Effect of NMN Supplementation on Organ System Biology
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STUDY TEAM

Principal Investigator: Samuel Klein, M.D.
Washington University School of Medicine in St. Louis
Campus Box 8031
660 South Euclid Avenue
St. Louis, Missouri 63110
Telephone: 314-362-8708
e-mail address: sklein@wustl.edu

Co-Investigators: Sarah Farabi, Ph.D.
Gordon Smith, Ph.D.
Shin-Ichiro Imai, M.D., Ph.D.

OVERVIEW OF THE STUDY

Study Title
Effect of NMN Supplementation on Organ System Biology

Objectives
To determine the effect of NMN supplementation on key health outcomes

Design and Outcomes
This study is a 16-week, randomized, placebo-controlled, double-blind trial to evaluate the efficacy of NMN supplementation on cardiometabolic function in middle-aged and older adult men and women who are overweight or obese and have evidence of metabolic dysfunction.

A total of 56 men and women will be enrolled and randomized to one of the study groups 1) Placebo (n=28) or 2) NMN supplementation (n=28). Men and women will be randomized separately to ensure number of men and women are similar in both groups.

This research study will be conducted at Washington University School of Medicine (WUSM) with weekly check-ins via phone or in person during the 16-week intervention.

1. **Screening.** All participants will undergo a comprehensive outpatient screening visit, including review of medical history and blood tests, to determine eligibility for the study. Participants who meet the inclusion criteria will be admitted to the Clinical Translational Research Unit to complete baseline metabolic studies.
2. **Baseline Testing.** There are three test visits. Visit 1 is an inpatient visit that includes a hyperinsulinemic-euglycemic clamp procedure, adipose tissue and muscle biopsies, Arginine stimulation test, and a 12-h urine collection. An activity monitor will be provided to the participant that will be worn for the proceeding 7 to 10 days. Visit 2 is an outpatient visit where dual-energy X-ray absorptiometry and magnetic resonance imaging/elastography scans will be performed and muscle strength will be assessed. Visit 3 is another inpatient visit that involves a modified 3-h oral glucose tolerance test and the first dose of study pill ingestion. A stool sample will also be returned to the study team at Visit 3. The scans performed during visit 2 may be performed at a different or separate visit, depending on scheduling availability and participant preference.
3. **Randomization.** Randomization will be conducted in a double-blind fashion with both the participants and members of the research team blinded to group assignment.

4. **Intervention period.** During the 16 weeks of the treatment period, participants will be seen by the research coordinator and/or one of the study investigators each week. At each visit at WU, compliance will be recorded based on an unused pill count.
5. **Repeat testing.** All baseline testing will be repeated after participants complete 16 weeks of treatment.

A. Background and Significance

Nicotinamide mononucleotide is a key intermediate for nicotinamide adenine dinucleotide (NAD^+) biosynthesis

Nicotinamide adenine dinucleotide (NAD^+) is a co-substrate for NAD^+-consuming enzymes that are essential in the regulation of diverse biological processes, including metabolic function, inflammation and aging [1-4]. In mammals, NAD^+ is synthesized from nicotinamide (NIC) and nicotinic acid (two different forms of vitamin B_3) and tryptophan (Figure 1). Nicotinamide is considered the major precursor of NAD^+. Nicotinamide mononucleotide (NMN) is a key NAD^+ intermediate which is synthesized from NIC by nicotinamide phosphoribosyltransferase (NAMPT), a rate-limiting NAD^+ biosynthetic enzyme. Nicotinamide riboside (NR) is also converted to NMN by nicotinamide riboside kinases (NRKs). NMN is converted to NAD^+ by three NMN adenylyltransferases (NMNATs). Synthesized NAD^+ is used by NAD^+-consuming enzymes, such as sirtuins (SIRT1-7), poly (ADP-ribose) polymerases (PARPs), and cyclic ADP ribose hydrolase (CD38), and redox reactions.

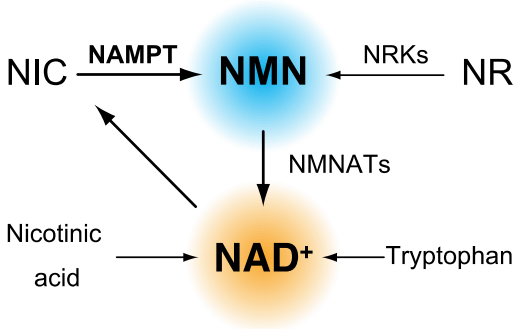

Figure 1. Nicotinamide mononucleotide (NMN) and NAD^+ biosynthetic pathways

Inadequate NAD^+ biosynthesis is associated with metabolic abnormalities in rodent models

