LSMEANS	Least-squares Means
MedDRA	Medical Dictionary for Regulatory Activities
MOP	Manual of Procedures
MSDS	Material Safety Data Sheet
NCT	National Clinical Trial
NIH	National Institutes of Health
NIH IC	NIH Institute or Center
OHRP	Office for Human Research Protections
PI	Principal Investigator
QA	Quality Assurance

QC	Quality Control
SAE	Serious Adverse Event
SAP	Statistical Analysis Plan
SMC	Safety Monitoring Committee
SOA	Schedule of Activities
SOC	System Organ Class
SOP	Standard Operating Procedure
T2D	Type 2 Diabetes/Diabetics
UP	Unanticipated Problem
US	United States

Introduction

This document is a protocol for a human research study. This study is to be conducted in accordance with applicable federal regulations and institutional research requirements and procedures.

The purpose of this study is to determine the effects of oral abscisic acid (ABA) on glucose metabolism in subjects with defined prediabetes and type 2 diabetes (T2D). To this end, a randomized, cross-over, placebo-controlled trial will be employed to evaluate the effects of ABA (95ug) twice a day (190ug total per day) for 14 days on tolerability and insulin sensitivity during a hyperinsulinemic euglycemic clamp. Participants will be randomized to either ABA (95 µg ABA and 300 mg of corn starch) or placebo (300 mg cornstarch) twice a day before meals for 14 days.

The primary aim is to determine the tolerability and efficacy of ABA supplementation on insulin sensitivity in prediabetic and T2D men and women. The secondary aim is to assess changes in insulin-stimulated signaling and glucose transporter proteins, insulin, glucose, pyruvate, and fatty acid oxidation levels in skeletal muscle tissue samples from biopsy in addition to inflammatory markers and fasting ABA concentrations in plasma.

Background Information and Scientific Rationale

Prediabetes afflicts 86 million Americans and has recently risen to more than 3 million new cases per year in the U.S. alone. A prediabetic person is likely to become diabetic within 10 years, because many people with prediabetes have no symptoms and do not adhere to lifestyle and diet changes or medical treatment. According to the CDC, 9 out of 10 Americans with prediabetes are not aware that they have it. Furthermore, approximately 1 in 10 Americans (~37 million) have type 2 diabetes. Insulin resistance within skeletal muscle is considered to be a key instigator of prediabetes and T2D. Fortunately, skeletal muscle insulin resistance is reversible and/or mitigated with lifestyle changes and the right diet; it is possible to restore andor manage glucose homeostasis. There are currently no specific drugs that target skeletal muscle insulin sensitivity. Identifying natural ingredients that can be produced and marketed to enhance insulin sensitivity could significantly impact the nearly 110 million people in the U.S. with either prediabetes or T2D.

Abscisic acid (ABA) has been identified as a putative sugar control hormone. The plasma ABA concentration (ABAp) increases after OGTT in healthy subjects (1). ABA stimulates glucose-dependent insulin release from human and rodent pancreatic β-cells (2). It increases both translocation of the glucose transporter GLUT-4 to the plasma membrane and GLUT-4-dependent glucose uptake by cultured adipocytes and myoblasts (3). The increase of ABAp that occurs after an OGTT is impaired in patients with T2D and in women with gestational diabetes (GDM) (4). Glucose tolerance after childbirth is paralleled by restoration of both the ABAp response to oral glucose and normal fasting ABAp levels (4). The impaired response of ABAp to hyperglycemia is a common feature in T2D and prediabetic subjects, pointing to a critical role

for ABAp in maintaining glycemic control. Based on studies in animals and humans (5), developing an ABA-based medical food to help prediabetic individuals may be highly beneficial.

Significance of generating GRAS (Generally Recognized as Safe) ABA. GRAS is a strict safety standard established by the FDA, requiring technical demonstration of non-toxicity and safety, as well as a general recognition and agreement by experts that the ingredients are safe for consumption. The FDA has determined many ingredients are GRAS; they are listed as such by regulation, in Volume 21 Code of Federal Regulations (CFR) Sections 182, 184, and 186. Other ingredients may achieve self-affirmed GRAS status via a panel of independent experts in the pertinent field who co-author a GRAS report. The process of attaining GRAS status is one of self-determination, whereby a panel of experts (the Expert Panel) critically evaluates the documentation supporting the safety of the ingredient under the conditions of intended use before providing a signed opinion. The material reviewed by the Expert Panel includes information pertinent to product manufacturing, specifications, and analyses of the substance, proposed uses and levels of use in foods, estimated dietary exposure, safety studies, and other information to support safety, as available. Following a successful GRAS determination, the ingredient can be marketed immediately.

Many U.S. food manufacturers will only purchase foods/beverages if they have, at a minimum, an FDA "No Objection" letter to a GRAS Notification. Regardless of the pathway to GRAS, the manufacturer maintains documentary evidence of the safety of the ingredient, including its natural or artificial derivation, the related processing, and the effects of its consumption under specified intended uses. Examples of successful commercial development of natural products as medical foods include: 1) Ensure® and Glucerna® for glucose control (Abbott), 2) Axona® for Alzheimer's patients, 3) Fosteum® (genistein aglycone/citrated zinc bisglycinate/cholecalciferol) for osteopenia and osteoporosis, 4) Metanx® (l-methylfolate calcium/pyridoxal 5′-phosphate/methylcobalamin) for diabetic neuropathy, and 5) Theramine^{TM} (l-arginine, 5-htp, histidine, l-glutamine) for myalgia. During the Phase 1 SBIR period, Biotherapeutics Inc (BTI) has successfully secured GRAS for ABA in October 2016 and is planning to use this product for clinical testing and commercialization. This Phase 2 proposal will generate qualified health claims through translational and clinical studies that allow developing ABA as a functional food ingredient for managing blood sugar levels.

ABA is an innovative medical food for metabolic syndrome, prediabetes, and T2D. The first line of management for prediabetes, metabolic syndrome, and diabetes includes diet and exercise; however, patient adherence is exceedingly difficult. Therapeutic alternatives are increasingly preferred, especially options that do not require a clinical visit for administering treatment. Thus, a medical food for glycemic control and treating T2D is desirable. Further, developing a medical food that exerts anti-diabetic and anti-inflammatory effects with the potential to prevent prediabetes and metabolic syndrome is novel. Moreover, ABA, a fruit-derived compound, is well suited to be developed into a medical food. ABA obtained from normal dietary consumption (3)) is insufficient to exert glycemic control. U.S. adults with metabolic syndrome and prediabetes have suboptimal ABAp levels, thus there might be an unmet nutritional need for increasing ABA intake. Marketing ABA as a medical food

formulation, as opposed to a pharmaceutical, could increase patient interest and the overall public health impact of this product. Preclinical and clinical studies have revealed an outstanding safety profile for ABA at 10,000, 15,000 and 20,000 ppm. ABA stimulates glucose consumption by adipocytes and L6 myoblasts, and lowers glycemia in humans when supplemented with 1 µg/kg body weight (6). We validated that low-doses of ABA (1, 10, 40 and 200 µg/kg/d) significantly reduce fasting blood glucose and A1C levels in severely diabetic db/db mice when administered daily for 28 d. In two independent preliminary human Phase 1 studies, a single dose of ABA administered concurrently with a caloric load improved glucose tolerance and reduced glycemic and insulin indices in healthy subjects. This project will move ABA to the forefront of translational and clinical research on metabolic syndrome, prediabetes, and diabetes.

ABA is an innovative ingredient that offers a natural solution to high sugar levels in blood. ABA is present in varying concentrations in fruits and vegetables. On average the concentration of ABA is 1.54 per mg/kg of wet weight of vegetable and 0.49 mg/kg of wet weight of fruit. According to the latest published dietary survey data from 2009–2012 (7), U.S. adults age ≥20 years consumed on average 2.79 servings/day of fruits and vegetables. The current recommendation is to eat ≥4.5 servings per day, which would lead to ≥718 µg/d of ABA in an ideal situation, but only 8% of the U.S. adult population is meeting the AHA 2020 Strategic Goals on dietary recommendations for fruits and vegetables (7). This means that 92% of the population might be obtaining less ABA from the diet than they should according to the fruit and vegetable recommendations. The addition of 2 doses of 95 µg each (total 190 µg/day) should bring plasma ABA levels closer to the recommendations for fruit and vegetable intake. Further, given its impact on glycemic control, a need to develop reference values for ABA exists.

LANCL2 is the proposed molecular target for the glycemic-controlling effects of ABA. The LANCL2 is a unique pathway that is not being exploited commercially by any other anti-diabetic medications. The mammalian ABA receptor has been proposed to be LANCL2 on the basis of, i) molecular modeling predictions (8), ii) direct and specific ABA binding to the purified recombinant protein (9), and iii) abrogation of the functional effects of ABA by silencing of LANCL2 expression in ABA-sensitive cells and deletion of the gene in mice. The signaling pathway downstream of LANCL2 includes a G-protein-mediated activation of adenylate cyclase, cAMP production and activation of protein kinase A (10). In addition, LANCL2 facilitates phosphorylation of Akt by mTORC2 via direct physical interactions (11). Active mTORC2 causes translocation of GLUT4 to the plasma membrane and stimulates glucose uptake (12). Increased Akt phosphorylation, GLUT4 translocation and GLUT4-dependent glucose transport occur in ABA-treated L6 myoblasts (3). Thus, the ABA/LANCL2 axis is emerging as a target for treating dysfunctional glucose homeostasis (13). ABA performs within 98% similarity to the type 2 diabetes drug, Avandia®, a PPAR gamma-agonist. Additionally, as opposed to TZDs, ABA does not bind to PPAR gamma (14). Biochemical analyses validated our finding that LANCL2 recognizes ABA (9, 10, 14).

LANCL2 expression in immune cells, skeletal muscle, adipose, and pancreas, and the potential to manipulate LANCL2 signaling and GLUT4 translocation with ABA make it a putative therapeutic and/or adjunct for metabolic syndrome, prediabetes and T2D. BTI has contributed to

elucidating the role of LANCL2 in inflammation and glucose metabolism. BTI performed the initial modeling studies and predicted that ABA binds to LANCL2 (8). This prediction was validated by biochemical assays (8, 9). We discovered that dietary ABA increases insulin sensitivity and suppresses obesity-related inflammation in obese/diabetic db/db mice (15, 16). Furthermore, µg amounts of ABA from fruit extracts improve glucose tolerance while also reducing insulinemia in both rats and humans (6, 17). Moreover, we provide mechanistic evidence in vivo that the molecular target of ABA might be LANCL2, as the loss of LANCL2 expression in LANCL2-/- mice diminishes the glucose-normalizing effects of oral ABA treatment. ABA increased glucagon-like peptide-1 (GLP1) levels in wild-type (WT) mice, stimulated GLP1 secretion from L-cells, and increased plasma GLP1 levels in rats (1). However, the loss of LANCL2 results in increased plasma insulin levels and decreased GLP1 levels in acute high fat feeding study.

BTI Secures GRAS for ABA. An independent expert panel using scientific procedures (U.S. FDA, 1997) determined that ABA, intended for use in certain foods within the general categories of functional beverages, foods and fruit juices, at a level of up to 95 µg/6-12 fl oz twice a day, is (i) exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act and (ii) is GRAS for such use. Given that ABA is naturally occurring in food, oral exposure to ABA at food comparable levels is not expected to cause toxicological effects. Consistent with its nature as a food component, ABA was well-tolerated and there were no signs of toxicity in several dietary studies with ABA in mice, rats, and humans. ABA was negative in an in vitro reverse mutation assay in bacteria, an in vitro chromosome aberration assay in Chinese hamster ovarian cells and an in vivo mouse micronucleus assay. ABA is not a developmental toxicant in rats. A two-generation reproduction study shows that ABA: 1) does not cause detriment to the reproduction rate or efficacy in rats and 2) does not cause numbers or follicle counts to regress. In sub-chronic toxicity studies following a 4-week and a 13-week dietary intervention with different concentrations of ABA in rats, no adverse toxicological effects were seen for 90 d at intakes of up to 20,000 ppm (around 1,500 mg/kg bw/d). Therefore, ABA has a no observable adverse effect level (NOAEL) of 20,000 ppm which corresponds to 1,500 mg/kg body weight in mice. ABA has an acute oral median lethal dose (LD_{50}) of more than 5,000 mg/kg body weight in mice. Furthermore, ABA is recognized as a substance in which a maximum permissible level is not applicable as no signs of toxicity have been observed in a multitude of toxicity studies. Pharmacokinetic (PK) analyses of ABAp levels following oral and i.v. administration demonstrate that ABA is systemically distributed has high bioavailability (BA). Specifically, a BA study comparing the area under the curve of plasma concentrations after oral and I.V. administration of 10 µg/kg gave a BA of >65%. Based on this PK profile of ABA, we determined the effects of one versus two doses of ABA daily. The latter provided superior glucose normalizing effects in db/db mice. Our ABA source is extracted from figs has shown efficacy and safety in mice and humans.

The <u>scientific impact</u> of this project is to develop the first ABA-containing medical food for glycemic control. We will conduct the translational and clinical studies that will provide the scientific basis for qualified health benefits of ABA in glycemic control.

Study Objectives

Primary Objective/Aim/Goal/Hypothesis

The primary objectives are to assess the tolerability (number of adverse events) and efficacy of ABA on insulin sensitivity compared to placebo.

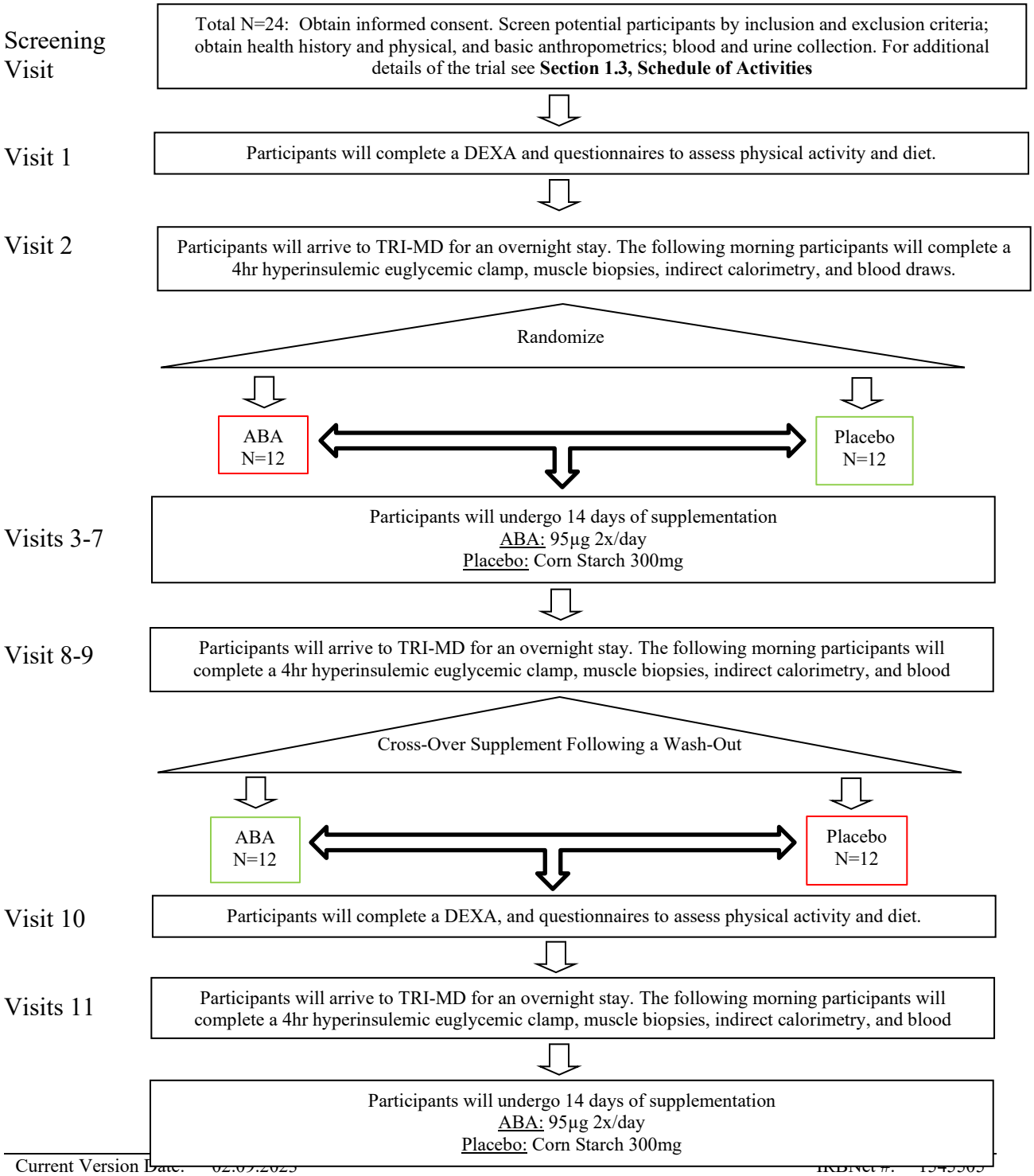
Secondary Objective/Aim/Goal/Hypothesis

Secondary objectives are to assess changes in insulin stimulated signaling and glucose transporter proteins in muscle biopsies, along with insulin levels, glucose, pyruvate, and fatty acid oxidation in skeletal muscle tissue samples, inflammatory markers and fasting ABA concentrations in plasma.