Data obtained from a series of studies we completed in rodent models have found inadequate NAMPT-mediated NAD^+ biosynthesis is involved in the pathogenesis of obesity- and age- associated metabolic abnormalities, including insulin resistance, β-cell dysfunction, mitochondrial dysfunction, inflammation, and NAFLD [1-4]: i) compared with regular chow-fed lean mice, high fat diet-fed obese mice have lower NAMPT protein and NAD^+ contents in the liver and adipose tissue [5]; ii) *Nampt* haplodeficiency impairs glucose tolerance and glucose-stimulated insulin secretion in pancreatic β-cells [6]; iii) adipocyte-specific deletion of *Nampt* causes adipose tissue inflammation, multi-organ insulin resistance, hypoadiponectinemia, dyslipidemia, and hepatic steatosis [4, 7]; and iv) NAD^+ content in multiple organs, including the pancreas, adipose tissue, and skeletal muscle decline with age [5, 8]. In addition, others have found muscle *Nampt* deletion impairs glycolytic metabolism and causes muscle mitochondrial respiratory dysfunction, inflammation, and severe degeneration [9, 10], and NAD^+ deficiency impairs liver fatty acid oxidation, mitochondrial oxygen consumption, and insulin sensitivity and increases intrahepatic lipid accumulation and inflammation [11-13]. Taken together, these findings demonstrate the importance of NAD^+ biology in maintaining cellular and multi-organ metabolic function.

NMN administration has beneficial metabolic effects in rodent models

We have found that acute and short-term enteral or parenteral NMN administration increases NAD^+ concentrations in key metabolic organs, such as skeletal muscle and liver, and improves cellular and whole-body metabolic function in different rodent models (i.e. diet and genetically induced obesity, and age-induced metabolic dysfunction) (Figure 2) [2-7, 14]. For example: i) a single intraperitoneal injection of NMN increases β-cell function and glucose-stimulated insulin secretion [5, 6, 15]; ii) 1-2 weeks of intraperitoneal NMN

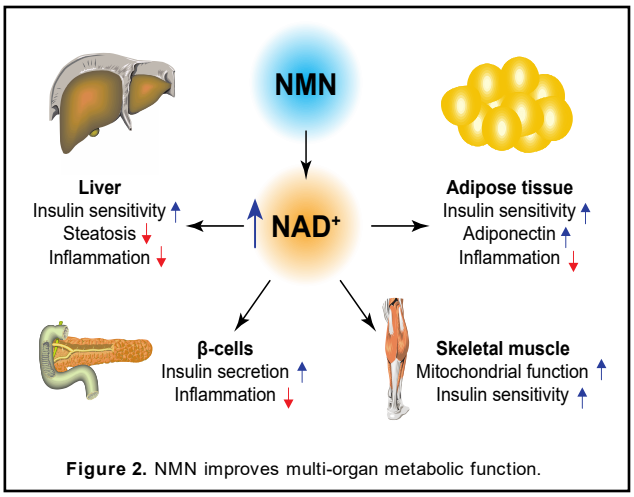

Figure 2. NMN improves multi-organ metabolic function.

administration markedly improved insulin resistance, glucose intolerance, and dyslipidemia, and decreased gene expression of inflammatory markers and protein content of acetylated NF-κB p65 in high-fat diet-induced obese mice [5]; and iii) 1-2 months of oral NMN administration reversed hypoadiponectinemia, hepatic steatosis, multi-organ insulin resistance, and adipose tissue inflammation induced by genetic *Nampt* ablation [7]. In addition, we recently found that long-term oral administration of NMN for 12 months was well-tolerated without any toxic or adverse effects in normal mice, and mitigated the normal age-associated decline in multi-organ function by improving whole-body insulin sensitivity, increasing skeletal muscle mitochondrial respiratory capacity, decreasing intrahepatic triglyceride content and decreasing markers of adipose tissue inflammation [14]. In concert with our findings, others have reported that NMN administration improves mitochondrial function, inflammation, and glucose metabolism in various mouse models [1, 11, 16-19].

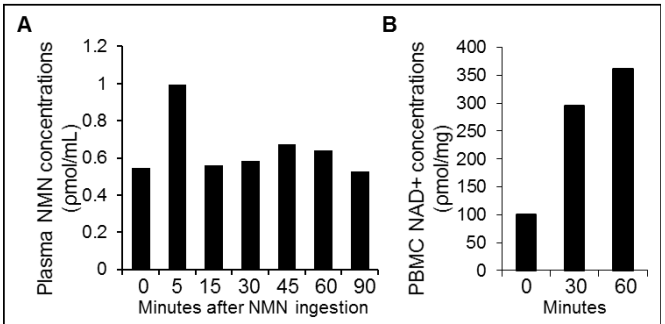

Figure 4. Plasma NMN and PBMC NAD^+ concentrations in a subject before and after ingesting 250 mg of NMN.