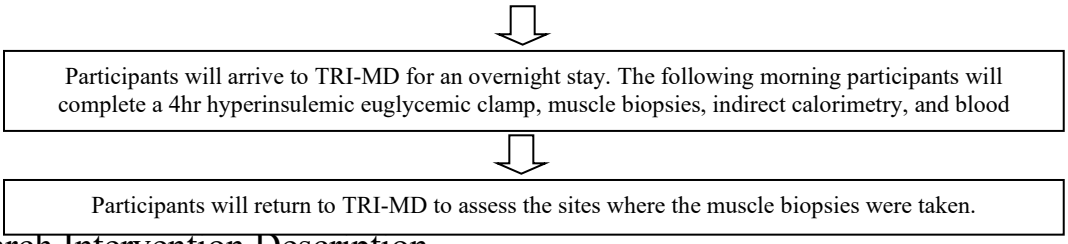
Study Design

Research Design

We propose to conduct a randomly assigned cross-over double-blind placebo-controlled trial (RCT) to evaluate the tolerability of ABA supplementation and efficacy of ABA to increase insulin sensitivity in subjects with prediabetes and T2D. Subjects will be randomly assigned to one of two groups: (1) ABA (95 ug 2x/day), or (2) Placebo (Corn Starch 300 mg) for 14 days. Subsequently, subjects will take part in a 4-5 week washout period then cross over and complete another 14-day treatment in the opposite study arm.


Screening Visit
Total N=24: Obtain informed consent. Screen potential participants by inclusion and exclusion criteria; obtain health history and physical, and basic anthropometrics; blood and urine collection. For additional details of the trial see **Section 1.3, Schedule of Activities**
Visit 1
Participants will complete a DEXA and questionnaires to assess physical activity and diet.
Visit 2
Participants will arrive to TRI-MD for an overnight stay. The following morning participants will complete a 4hr hyperinsulemic euglycemic clamp, muscle biopsies, indirect calorimetry, and blood draws.
Randomize
ABA N=12
Placebo N=12
Visits 3-7
Participants will undergo 14 days of supplementation
ABA: 95µg 2x/day
Placebo: Corn Starch 300mg
Visit 8-9
Participants will arrive to TRI-MD for an overnight stay. The following morning participants will complete a 4hr hyperinsulemic euglycemic clamp, muscle biopsies, indirect calorimetry, and blood
Cross-Over Supplement Following a Wash-Out
ABA N=12
Placebo N=12
Visit 10
Participants will complete a DEXA, and questionnaires to assess physical activity and diet.
Visits 11
Participants will arrive to TRI-MD for an overnight stay. The following morning participants will complete a 4hr hyperinsulemic euglycemic clamp, muscle biopsies, indirect calorimetry, and blood
Participants will undergo 14 days of supplementation
ABA: 95µg 2x/day
Placebo: Corn Starch 300mg

Visits 12-16



Research Intervention Description

Pharmacologic Intervention

This is a Phase 2 single-site study. The study population will include males and females of all races and ethnicities,18-75 years of age from the greater Orlando metropolitan areas who are pre diabetic (fasting glucose 100-125mg/dl/ 5.5-6.9mmol) and/or diagnosed T2D.

This is a randomly assigned cross-over double-blind placebo-controlled trial (RCT) design. As such, subjects will be randomly assigned to one of the groups (ABA or placebo) for 14 days, then after a 4-5 week washout period, will complete another 14-day treatment in the opposite study arm.

Subjects in the ABA study arm will be supplemented with ABA at a dose of 95 ug twice per day. ABA is a GRAS (generally recognized as safe) ingredient being developed for inclusion within health foods designed for improved glucose homeostasis. The placebo group will consume a control supplement (corn starch) 300 mg twice per day.

Drug preparation

Both study products (ABA and placebo) used for this trial will be packaged and clinically labeled by Biotherapeutics, Inc. The control and experimental products will be identified using a unique study number for randomization purposes, however all parties involved in the study will remain blinded. Both products will be delivered to the Research Pharmacy at the TRI.

Purified ABA is a white, crystalline solid. ABA may be isolated and enriched from fruits and vegetables, particularly apricots and figs. ABA is a small isoprenoid compound of 264.32 g/mol molecular weight. It has a boiling point of 160-161°C. ABA presents no health, fire or reactivity hazards and is recognized as safe for consumption by the FDA. ABA and placebo will be administered as a powder in capsules.

Study products are to be stored in an access-controlled environment at ambient temperature under conditions suitable for food storage (i.e., sanitary conditions, off the floor and organized to minimize the risk of product dispensing errors). The investigator agrees not to supply study product to any person not enrolled in the study or to any physician or scientist not named as an investigator. The investigator also agrees to use the study product only within the context of the

study protocol. Subjects will be asked to return any study product remaining after the completion of the study to the investigator.

Biotherapeutics, Inc will review the investigator's product accountability records. The investigator will return to Biotherapeutics, Inc or destroy [render product unusable by destroying primary packaging (i.e. packaging in direct contact with product)] all unused product after all study supply inventories have been reconciled at the conclusion of the study.

This study is a double blinded study. Neither the investigator(s), their staff, appropriate member of Biotherapeutics, Inc. staff, or subjects will be informed of the identity of any of the study products during the clinical portion of the study. The study center personnel will not analyze the contents of the study products or in any way seek to learn the identity of the study products. The investigator should ensure that if it becomes necessary, blinding is broken only in accordance with the protocol.

Each subject should consume two doses of product per day. Product will be administered and/or distributed by study staff and recorded on product consumption forms by study staff and subjects.

Study Site(s)/Location(s) and Number of Subjects

AdventHealth Orlando site locations (campus, physician offices, etc): Translational Research Institute 301 E. Princeton St., Orlando, Florida 32804
Estimated number of subjects at AdventHealth Orlando sites: 24

Total number of all sites: 1
Estimated number of subjects at all sites combined: 24

Multi-Site Research Logistics/Communication Plan

N/A

Research Conducted in a Foreign Country

N/A

Community-Based Participatory Research

N/A

Subject Selection

Vulnerable Populations (if applicable)

AdventHealth Employees: Recruitment efforts will follow AdventHealth recruitment Standard Operating Procedures (SOPs) for research. AdventHealth employees will not be individually targeted nor excluded from study participation based on employment. AdventHealth employees who engage the AdventHealth Translational Research Institute asking to participate in the study will be processed per standard consent procedures for participants. In addition, during the consent process, the study staff will review standard consent language stating that an employee's participation or lack of participation in the study will not affect their employment status or relationship with AdventHealth.

Inclusion Criteria

1. Provision of signed and dated informed consent form
2. Stated willingness to comply with all study procedures and availability for the duration of the study
3. Male or female, aged 18-75
4. In good general health as evidenced by medical history
5. Fasting glucose >/= 5.6 mmol/L (100 mg/dL) and/or HbA1c >/= 5.7
6. Ability to take oral medication and be willing to adhere to the study intervention regimen
7. Females currently on hormone replacement therapy (HRT) can participate in the study if they have been on HRT for at least 6 months and will continue to be on HRT during the study.
8. For males of reproductive potential: use of condoms or other methods to ensure effective contraception with partner
9. Agreement to adhere to Lifestyle Considerations throughout study duration.

Exclusion Criteria

1. 19<BMI>45.0 kg/m^2
2. Currently taking insulin
3. Blood pressure (BP) ≥ 150mmHg systolic and ≥95 mmHg diastolic
4. Current use of medications or antioxidant vitamins or supplements that would impact dependent variables, including glucose metabolism.
5. Past or current ischemic heart disease, stroke, respiratory disease, neurological disease, hematological-oncological disease and free of recent infection (prior 2 weeks)
6. Pregnancy or lactation
7. Treatment with another investigational drug or other intervention within 1 year.
8. Current smoker or tobacco use within the past year.
9. Disqualifying findings on physical examination include cardiac murmurs, diminished pulses or the presence of bruits in the lower extremities, lower extremity thrombophlebitis, evidence of peripheral neuropathy, paresis or edema.

10. History of a corn allergy.

Resources Available

We attest that all AdventHealth Translational Research Institute faculty and staff will be trained, and this training will be documented as described in AdventHealth Translational Research Institute Work Instruction 031.100.015 Documentation of Protocol Training.

We will implement regular, ongoing discussions between the PI and coordinator as per the AdventHealth Translational Research Institute SOP 030.000.002 Oversight of Research Studies at the Translational Research Institute. The coordinator will review source and communicate with all applicable study team members involved in the study on a regular basis regarding reportable new information, implementing amendments, study progress, and quality assurance/control.

With our internal database, electronic medical records and direct to consumer advertising we expect to recruit the required number of suitable participants within our planned enrollment period of approximately 24 months. The AdventHealth Translational Research Institute facilities are state of the art and we have within our building all required resources and staff to execute the study. We have a medical oversight team, Medical Oversight Committee, as well as a Quality Committee to appropriately monitor and address adverse events.

Study Procedures

Subject Recruitment and Screening

The study population will include males and females of all races and ethnicities,18-75 years of age from the greater Orlando metropolitan areas who are pre diabetic (fasting glucose 100-125mg/dl/ 5.5-6.9mmol and/or HbA1C >5.7 and <6.5%) or diagnosed T2D.

Recruitment methods utilized may include, but will not be limited to, the following: recruitment from within the AdventHealth TRI patient population, electronic medical records and database searches (including third party recruitment vendors); advertising in multiple media such as print ads, flyers, brochures, posters; radio ads; television spots; and internet/social media advertising. All advertising materials will be submitted to the AdventHealth Orlando IRB for review prior to using or publishing them. Recruitment efforts will follow AdventHealth Orlando Translational Research Institute Recruitment SOPs for research.

Participants can be re-screened and repeat screening laboratory measurements can be completed at the discretion of the PI or Sub-I.

Consent Process

We attest that all study staff delegated the authority to obtain informed consent will follow **"**Informed Consent Process and Written Documentation of Informed Consent" (SOP401.116).

Potential subjects will be given time to read the consent document and will be interviewed by a clinical coordinator. The coordinator will give a detailed overview of the study and then ask if the potential subject has questions and if they might be interested. The candidate is then encouraged to ask questions. After all questions have been addressed and interest remains, the candidate then signs the consent if he/she chooses to do so. After the subject has signed the consent, inclusion and exclusion criteria will be reviewed. The new subject is given a photocopy of the signed consent form for his/her records. The PI/Sub-I will review all participant Inclusion Exclusion Worksheets. Once deemed eligible, the PI/Sub-I will sign and date the Inclusion Exclusion Worksheet. Participants will be free to withdraw at any time for any reason without consequence.

Subjects who are not yet adults (infants, children, teenagers)

N/A

Cognitively Impaired Adults

N/A

Adults Unable to Consent

N/A

Documentation of Informed Consent Process

Documentation of the informed consent process is required to establish that the subject was accurately and adequately informed and that no study-related procedures were initiated prior to obtaining informed consent. A research team member will note in the source documentation the consent process, date consent was obtained, and that consent was obtained prior to initiating any research procedures.

Waiver of Written Documentation of Consent or Waiver of Consent

Waiver of Written documentation of Consent (consent will be obtained but signatures will not be required)

N/A

Waiver or Alteration of the Consent Process (consent will not be obtained, required information will not be disclosed, or the research involves deception)

N/A

Non-English Speaking Subjects

The participant population is primarily English speaking. In the unlikely event that a non-English speaking participant meets criteria for enrollment, then IRB Short Form procedures will be initiated with the assistance of a Qualified Medical Interpreter, or Video Remote Interpretation will be utilized when there is a need of sight translation. A Qualified Bilingual Staff (QBS), or if not available, a Qualified Medical Interpreter or Video Remote Interpretation will be utilized if only interpreting for another staff member is required.

Randomization

This trial will have a triple-masked design, so that the investigators, study staff and the participants are masked to the drug intervention assignment. Randomization will be performed at each site by the investigational pharmacy based upon a randomization structure provided by the study biostatistician. The randomization scheme will be concealed to investigators and study staff until the completion of the study. Randomization will be performed 1:1.

Only under emergent circumstances and with all appropriate approvals (i.e. study physician and/or DSMB) will the blinding be broken regarding the treatment group. If the request to unblind is granted, the ID, the reason for unblinding, the study staff member who performs the unblinding and a list of the persons who are not blinded will be recorded.

Study Visits

All study visits will be conducted at the AdventHealth Translational Research Institute.

		Baseline Period			**Intervention Period**					**Follow-Up Period**		**Drug Washout Period**	
Visit	SV1	V1	V2		V3	V4	V5	V6	V7	V8		V9	
Day	D-28	D-9	D-2	D-1	D0	D2	D5	D8	D11	D13	D14	D15	D15-43
Windows	Within 7 days of Visit 1	±7			±1	±1	±1	±1	±1			±1	±7
HIPPA	X												
Informed Consent	X												
Assessment of Eligibility	X												
Vital Signs[a]	X	X	X	X	X	X	X	X	X	X	X	X	
Weight	X	X	X	X	X	X	X	X	X	X	X	X	
Height	X												
Waist Circ	X												
Screening Blood Draw[b]	X												
Urinalysis[c]	X												
Review AEs and Con Meds	X	X	X	X	X	X	X	X	X	X	X	X	
Med Hx, Physical	X												
DEXA		X											
Physical Activity Questionnaire[d]		X											
Physical Activity Monitor On[e]		X											
Physical Activity Monitor Off			X										
4d Diet Recall		X											
Overnight Stay			X							X			
Hyperinsulemic Euglycemic Clamp[f]				X							X		
RMR[g]				X							X		
Muscle Biopsies[h]				X							X		
Blood Draws[i]				X							X		
Randomization				X							X		
Post Procedure Lunch				X							X		
Biopsy Check					X							X	
Drug Washout[j]												X	
Drug Distribution[k]					X	X	X	X	X				
Drug Compliance					X	X	X	X	X				
Issuance of Stipend					X						X		

	Crossover Baseline Period			Crossover Intervention Period					Follow-Up Period		
Visit	V10	V11		V12	V13	V14	V15	V16	V17		V18
Day	D44	D52	D53	D54	D56	D59	D62	D65	D67	D68	D69
Windows	±7			±1	±1	±1	±1	±1			±1
HIPPA											
Informed Consent											
Assessment of Eligibility											
Vital Signs[a]	X	X	X	X	X	X	X	X	X	X	X
Weight	X	X	X	X	X	X	X	X	X	X	X
Height											
Waist Circ											
Screening Blood Draw[b]										X	
Urinalysis[c]										X	
Review AEs and Con Meds	X	X	X	X	X	X	X	X	X	X	X
Med Hx, Physical[d]											
DEXA[e]	X										
Physical Activity Questionnaire[f]	X										
Physical Activity Monitor On[g]	X										
Physical Activity Monitor Off		X									
4d Diet Recall[h]	X										
Overnight Stay		X							X		
Hyperinsulemic Euglycemic Clamp[i]			X							X	
RMR[j]			X							X	
Muscle Biopsies[k]			X							X	
Blood Draws[l]			X							X	
Randomization			X							X	
Post Procedure Lunch			X							X	
Biopsy Check				X							X
Drug Washout											
Drug Distribution				X	X	X	X	X			
Drug Compliance				X	X	X	X	X			
Issuance of Stipend				X						X	

Screening Visit; Day -28

Participants will arrive at the clinic in the morning of the screening visit (~7-8 AM) following a ≥ 10-hour overnight fast. Participants will be encouraged to stay hydrated prior to this visit. The following is a list of the tests the participants will undergo after signing the Informed Consent Form as part of the screening procedure prior to participation in the protocol. Any participant who does not match the listed inclusion criteria or has any of the listed exclusion criteria (see above) will not be eligible to participate in the study:

[a]Vital Signs: Blood pressure and pulse rate, respiration rate, temperature

Height

Weight

Waist Circumference

[b]Screening Blood Draw: Complete hemogram (RBC, Hb, MCHC, Htc, MCW, WBC, PLT), comprehensive chemical chemistry profile (BUN, creatinine, ALT, AST, ALP, GGT, bilirubin, plasma protein electrophoresis, fasting glucose, triglycerides and cholesterol, ions and anion gap), and HgbA1C.