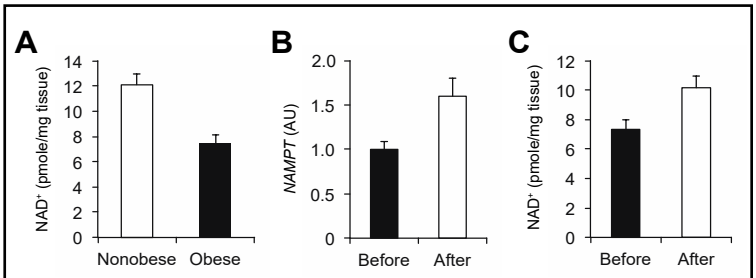

Figure 3. NAD^+ metabolism in people with obesity. (**A**) Adipose tissue NAD^+ contents in people who are non-obese or obese. (**B**) Skeletal muscle NAMPT gene expression before and after 16% weight loss. (**C**) Adipose tissue NAD^+ content before and after 20% weight loss.

Sexual dimorphism in NAD^+ biology in mice

We have found unique sex differences in the effect of NAD^+ depletion and NMN therapy in mice. In *Nampt* heterozygous knockout mice and adipocyte-specific *Nampt* knockout mice, only female mice, but not male mice, display impaired glucose tolerance [5, 7]. In addition, although NMN administration improves glucose metabolism in both female and male obese mice, NMN increases insulin sensitivity but not insulin secretion in female mice, whereas NMN increases insulin secretion but not insulin sensitivity in male mice. These findings suggest NMN therapy could have sex-specific metabolic effects in people, and is why we will study a balanced number of men and women in each group in this study.

NAD^+ biology and NMN supplementation in people

Little is known about NAD^+ biology and NMN therapy in people. Data from preliminary studies we recently conducted in human subjects show that enhanced NAD^+ biosynthesis is associated with metabolic health in people: i) adipose tissue NAD^+ content is lower in people with obesity than people who are not obese (Figure 3A); ii) 16% weight loss induced by lifestyle (diet and exercise) therapy improved insulin sensitivity and β-cell function and decreased intrahepatic triglyceride (IHTG) content and caused a concomitant increase in skeletal muscle gene expression of a key NAD^+ biosynthetic enzyme, NAMPT, in people with obesity and T2D (Figure 3B); and iii) 20% bariatric surgery-induced weight loss, which improved insulin sensitivity, β-cell function, and adipose tissue inflammation and decreased IHTG content in people with extreme obesity [20], also increased NAD^+ concentrations in adipose tissue (Figure 3C). Although NMN is present in a variety of natural foods, including broccoli, tomatoes, avocado, and beef [14], the daily amount of NMN that is normally consumed as part of a healthy diet is very small. We found that ingestion of a single 250 mg dose of NMN, which is at the upper end of the dose typically taken by consumers, caused a rapid but transient increase in plasma NMN concentrations (Figure 4A) but caused more than a 3-fold increase in peripheral blood mononuclear cell (PBMC) NAD^+ content within 60 minutes (Figure 4B). The dose of NMN used in the proposed study is 300 or 450 mg/day.

Summary. Data obtained from preclinical studies we conducted in rodent models and our preliminary studies conducted in people support the therapeutic potential of enhancing NAD^+ biosynthesis to ameliorate metabolic dysfunction in people. In fact, NMN supplementation is commercially available, and is advertised to improve health and reverse aging, without evidence of these effects in people. The goal of this proposal is to fill this important gap in our knowledge by using a systems biology research approach, within the context of a randomized, placebo-controlled trial, to determine whether the beneficial effects of NMN on metabolic function, mitochondrial function and inflammation observed in rodents applies to people. In addition, we will evaluate for potential sex differences in the efficacy of NMN supplementation (observed in studies conducted in rodents) by studying a balanced number of men and women in each group.

B. Objectives/Specific Aims/Hypotheses

The overall goal of this proposal is to expand our pilot clinical study and further evaluate the translation of our preclinical findings of NMN supplementation on metabolic health and inflammation in rodents to people. To this end, we will conduct a randomized placebo-controlled trial in men and women with prediabetes to assess the effect of NMN supplementation on key metabolic, mitochondrial and inflammation outcomes, specifically: 1) body composition; 2) multi-organ (adipose tissue, liver, skeletal muscle) insulin sensitivity; 3) β-cell function; 4) putative cellular mediators of NAD^+ biosynthesis in skeletal muscle and adipose tissue; 5) skeletal muscle mitochondrial function; and 5) systemic and tissue inflammation. We hypothesize that, compared with placebo, NMN supplementation will improve all outcome measures in people with prediabetes. The results obtained from this study will provide important insights into the potential use of NMN as a novel therapy to improve metabolic health in people who have metabolic dysfunction and are at high risk for developing T2D.