[c]Complete Urinalysis

Review Adverse Events and Concomitant Medications

[d]Medical History/Physical Examination:

- Participant history: Demographics including age, marital status, education, ethnic group, employment status, smoking history (if applicable), and past medical history will be collected on a participant history form
- Medication and Supplement Use

Visit 1 (Day -9)

Vital Signs

Weight

Review AEs and Concomitant Medications

[e]DEXA: DEXA Scans will be performed to measure body fat and estimate muscle mass using a GE Lunar iDXA whole-body scanner. The participant will remove all metal accessories and may be asked to change into a hospital gown. The participant will lie on the DEXA table while the scanner arm emits low energy X-rays as it passes along the body. The scan takes up to 15 minutes and the radiation dose is less than 1 mrem, less than half the average daily radiation dose in America. A urine pregnancy test will be completed on all women of childbearing potential (all women except those with prior hysterectomy, tubal ligation, or absence of menses for $\geq$2 years) prior to the DEXA scan for safety.

[f]Physical Activity Questionnaire: International Physical Activity Questionnaire (IPAQ)

[g]Physical Activity Monitor On: ActiGraph Monitor. **Physical Activity Level** will be measured objectively using an accelerometer (GT3X-BT, Actigraph Inc.) on both the wrist and waist for the entire duration of the study to assess baseline physical activity and to account for any changes in physical activity during the trial. When/if limited availability of accelerometers arises, one accelerometer will be worn on the wrist.

[h]4-day Diet Recall: **Habitual Dietary Intake** including average total calorie and macronutrient intake will be obtained prior to randomization from detailed 4-d diet records. Subjects will be instructed on procedures for weighing and recording food intake on 3 weekdays and one weekend day. The ProNutra (version 3.5.0.1) will be used to calculate energy intake and macronutrient composition.

Unscheduled Clinic Visit (2-5 days prior to Visit 2):

- COVID-19 Nasal Swab to be completed prior to next visit.

Visit 2 (Day -2)

Vital Signs
Weight
Review AEs and Concomitant Medications
Physical Activity Monitor Off
Overnight Stay: Participants will be admitted to the Clinical Research Unit (CRU) in the evening and given a standard dinner of 7 kcal/kg consisting of 50% carbohydrate, 20% protein and 30% fat.

Visit 2 (Day -1)

Vital Signs

Weight

Review AEs and Concomitant Medications

[i]Hyperinsulinemic Euglycemic Clamp: Following a 12-hour overnight fast at the CRU (including abstinence from caffeine containing foods and beverages), insulin sensitivity will be measured using the criterion method of the euglycemic clamp. Participants will have not performed vigorous physical activity for at least the previous 24 h, and be free of acute illness for the prior 2 weeks. Subjects will undergo key laboratory visits at baseline and at prescribed time points during the dietary intervention. All female participants will be eumenorrheic and will begin the study during the early follicular phase (low estrogen and progesterone) of the menstrual cycle to minimize the impact of menstrual phase on substrate metabolism. An intravenous catheter will be placed in an antecubital vein for subsequent insulin (40 mU/m^2-min) and glucose infusions and for stable isotope (6, 6-$2H^2$) infusions to measure insulin sensitivity. Insulin and FFA samples will be drawn at various time points throughout the clamp.

[j]RMR: Taken at baseline and last 30 minutes of the clamp using the AEI system.

[k]Muscle Biopsy: One biopsy taken prior to clamp and a second biopsy taken 30-45 min after the start of the clamp. A biopsy of the Vastus Lateralis muscle will be performed on the leg/thigh using the Bergstrom technique. Subjects will be placed in a supine position and the skin will be cleansed with chlorhexidine solution. After a sterile drape is placed over the skin, local anesthesia will be administered using 2% lidocaine. The skin will be incised (approximately 0.75 cm) with a #11 scalpel, and the Bergstrom needle will be inserted into the Vastus Lateralis. Approximately 220 mg of muscle tissue will be obtained under suction. After the biopsy, pressure will be applied to stop bleeding and the skin will be closed with Steri strips (suture(s) if allergy to steri strips). A sterile dressing will be applied. An MD, DO, NP, or PA will conduct this procedure.

Muscle specimens will be trimmed of adipose and connective tissues using a dissecting microscope, and analyzed as follows:

- *High-Resolution Respirometry.* Mitochondrial respiratory capacity will be measured in permeabilized muscle fibers from vastus lateralis muscle biopsies (Oroboros Oxygraph 2K, Innsbruck, Austria). The instrument has two chambers, which permits high-resolution respiration measurements to be made in duplicate with low amounts of muscle tissue (2mg). The instrument employs a polarographic oxygen sensor with a high signal to noise ratio and accurate steady-state measurement of O2 levels and respiration with resolution > +/- 1 nmol O2 per liter. The O2K assay allows a comprehensive evaluation of mitochondrial respiratory function upon titration with ETC complex-specific inhibitors and substrates with both carbohydrate and fat as substrates (25, 26).
- *Protein and Gene Expression.* Several proteins and genes related to insulin signaling (IRS, Akt, mTORC2, AS160, GLUT4) will be measured via Western blot quantification and Real-Time Quantitative PCR (RT-qPCR). Protein expression will be performed in duplicate in ~20 mg of the biopsy specimen obtained during both fasting and insulin-stimulated conditions. Optimal antibody conditions for each protein have been established using aliquots from human muscle maintained in our lab for such purposes.
- *Immunohistochemistry.* Histochemical analyses will be performed on serial sections using methods previously described (27). Histochemical analysis of IMCL (Oil Red O Staining), fiber-type and cross-sectional area will be measured in the fasted biopsy sample. In addition, LANCL2, GLUT4 and TXNIP translocation will be measured in sections from both the fasting and insulin stimulated biopsies (28).

[l]Blood Draws: Baseline blood to be taken prior to the clamp and taken throughout the clamp at -30, 0, 30, 60, 100, 110, and 120 min, plus every 10 min during the last 30 min of the clamp (220, 230 and 240 min) for the GCMS determination of the [6, 6-$2H^2$] glucose enrichment. Insulin and FFA samples will be drawn at various time points throughout the clamp.

Randomization

Post Procedure Lunch

Visit 3 (Day 0)

Vital Signs
Weight
Review AEs and Con Meds
Biopsy Check
Drug Distribution
Drug Compliance
Issuance of Stipend

Visit 4 (Day 2), Visit 5 (Day 5), Visit 6 (Day 8), Visit 7 (Day 11)

Vital Signs
Weight
Review AEs and Concomitant Medications
Drug Distribution
Drug Compliance

Visit 8 (Day 13)

Vital Signs
Weight
Review AEs and Concomitant Medications
Physical Activity Monitor OFF
Overnight Stay

Visit 8 (Day 14)

Vital Signs
Weight
Review AEs and Concomitant Medications
Hyperinsulinemic Euglycemic Clamp
RMR
Muscle Biopsies
Blood Draws
Randomization
Post Procedure Lunch
Issuance of Stipend

Visit 9 (Day 15)

Vital Signs
Weight
Review AEs and Concomitant Medications

Biopsy Check
Drug Washout

Visit 10 (Day 44)

Vital Signs
Weight
Review AEs and Concomitant Medications
DEXA
Physical Activity Questionnaire
Physical Activity Monitor On
4-day Diet Recall

Unscheduled Clinic Visit (2-5 days prior to next visit):

- COVID-19 Nasal Swab to be completed prior to next visit.

Visit 11 (Day 52)

Vital Signs
Weight
Review AEs and Concomitant Medications
Physical Activity Monitor Off
Overnight Stay

Visit 12 (Day 53)

Vital Signs
Weight
Review AEs and Concomitant Medications
Hyperinsulinemic Euglycemic Clamp
RMR
Muscle Biopsies
Blood Draws
Randomization
Post Procedure Lunch

Visit 12 (Day 54)

Vital Signs
Weight
Review AEs and Concomitant Medications
Biopsy Check
Drug Distribution
Drug Compliance

Issuance of Stipend

Visit 13 (Day 56), Visit 14 (Day 59), Visit 15 (Day 62), Visit 16 (Day 65)

Vital Signs
Weight
Review AEs and Concomitant Medications
Drug Distribution
Drug Compliance

Unscheduled Clinic Visit (2-5 days prior to next visit):

- COVID-19 Nasal Swab to be completed prior to next visit.

Visit 17 (Day 67)

Vital Signs
Weight
Review AEs and Concomitant Medications
Overnight Stay

Visit 17 (Day 68)

Vital Signs
Weight
Screening Blood Draw
Urinalysis
Review AEs and Concomitant Medications
Hyperinsulinemic Euglycemic Clamp
RMR
Muscle Biopsies
Blood Draws
Physical Activity Monitor OFF
Post Procedure Lunch
Issuance of Stipend

Visit 18 (Day 69)

Vital Signs
Weight
Review AEs and Concomitant Medications
Biopsy Check

Measuring ABA in plasma. Plasma will be isolated from blood samples collected from fasted subjects prior to the clamp and stored at –80 °C. Blood samples will be sent to BioTherapeutics

Inc. after obtaining the Material Transfer Agreement, which will govern the transfer and chain of custody of the biospecimens outside of AH. Investigators at BioTherapeutics Inc. will measure ABAp as described (3), samples will be spiked with d-ABA (internal standard) in methanol, stored at –20 °C for 24 h, centrifuged for 10 min at 2,000 g and concentrated in a rotovaporator. The residue will be redissolved in DI water with the pH adjusted to ~2.5 with trifluoroacetic acid. Three extractions with diethyl ether will be performed; the ether layer will be collected and concentrated; the residue will be dissolved in DI water. Samples will be analyzed by LC/MS to quantify ABA levels.

Study Duration

All research visits will occur at the AdventHealth Translational Research Institute, Orlando. The project is planned to begin in October 2020, initiated by the recruitment of participants. For each participant undergoing the whole study protocol (including enrollment, following all the visits and related procedures), the total study duration is approximately 69 days (10 weeks). The estimated duration of the whole study completion including recruitment, clinical protocol, data collection, sample analyses, statistical analysis is about 2 years.

Materials of Human Origin: Collection, Preparation, Handling and Shipping

Materials of human origin will be collected in the manner described in the specific study visits section of this protocol.

Biospecimen samples will be stored in ultralow temperature freezers and liquid nitrogen drawers or other storage units located at the TRI Laboratory Room 2404. The TRI facility is secured via key card and equipped with a back-up generator system. Laboratory personnel in the facility have 24/7 key-controlled access to the laboratory. Chain of custody of biospecimen samples is maintained through requisition forms and in the StarLIMS database. Specimen tubes are coded, and specimen requests and distribution are documented.

Muscle Biopsy Specimens. Muscle specimens will be trimmed of any visible adipose and connective tissue using a dissecting microscope and processed and assays will be run as previously described. Any remaining tissue will be flash frozen in liquid nitrogen for future biochemical, molecular or genetic analyses.

The muscle biopsy specimens and plasma/serum samples will be stored indefinitely, or until a sample is fully used. All biological specimens will be stored without identifiers or linkage codes.

Biospecimens collected will be stored at AdventHealth TRI until shipment to outside laboratories/institutions. Laboratories that will analyze de-identified samples may

include but is not limited to BioTherapeutics Inc. and Quest Laboratory. When testing and analysis cannot be conducted at AdventHealth Orlando and will be conducted at outside laboratories, a Materials Transfer Agreement will be obtained. The agreement will govern the transfer and chain of custody of the biospecimen to outside entities for testing and analysis.

After study aims have been achieved and study related endpoints have been measured and analyzed, any remaining biospecimens will be stored at the TRI Laboratory Room 2404 and will then be considered as "archived biospecimens." Archived biospecimens will be used for any additional hypothesis-related experimentation or testing for the purposes of this study, consistent with the original aims, which cannot be predicted at the time the protocol is developed due to the evolving nature of scientific exploration.

Additionally, archived biospecimen samples may be stored indefinitely for future research.

Archived biospecimens could be used for separate research by both AdventHealth scientists and scientists outside of AdventHealth. This would be allowed for research of any type (without limitation to disease, process, or research methods) if it has scientific merit as determined by the Principal Investigator, with an additional review by the respective Program Director. For research outside of AdventHealth, a Material Transfer Agreement will be obtained, which will govern the transfer and chain of custody of the biospecimens outside of AH.

Study Outcome Measures (Endpoints)

The primary outcome tolerability will be assessed via monitoring adverse events at each visit throughout the duration of the study. Additionally, before and immediately after the end of the trial the following examinations will be completed: complete physical exam, complete hemogram, comprehensive clinical chemistry profile, complete urine analysis. The second primary outcome (insulin sensitivity) will be measured using hyperinsulinic euglycemic clamp at visits 2, 8, 11, and 17.

Secondary outcomes, including skeletal muscle biopsy-derived assessments and plasma-derived inflammatory, ABA and glucose control concentrations will be measured at visits 2, 8, 11, and 17.

Data Management and Quality Plan

Data De-identification

Participants will be enrolled using Cerner's Patient Protocol Manager; the application assigns each participant a unique participant identifier, or "PID". This PID is a code consisting of a combination of numerals and letters, which serves as the identifier for this participant for this research study and links them back to their hospital medical record and their protected health information (PHI). Access to the "link" between the PIDs, the PHI, and to the clinical data are only granted to the clinical research team as assigned on the Delegation of Authority Log. All the clinical research data is recorded in a de-identified fashion onto our paper source documents, which is then transcribed into our electronic case report forms, (CRF). The CRF is used for storage (a database) and facilitates analysis. Clinical data generated by research devices also uses the PID, and once the data has been transformed into interpretable results it is stored into the clinical research database. Both storage locations are secured and only assessable to the assigned clinical research team. The "link" will not be used to re-identify participants except in the event of a serious adverse event (SAE) requiring "unblinding" to treat the participant. The "link" will be stored in the Patient Protocol Manager and in the clinical research database, where only the AdventHealth Translational Research Institute research team has access. These secure databases are stored/accessed on the AdventHealth password-protected computer network. No one outside of AdventHealth investigators or researchers will have access to the databases.

Data Confidentiality, Storage, and Retention

The identity and personal health information will be kept confidential to the extent permitted by the applicable laws and/or regulations and will not be made publicly available. If results of this study are published or presented, the identities will not be revealed. Confidentiality will be maintained during and after the study. This information is also included in the informed consent, which is discussed with participant prior to enrollment.

Study documentation and paperwork will be stored in our locked medical records room. The data records will also be stored in as electronic records. This data will be safeguarded so that only those on the research team have access to any of the clinical data (both source documentation and data warehouse storage). The electronic data is maintained by Adventist Information Technology (AIT) security controls.

The duration of study data retention will be determined by governing FDA regulations and/or sponsor contract mandate (if applicable). AdventHealth Translational Research Institute retention policy is maintained in the Records Management Policy. Electronic de-identified data will be kept indefinitely in our data warehouse.

Per the institutional policy, investigator records must be kept for a minimum of 7 years after completion of discontinuation of the study, or for longer if required by applicable local regulations, or what is applicable per the Sponsor.

Data Quality

Data quality control will occur according to our SOPs on Data Entry, Quality Control Procedures and Query Management. All data will be entered into an electronic data capture (EDC) system and checked against the paper source for accuracy by a second party (Data Entry SOP) and errors resolved through the Query Management SOP. Ten percent of the data points will be routinely checked at the beginning, middle, and close of a study for quality control (Quality Control SOP). Finally, all critical endpoints (as determined by the PI or Sub-I) will be assessed using quality control analyses. The data will be loaded into the clinical research database. Data in the warehouse will also be routinely monitored over time.

Data Sharing (outside of AdventHealth Orlando)

Sharing of anonymized data with other organizations would be allowed, as defined in respective Agreements, for research of any type if the research has scientific merit as determined by the TRI Scientific Review Committee.