The effect of NMN supplementation on the following specific outcomes will be evaluated:

Aim 1. Body composition and metabolic function: i) body composition (body fat and fat-free masses, intra-abdominal adipose tissue volume, intrahepatic triglyceride [IHTG] content, and liver fibrosis), assessed by using dual-energy x-ray absorptiometry (DXA), magnetic resonance imaging (MRI), and magnetic resonance elastography (MRE); ii) *in vivo* multi-organ insulin sensitivity in adipose tissue and skeletal muscle (insulin-mediated suppression of palmitate production and insulin-mediated stimulation of glucose disposal), assessed by using a one-stage hyperinsulinemic-euglycemic clamp procedure in conjunction with stable isotope tracer infusion; iii) β-cell function, assessed by using a modified 3-hour oral glucose tolerance test; and iv) cellular analyses of NAD^+-related metabolites, downstream targets of NAD^+ biosynthesis, and markers of insulin resistance and insulin signaling in adipose tissue and skeletal muscle biopsies by using high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), RT-PCR and Western blot analyses.

Aim 2. Skeletal muscle mitochondrial function i) *ex vivo* respiratory capacity by using high-resolution respirometry; and ii) global and targeted cellular analyses of muscle biopsy samples to assess proteins and metabolites involved in mitochondrial electron transport chain and oxidative metabolism, and both NAD^+ and ATP contents, by using RNA-seq, RT-PCR, Western blot, metabolomics, and HPLC.

Aim 3. Systemic and tissue markers of inflammation: i) plasma concentrations of IL6, TNFα, MCP-1, IL1β, PAI-1 and C-reactive protein; ii) both adipose tissue and muscle gene expression of *IL6, TNF, MCP-1, IL1β, EMR1, CD68*, and iii) protein content of acetylated NF-κB p65, a key regulator of inflammation and downstream target of NAD^+-dependent protein deacetylase SIRT1.

Aim 4. Gut microbiome: Stool samples will be collected before and after 16 weeks of treatment to assess the composition and diversity of the gut microbiome.

In addition, we will store a repository of blood, urine, stool, adipose tissue, and muscle tissue samples collected before and after the intervention, which can later be used to valuate additional potential cellular and systemic mechanisms based on the results of the clinical trial.

C. Study Design

C.1. Overview. A 16-week, randomized, placebo-controlled, double-blind trial will be conducted in men and women with prediabetes to evaluate the efficacy of NMN supplementation (300 or 450 mg/day) on: 1) body composition; 2) multi-organ insulin sensitivity; 3) β-cell function; 4) mitochondrial function; and 5) systemic and adipose tissue inflammation (Figure 5).

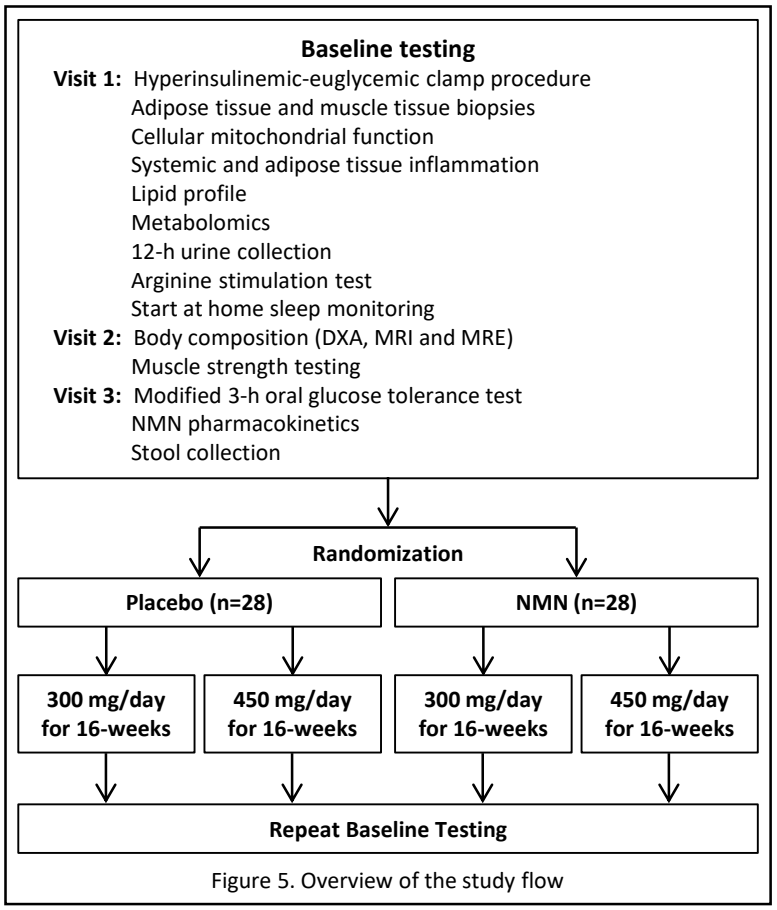

Figure 5. Overview of the study flow

C.2. Study population. A total of 56 men and women who are overweight or obese (body mass index [BMI]=25.0-50.0 kg/m^2; 45-75 years old) with prediabetes will be enrolled in this study. By limiting our subjects to middle-aged men and postmenopausal women, we eliminate the confounding effect of potential differences in age, menopausal status and menstrual cycle phase between groups on the outcome measures [29].