Some of the endpoint testing will be conducted at outside laboratories/institutions. To perform these analyses/testing/etc. and to interpret results, certain data elements will need to be shared along with the biospecimen samples. Data Use Agreements will be obtained, which will identify the specific data elements to be shared and will govern the sharing of data related to this study. Data will be de-identified, but a link/code is managed within an electronic research management system and maintained by a study coordinator.

Should archived biospecimens be needed for research outside AdventHealth Orlando and certain data elements that are connected to the archived biospecimen samples are needed to conduct the research, then Data Use Agreement(s) will be obtained. The Data Use Agreement(s) will identify the purpose for data sharing, the specific data elements to be shared, and will govern the sharing of data related to this study. Data will be de-identified, but a link/code is managed within an electronic research management system and maintained by a study coordinator.

Sample Size Determination

Power analyses has been conducted for repeated measures ANOVA. Based on published findings (3), we presume a minimum mean difference between the two treatments of 10.3 with a SD of 11.3 and 11.9 at each of the data collection points. The correlation among the repeated measures is expected to be 0.8. Results of these simulations indicated that at alpha=0.05, a sample size of 24 per treatment will achieve >80% power. We believe it is prudent to plan for some level of dropping

out. We will conservatively plan for a 20% dropout and recruit 30 total participants. We will continue to recruit and study subjects to achieve our desired sample size if dropouts exceed this estimate.

Statistical Analysis Plan

Analysis datasets will include:

- Intention-to-Treat (ITT) Analysis Dataset (i.e., all randomized participants)
- Modified Intention-to-Treat Analysis Dataset (e.g., participants who took at least one dose of study intervention and/or have some particular amount of follow-up outcome data)
- Safety Analysis Dataset: defines the subset of participants for whom safety analyses will be conducted (e.g., participants who took at least one dose of study intervention)
- Per-Protocol Analysis Dataset: defines a subset of the participants in the full analysis (ITT) set who complied with the protocol sufficiently to ensure that these data would be likely to represent the effects of study intervention according to the underlying scientific model (e.g., participants who took at least 80% of study intervention for 80% of the days within the maintenance period)
- Other Datasets may be used for sensitivity analyses

Prior to all analyses, the assumption of normality will be checked using histograms and normal probability plots. If normality is questionable, the nonparametric Wilcoxon rank sum test will be used. Multiple comparisons will be adjusted using Holm–Bonferroni method step down method.

Primary Objective Analysis

All analyses will be conducted using SAS or R package. We will begin by testing for any order effects in the randomized sequences (ABA vs. placebo) by using a two-sample t-test or non-parametric rank sum test (i.e. H_0: $\mu_{AB} - \mu_{BA} = 0$). If the order effects are insignificant, data will be pooled from both arms to estimate treatment effects. We will use a multiple-sample repeated measures mixed model with a between-subject factors approach. Because the data are repeated, we will treat the multiple observations as nested within individuals. This will allow us to directly compare time points while accounting for the correlation in the data to make the correct inferences regarding differences between two conditions. Here, with $h = 1, .., s$ groups, $i = 1, \ldots N_h$ subjects in group h and $j = 1, \ldots, n$ time points, the repeated measures model is: $\gamma_{hij} = \mu + \gamma_h + \tau_j + (\gamma\tau)_{hj} + \pi_{i(h)} + e_{hij}$, where μ = grand mean; γ_h = effect of group h; τ_j = effect of time j; $(\gamma\tau)_{hj}$ = interaction effect of time j and group h; $\pi_{i(h)}$ = individual difference component for subject i nested in group h, and e_{hij} = error for subject i in group h at time j. Confidence intervals and P-values will be adjusted for multiple comparisons of the individual effects.

Missing Data. We will use an intention-to-treat analysis as our primary analytic approach and rely on this analysis for interpreting the data. We will begin by examining the missing data patterns. Next, we will utilize maximum likelihood algorithms with the mixed linear model ANOVAs to longitudinally compare our outcomes across the three groups. Maximum likelihood algorithms estimations use all available data to construct weighted averages across the different patterns of missing data to provide valid point estimates and confidence intervals for population parameters as opposed to list-wise deletion. As a secondary analysis, we will conduct a completers-only analysis. However, in our experience both approaches yield similar conclusions.

Secondary Objective Analysis

Similar approaches will be uses as for primary endpoints.

Potential Risks and Benefits

Potential Benefits

Importantly, the proposed project might have direct, tangible benefits for participants. These benefits include information about their health. All study participants will be encouraged to communicate the results from the study to their primary care providers.

Potential Risks

Potential risks are those associated with health information privacy, venipuncture, ABA supplementation and radiation from DEXA.

Blood draws: There is a risk of pain, vasovagal syncope, hematomas, and/or infection at IV insertion/blood draw site (low risk of serious AEs).

ABA Supplementation: The risks presented to subjects are minimal, and information gained from this project will be useful in assessing how metabolism in prediabetes and T2D is influenced by nutrition. Abscisic acid is endogenously produced and consumed within the standard diet. Studies assessing the presence of abscisic acid within blood and tissue have identified nanogram amounts. We have determined ABA supplementation is generally recognized as safe (GRAS) in accordance with FDA guidance. The matching placebo supplementation is made using corn products, which could pose a mild risk to those participants that experience a corn allergy. Participants will be screened for corn allergies and excluded if appropriate.

DEXA Scan: There is a very small risk of cancer with excessive exposure to any radiation. There are also risks for unborn children associated with radiation exposure. The radiation dose from the scan is less than a chest x-ray, or less than half the average amount a person would receive in a day in America.

Muscle Biopsy. There is a risk of pain from the local anesthesia, and a risk of bruising, bleeding (hematoma) and infection at the site of the biopsy. In addition, there is a risk of skin irritation due to the steri strips and dressing. Local sensory loss may occur by cutting a subcutaneous sensory nerve (<1 in 100), which is almost always temporary but occasionally can become permanent. On rare occasion (<1 in 1000) bleeding, requiring hospitalization, may occur. The procedures are well tolerated when performed properly.

RMR (Resting Metabolic Rate): The measure of your metabolic rate using a ventilated hood carries no risk. The only adverse factor about this testing may be a feeling of claustrophobia. A member of the study staff will be at the bedside at all times and will check to see that you are comfortable. The transparent hood can easily be removed, if necessary

Activity Monitor: There are no anticipated risks with the activity monitor itself, however it is possible you may experience some minor skin irritation from the monitor strap. If you have allergies to metal (especially nickel) you will not be required to wear the monitor.

Glucose clamp: Minor risks of this procedure include bruising at the site of the needle insertion in your veins. The major risk of this procedure is low blood sugar due to the insulin given. Low blood sugar can make you feel nauseous, sweaty, irritable and sometimes confused. To prevent this, your blood sugar will be checked every 5-10 minutes, and you will be given glucose through the IV line if needed.

Mitigation of Risks

Blood draws (lab samples, e.g.). There is a risk of pain, vasovagal syncope, hematomas, and/or infection blood draw site (low risk of serious AEs).

Protection Against Risk:
All venipuncture will be conducted by qualified staff using aseptic techniques. Total blood volume throughout the entire study will be approximately 110 mls.

DEXA scan. There is a very small risk of cancer with excessive exposure to any radiation. There are also risks for unborn children associated with radiation exposure. The radiation dose from the scan is less than a chest x-ray, or less than half the average amount a person would receive in a day in America.

Protection Against Risk:
A urine pregnancy test will be done prior to scans of all women of childbearing potential (all women except those with prior hysterectomy, tubal ligation, or absence of menses for ≥2 years).

Muscle Biopsy. There is a risk of pain from the local anesthesia, and a risk of bruising, bleeding and infection at the site of the biopsy. In addition, there is a risk of skin irritation due to the steri strips and dressing.

Protection Against Risk:
Muscle biopsies will be conducted by qualified staff following institutional policies and procedures including sterile techniques and sterile dressing to the site. Prior to the biopsies, participants will be asked for allergies including to lidocaine and the use of anticoagulants. Subjects allergic to lidocaine or that used anticoagulants in clinically significant amounts will be excluded. Sutures will be used to close the site, for those with a known allergy to steri strips.