We will recruit subjects from the clinics and research volunteer database at Washington University School of Medicine. An outpatient screening visit will be scheduled at the Clinical Translational Research Unit (CTRU) at Washington University School of Medicine. During the screening visit, objectives of the project, all experimental procedures, all the requirements for participation, and possible discomfort and risks and benefits of participation will be clearly explained in writing and orally in lay terms by the PI, one of the study investigators or research coordinator. After all questions have been answered, and the participants agree to participate, written informed consent will be obtained. All potential participants will then undergo a medical history, physical exam, and standard blood tests to determine eligibility. A dual energy X-ray absorptiometry (DXA) scan to assess total body fat mass and fat-free mass will also be performed at the screening visit, Participants who meet the study inclusion/exclusion criteria will proceed with the rest of the study.

Potential participants must meet the following inclusion and exclusion criteria:

Inclusion Criteria:

a. Age 45-75 years old
b. BMI 25.0-50.0 kg/m^2
c. Prediabetes defined as fasting plasma glucose of ≥100 mg/dL, or HbA1C ≥5.7, or HOMA-IR ≥2.5.

Exclusion Criteria:

a. Persons who take niacin, nicotinamide, or other vitamin B3-related supplementation and are not willing to discontinue supplementation for 3 weeks before medical screening and during the entire study period
b. Persons who consume moderate-large amounts of caffeine daily (>2 cups of coffee or 8 oz caffeinated drinks per day) or consume less amounts of caffeine but believe withdrawal symptoms (e.g. headache) are likely if caffeine is stopped
c. Structured exercise: ≥75 min/wk of vigorous exercise (e.g., jogging, activity that causes heavy breathing and sweating) or ≥200 min/wk of low intensity physical activity (e.g., brisk walking)
d. Unstable weight (>3% change during the last 2 months before entering the study)
e. Coagulation disorders (platelets <100,000, Prothrombin Time >2 seconds above control or INR >1.5)

f. Anemia (hemoglobin <10.5 g/dL in women and <11.0 g/dL in men)
g. Significant organ system dysfunction or disease including cirrhosis, severe coronary heart disease (e.g. unstable angina, severe heart failure)
h. Cancer or history of cancer that has been in remission for <5 years
i. Polycystic ovary syndrome
j. Major psychiatric illness
k. Use of medications known to affect study outcome measures (e.g., steroids) or increase the risk of study procedures (e.g., anticoagulants such as warfarin and non-vitamin K oral anticoagulants) that cannot be temporarily discontinued for the study
l. Use of dietary supplements and/or medications known to affect sleep (e.g., sleeping pills)
m. Metal implants which prevent magnetic resonance imaging
n. Regular use of tobacco products or illegal drugs determined by medical history
o. Women who are still having menses
p. Women who consume >14 units of alcohol per week and men who consume >21 units of alcohol per week
q. Unable or unwilling to follow the study protocol or who, for any reason, is considered an inappropriate candidate for the study by the research team

C.3. Study Procedures

This study will be conducted as a randomized, placebo-controlled, double-blind trial, and both the participants and members of the research team directly involved with study participants and data analysis will be blinded to group assignment. Participants will be admitted to the inpatient CTRU on two occasions to complete baseline testing. Each of these visits will be scheduled ~5-10 days apart. Subjects will be asked to avoid caffeine and alcohol for at least 24 h, and to abstain from any structured intense exercise for 3 days before each admission. After participants complete baseline testing, they will be randomly assigned to treatment with either placebo or NMN, taken daily after breakfast for 16 weeks. *Placebo and NMN capsules will be provided at no charge by Shin-Kowa Pharma Company (Tokyo, Japan) (see letter from company president Megumi Tanaka).* During the treatment period, participants will be seen by the research coordinator and/or one of the study investigators every week. At each check in meeting, treatment compliance will be documented in a supplement accountability log based on the unused pill count and participant interview. These visits will also be used to address any study-related issues and help ensure body weight and physical activity remain constant during the study to avoid any potential confounding effects on the outcome measures. All testing conducted at baseline will be repeated after 16 weeks of treatment, and treatment will be continued until all repeat testing is completed. Metabolic testing performed during baseline visits 1 and 3 will be repeated in the same order after the 16-week treatment period.

Participants will be assigned a subject ID number upon entry to the study. The study ID is not related information about the individual and is not capable of being translated so as to identify the individual. Data and biological samples collected from study participants during the study participation will be labeled with the study ID and will *not* be used in combination with any personal identifiable information (PII). All data collected during the study will be de-identified and will not be used with any personal identifiable information.