RMR (Resting Metabolic Rate): The measure of your metabolic rate using a ventilated hood carries no risk. The only adverse factor about this testing may be a feeling of claustrophobia. A member of the study staff will be at the bedside at all times and will check to see that you are comfortable. The transparent hood can easily be removed, if necessary

Protection Against Risk:
A member of the study staff will be at the bedside at all times and will check to see that you are comfortable. The transparent hood can easily be removed, if necessary

Activity Monitor: There are no anticipated risks with the activity monitor itself, however it is possible you may experience some minor skin irritation from the monitor strap. If you have allergies to metal (especially nickel) you will not be required to wear the monitor.

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Protection Against Risk:
To prevent this, your blood sugar will be checked every 5-10 minutes, and you will be given glucose through the IV line if needed.

Again, written informed consent will be obtained after explanation to subjects about all procedures and time commitments. The study interviewers will explain to prospective participants the purpose, methods and extent of the study. Potential participants will be asked to read the informed consent form and to ask questions. The form will be written in simple, easy-to-understand language. Staff members will also review all key aspects of the study verbally. Confidentiality of data will be maintained by using research identification numbers that uniquely identify each individual. Data will be used only in aggregate and no identifying characteristics of individuals will be published or

presented. Finally, the study protocol has been registered at www.ClinicalTrials.gov (NCT#03295734) and will be updated as required.

Provisions to Protect the Privacy Interest of Subjects

Subjects will be assigned unique identifiers for study-related records. All precautions will be taken to make sure that only authorized individuals will access subject research records. Consenting, eligibility criteria, research intervention and procedures are conducted in a private room. The collection of personal and sensitive information is limited to only necessary information. Private health information will be collected, accessed, and stored according to HIPAA guidelines. Data on subjects will be kept in locked areas and/or in secure research databases to ensure confidentiality. Blood and tissue specimens, when applicable, will be bar coded in research storage freezers. Code numbers will be assigned to patients for data entry purposes, and the data will be kept on a password protected server.

Early Withdrawal of Subjects

Investigator Withdrawal of Subjects

The participation in this study may be stopped at any time by the study PI without the participant's consent because:

- The study Medical investigator thinks it necessary for subject's health or safety;
- Participant has not followed study instructions;
- The AdventHealth Translational Research Institute has stopped the study; or
- Administrative reasons require the participant's withdrawal.

Subject Request for Withdrawal from Study

Participation in this study is voluntary. Participants may decide not to participate in this study or may withdraw from this study at any time without penalty or loss of benefits. If a participant leaves the study before the final regularly scheduled visit, she/he may be asked by the study doctor to make a final visit for some 'end-of–study' procedures. This is to make sure that there are no safety concerns.

Data Collection and Follow-up for Withdrawn Subjects

Participants who request withdrawal or who are withdrawn by the PI from the study will have their data maintained in the research database up to the point of withdrawal. The available data will be included in subsequent analysis because a participant may have withdrawn due to possible drug side effects (if applicable) and keeping these participants in the analysis is essential for study validity.

Adverse Event Reporting

An adverse event (AE) is defined as both an expected side effect that is of a serious nature, or an unexpected side effect/event regardless of severity. Each participant is evaluated for adverse events at every study visit. Any event that is reported to the study staff and which meets the criteria of an adverse event will be documented as such and graded as to its attribution (unrelated to protocol, or possibly, probably, or definitely related to protocol) and severity (mild, moderate, or severe). Any severe and/or unanticipated adverse event will be immediately reported to the IRB according to FH IRB guidelines.

At each contact with the subject, the study team will seek information on adverse events by specific questioning and, as appropriate by the Provider through examination. Information on all adverse events will be recorded immediately in the source document, also in the appropriate adverse event module of the case report form CRF. All clearly related signs, symptoms, and abnormal diagnostic procedures results will be recorded in the source document. All adverse and unexpected events will be reported according to AdventHealth IRB guidelines.

Adverse events will be documented and reported by the study coordinator, Medical Investigator or other TRI staff. Research and safety data will be reviewed by the PI. This review will take place at regular meetings with the research coordinator and Medical Investigator. Other items discussed will include progress or adverse events occurring in the following: participant confidentiality, participant recruitment, and consent process. All adverse events and unexpected and/or severe adverse events will be reported to the AdventHealth Orlando IRB. The TRI has a standing committee that meets monthly to review all adverse events in our clinical trials and will additionally be charged with review of the study
All adverse events will be reported according to AdventHealth IRB guidelines.

Safety Monitoring Plan

Safety Monitoring

Safety of participants is our primary concern. Prior to study entry, prospective participants will be advised to consult with their physician. Numerous safety procedures will be utilized to ensure participant safety.

First, many of the exclusion criteria are designed to exclude those at moderate to high risk. Participant education about safety begins with the consent process and continues throughout the study. Potential adverse events for study related activities and interventions are explained to each participant by trained study personnel during the informed consent process. Participants will be encouraged to notify study staff immediately if they have any adverse experiences that could be related to the study drug. Abnormal symptoms or vital signs detected during intervention visits will be discussed with the study physician in real time, who will dictate the appropriate course of action.

Center-based assessments and interventions will be conducted and supervised by trained staff who will monitor potential adverse experiences and symptoms. All assessors and interventionists receive CPR training and training on management of acute events including syncope, chest pain, acute dyspnea, focal neurological symptoms and abnormal vital signs. Portable defibrillators are available, and all study staff have on-call access to the study physician and contact numbers for emergency services are. Institutional and community EMS services will be activated if needed.

Any significant adverse events will be reported promptly to the IRB and to the Data Safety Monitoring Board (DSMB). Again, written informed consent will be obtained after explanation to subjects about all procedures and time commitments. The study interviewers will explain to prospective participants the purpose, methods and extent of the study. Potential participants will be asked to read the informed consent form and to ask questions. The form will be written in simple, easy-to-understand language. Staff members will also review all key aspects of the study verbally. Confidentiality of data will be maintained by using research identification numbers that uniquely identify each individual. Data will be used only in aggregate and no identifying characteristics of individuals will be published or presented.

The muscle biopsy procedure will be performed by a trained and experienced study healthcare provider. The muscle biopsy procedure will be performed under sterile conditions using aseptic techniques. Local anesthesia is obtained using subcutaneous lidocaine. The incision will be closed with a sterile suture and covered with a pressure dressing. The risk of the participants experiencing an allergic reaction is this injection is extremely low, and to reduce the risk of any potential adverse reactions occurring, participants will be queried prior to the procedure to verify that they do not have a known allergy to lidocaine.

Data and Safety Monitoring Board (DSMB) or Equivalent

Among the most comprehensive and effective method of monitoring risks in studies with relatively few subjects is a weekly and biweekly individual case that will be reviewed by the PI, one of the study physicians, and/or research coordinator including progress or adverse events occurring in the following: subject confidentiality, subject recruitment, and consent process. All will monitor response to tolerance of and effectiveness of the exercise program. Files will be kept in a locked file cabinet in the laboratory of the PI to assure individual privacy and confidentiality of subject records. The research coordinators will monitor the following items: the timeframe of recruiting subjects, quality of data being entered, any external factors relevant for the safety of participants throughout the entire study, and is also instructed to make the PI aware of all events, expected or unexpected.

Ethical Considerations

Participation in this study is voluntary. Subjects may decide not to participate in this study or may withdraw from this study at any time without penalty or loss of benefits. No vulnerable populations will be studied in this protocol.

No other specific ethical considerations are identified for this study.

Sharing of Results with Subjects: Participants will be offered the opportunity to meet with the Principal Investigator or designated medical staff to review the results of their lab assessments or other standard clinical data. Copies of their testing results will be made available to the participants upon request when appropriate and approved by the PI. In addition, the Principal Investigator or designated study staff will provide an overview of the study's outcome to the participant if he or she requests the information.

Funding Source

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Subject Stipends or Payments

Participants will receive a total of $1200 upon completion of all study visits. If a participant is unable to complete all study visits, the stipends will be prorated to the following:

Visit	Prorated Amount
After completion of V2	$400
After completion of V8	$400
After completion of V18	$400

A Payment Card will be the method of payment and will be requested upon completion of the study or on their last day of participation, should they find they cannot complete the study.

Publication Plan

We attest that the TRI faculty and staff will adhere to POLICY-TRI-ADM-005 (Access to Clinical Trial Data for Publication Purposes). The goal will be to publish novel and interesting findings from this research. Assignment of authorship and the contributions of each author will be determined by the International Committee of Medical Journal Editors (ICMJE) policy to guide authorship.

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