C.3.1. Study outcomes. The effect of NMN therapy on the following outcomes will be assessed:

a. *Primary outcome: in vivo* muscle insulin sensitivity*:* Skeletal muscle (stimulation of glucose disposal) insulin sensitivity, assessed by using a hyperinsulinemic-euglycemic clamp procedure in conjunction with stable isotopically-labeled glucose tracer infusions [30-32].

b. *Secondary outcomes:*

i) Body composition analyses: Total body fat mass and fat-free mass will be determined by using dual energy X-ray absorptiometry (DXA), and intra-abdominal adipose tissue volume, intrahepatic triglyceride

content, and hepatic fibrosis will be assessed by using magnetic resonance imaging (MRI) and magnetic resonance elastography (MRE).

ii) β-cell function: Insulin secretion rate and plasma insulin concentration assessed by using modified 3-h oral glucose tolerance test, in conjunction with a measure of insulin sensitivity obtained by using the hyperinsulinemic-euglycemic clamp procedure. β-cell function in response to non-glucose stimuli will be assessed by using the Arginine stimulation test.

iii) Pharmacokinetics of NMN and NAD^+: NMN concentration or NMN metabolites in plasma, and NAD^+ concentration in PBMCs, muscle and adipose tissue will be obtained at baseline and after 16 weeks of placebo or NMN treatment. Blood for plasma NMN, PBMC, muscle and adipose tissue will be obtained 60 minutes after ingesting placebo or NMN capsules during basal stage of post-treatment clamp procedure. NMN pharmacokinetics after the first dose of NMN will be assessed after completing all metabolic testing during baseline visit 2 and will be repeated at the end of the study.

iv) Targeted metabolomics: Plasma, muscle and adipose tissue metabolites: a) NAD^+ content and NAD^+-related metabolites (nicotinamide, nicotinic acid, NMN, NR, tryptophan, N-methylnicotinamide, ATP), b) markers of insulin resistance (branched-chain amino acids, total amino acids, and acylcarnitines) will be determined by using established high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) based methods.

v) Mitochondrial function: i) *ex vivo* muscle mitochondrial respirometry; and ii) cellular analysis of mitochondrial proteins (Western blot), NAD^+ and ATP contents (HPLC/LC-MS), and gene expression (Real-time PCR and RNA-seq) of proteins involved in mitochondrial electron transport chain activity and oxidative metabolism in skeletal muscle biopsy samples.

vi) Plasma and tissue markers of inflammation: Plasma concentrations (IL6, TNFα, MCP-1, IL1β and C-reactive protein), and both adipose tissue and muscle gene expression (*IL6, TNF, MCP-1, IL1β, EMR1, CD68*) of selected markers of inflammation.

vii) Cellular insulin signaling and putative downstream targets of NAD^+: Cellular analyses of specific components of the insulin signaling cascade (phosphorylation of AKT and AMPK) and downstream targets of NAD^+-dependent protein deacetylase SIRT1 (global lysine acetylation and acetylated NF-κB p65).

viii) Gut microbiome: DNA extracted from all fecal samples will be subjected to multiplex sequencing of PCR amplicons generated from variable region 4 of bacterial 16S rRNA genes to determine relative taxonomic abundances and to assess diversity and the overall configurations (using an unsupervised, phylogenetic method [UniFrac-based] and a supervised, non-phylogenetic method) of the fecal microbiota in each treatment group.

ix) Muscle strength: Muscle strength will be assessed by one-repetition maximum (1RM) test and hand grip testing.

C.3.2. Experimental procedures and preliminary data. The following procedures will be performed during two inpatient admissions and one outpatient visit to the CTRU at Washington University before and after the treatment intervention. The visits/studies might be broken up into multiple visits if it better accommodates participants' schedule. The timing, tests, and visits might also be performed in a different order if that is more convenient for participants:

C.3.2.1. *Baseline Visit 1*. Subjects will be admitted to the inpatient CTRU at ~1400 h and stay for ~24 h to complete a series of cardio-metabolic tests described below. Subjects will be given a standard meal at 1900 h

on the day of admission containing one-third of their estimated daily energy requirements (calculated as 1.25 x estimated resting energy expenditure [33] and comprised of 50% carbohydrate, 35% fat, and 15% protein) and initiate ~12-h urine collection until the next morning.

a. Hyperinsulinemic-euglycemic clamp procedure. At ~0700h in the morning after admission, after subjects have fasted for ~11 hours overnight, an intravenous catheter will be inserted into an antecubital vein to infuse stable isotope tracers, insulin and dextrose. A second catheter will be inserted into a radial artery to obtain arterial blood samples. A primed-constant infusion of [6,6-$^{2}H_2$]glucose and a constant infusion of [U-^{13}C]palmitate will be started and maintained for 3.5 h to obtain basal glucose and fatty acid kinetics. At ~1030h, insulin will infused for ~ 3.5 h at 50 mU/m^2 BSA/min (initiated with a two-step priming dose of 200 mU/m^2 BSA/min for 5 min followed by 100 mU/m^2 BSA/min for 5 minutes). Euglycemia (~100 mg/dL) will be maintained by variable rate infusion of 20% dextrose enriched to 2.5% with [6,6-$^{2}H_2$]glucose. Adding glucose tracer to the dextrose infusion provides a more accurate measure of glucose kinetics by minimizing changes in plasma glucose enrichment [35]. The infusion rates of [6,6-$^{2}H_2$]glucose and [U-^{13}C]palmitate will be decreased by 75% during insulin infusion, because of the expected decreases in endogenous glucose production and palmitate release into the circulation. Blood samples will be obtained immediately before starting the tracer infusion and every 10 min during the final 30 min (4 samples) of the basal period and clamp procedure, to determine glucose and insulin concentrations and substrate kinetics. We have found this infusion protocol is well-tolerated by study subjects and is needed to achieve steady-state conditions in plasma glucose tracer enrichment during each stage.

b. Muscle and adipose tissue biopsies. Abdominal subcutaneous adipose tissue and vastus lateralis muscle tissue and thigh fat samples will be obtained during the basal period, and subcutaneous adipose tissue and vastus lateralis muscle tissue samples from the opposite leg will also be obtained during insulin infusion of the clamp procedure. To obtain abdominal subcutaneous adipose tissue, the periumbilical area on one side of the body will be cleaned and anesthetized with 1% lidocaine, then a small skin incision (~0.5 cm) will be made in the skin and a small liposuction cannula will be inserted into the incision to aspirate ~2-4 grams of subcutaneous adipose tissue under sterile conditions. The samples will be rinsed immediately in ice-cold saline and cleaned of connective tissue and blood, and submerged in liquid nitrogen and stored at -80 °C until further processing. To obtain skeletal muscle tissue, the site will be cleaned and anesthetized with 2% lidocaine, and then a small incision (~0.5 cm) will be made in the skin. A muscle sample will be obtained by percutaneous biopsy under sterile conditions. Muscle tissue samples will be rinsed immediately in ice-cold saline and cleaned of connective tissue and blood. Most of the tissue will be submerged in liquid nitrogen, and then stored at -80 °C until further processing for protein content, gene expression and metabolomics. One aliquot of fresh muscle tissue obtained during the basal period will be used for high resolution respirometry, conducted on permeabilized muscle fibers by using the Oroboros Oxygraph-2k system. We found that NMN-treated mice increase mitochondrial respiratory function in skeletal muscle by using the same assay system [14]. Respiration in the non-phosphorylative, "Leak", state is measured in the presence of malate (1 mM), glutamate (10 mM) and pyruvate (5 mM). After stable Leak state measurements are made, oxygen consumption during the oxidative phosphorylation state (OxPHOS) is determined by adding ADP (5 mM) and succinate (10 mM).

If taking certain medications that affect study outcome measures (e.g., steroids) or increase the risk of study procedures (non-steroidal anti-inflammatories e.g. ibuprofen), participants will be asked to temporarily (approximately 7-14 days) discontinue the medications for the study visits.

c. NMN pharmacokinetics. NMN concentration or NMN metabolites in plasma, and NAD^+concentration in PBMCs, muscle and adipose tissue will be obtained at baseline and after 16 weeks of placebo or NMN treatment. Blood for plasma NMN, PBMC, muscle and adipose tissue will be obtained 60 minutes after

ingesting placebo or NMN capsules during the basal period of the hyperinsulinemic-euglycemic clamp procedure performed after 16-week treatment period.

d. Arginine stimulation test. After completing the hyperinsulinemic-euglycemic clamp procedure, an Arginine bolus infusion will be given over 1 minute, followed by blood sampling every 2 min for 10 min to assess maximal β-cell function.

Sleep monitoring. Wrist actigraphy with concurrent light exposure (Actiwatch Spectrum Pro; Philips Respironics) will be used to assess total sleep time during the 7-10 day period before and after 16 weeks of treatment period. Participant will also be asked to complete a sleep journal during this monitoring period.

Sleep questionnaire. Participants will be asked to take answer a questionnaire about their sleep quality.

C.3.2.2. *Baseline Visit 2.*
a. Body composition analyses. In the afternoon of admission, intra-abdominal adipose tissue volume, intrahepatic triglyceride content, and liver fibrosis will be assessed by magnetic resonance imaging (MRI) and magnetic resonance elastography (MRE) [34]. These tests might be scheduled on different days as separate visits or in combination with another visit if necessary.

If a participant has claustrophobia, they will be given the option of taking a benzodiazepine (Ativan or Xanax) pre-procedure which will be dispensed through BJC Pharmacy. If a participant does agree to take the benzodiazepine, prior to the prescription his/her medical history will be reviewed by a study physician. If a participant does take a benzodiazepine during MRI scan, he/she will be required to have a ride home by a caregiver after the visit and to avoid alcohol or other sedating agents overnight.

b. Muscle strength testing. Muscle strength will be measured by one-repetition maximum (1RM) test and hand grip testing. 1RM will be measured on each of five different exercises (leg press, knee extension, knee flexion, seated row, chest press) which are performed bilaterally on a Hoist weightlifting machine (Hoist Fitness Systems, San Diego, CA). Grip strength will be measured by using a Jamar dynamometer, which will be adjusted to accommodate for difference in hand size. At baseline, subjects will be asked to participate in an orientation session during one of the study visits to become familiar with the exercise equipment and testing procedures. After approximately 7-14 days, the testing will be repeated to obtain each subject's baseline measurements. The testing might be scheduled on different day as separate visits during a baseline study period.

C.3.2.3. *Baseline Visit 3*. Subjects will be admitted to the inpatient CTRU at ~1800 h and stay for ~20 h to complete the following tests described below. Subjects will be given a standard meal at 1900 h on the day of admission containing one-third of their estimated daily energy requirements (calculated as 1.25 x estimated resting energy expenditure and comprised of 50% carbohydrate, 35% fat, and 15% protein).

a. Modified 3-hour Oral Glucose Tolerance Test. At ~0500 h in the morning, after subjects have fasted for ~11 hours overnight, an intravenous catheter will be inserted into an antecubital vein to infuse isotope tracers. A second catheter will be inserted into a hand vein for blood sampling with the hand heated using a thermostatically controlled box, to obtain arterialized venous samples [36]. If a hand vein cannot be placed a line will be inserted into a radial artery to obtain arterial blood samples. A primed-constant infusion of [6,6-$^{2}H_2$]glucose will be started and maintained for 3.5 h to obtain basal glucose kinetics. After three baseline blood samples are obtained (time -10, -5 and 0 minutes), subjects will consume a 75-gram glucose solution labeled with [U-^{13}C]glucose. Blood samples will then be obtained at 10, 20, 30, 60, 90, 120, 150, and 180 minutes after glucose ingestion to determine plasma glucose, insulin, and C-peptide concentrations, and to calculate indices of β-cell function (see section C.3.6.b. for calculations used to assess β-cell function).

b. NMN pharmacokinetics after the first dose of NMN. After subjects complete all baseline metabolic testing, NMN concentration or NMN metabolites in plasma and NAD^+concentration in PBMCs will be analyzed. Blood samples will be obtained before and 10, 20, and 60 minutes after the first dose of placebo or NMN.

c. Stool sample collection. Sujbects will bring a frozen stool sample (obtained from home within 36 h of the study visit) by using a stool collection kit dispensed by the study coordinator during Visits 1 or 2.

C.3.3. Randomization procedure. After participants complete baseline metabolic testing, they will be randomly assigned to one of two groups: 1) placebo (two or three study pills made from food grade starch) once a day with breakfast for at least 16 weeks or 2) NMN (300 mg or 450 mg) once a day with breakfast for at least 16 weeks. A randomization scheme stratified by BMI (two strata: 25.0 kg/m^2 to 34.9 kg/m^2, and 35.0 kg/m^2 to 50.0 kg/m^2) and sex (men and women) will be generated by using the randomization module of Research Electronic Data Capture (REDCap), a secure, web-based application designed to support data capture for research studies [37] and a randomization scheme to account for the potential imbalance in randomization. The study will be conducted in a double-blind fashion and both the participants and members of the research team directly involved with study participants and data analysis will be blinded to group assignment. An individual from the Washington University Center for Human Nutrition, who is not directly involved with the study participants and data analysis, will have access to a master list to unblind participants, if necessary.

C.3.4. Intervention. Placebo and NMN capsules will be provided at no cost by Shinkowa Pharma (Mirailab Bioscience Inc.) (Tokyo, Japan) under a Material Transfer Agreement (MTA) between Washington University School of Medicine (WUSM) and Shinkowa Pharma Co. Ltd. Capsules (placebo and NMN) will be shipped to us in sealed bottles and upon receipt stored in a locked room access limited to a small number of research team members under the responsibility of the PI. Study participants will be provided with either placebo or NMN pills by CTRU metabolic kitchen at WUSM after completing baseline testing. Daily consumption of the placebo or NMN pills will continue throughout repeat testing after 16 weeks of treatment. Study participants will be contacted via phone by the research coordinator weekly to help ensure compliance with the study protocol and problem-solve any barriers (such as introducing use of a pill organizer and daily log sheet if study pills are not being taken on a regular basis). Monthly follow-up study visits for participants will be held at WUSM. Medical monitoring of participants will be performed by the study Research Nurse Practitioner in consultation with the PI, as needed throughout the entire study period. In addition, a mid-intervention blood test (CBC and CMP) will be performed at approximately week 8 of intervention at WUSM. Participants will be provided with dietary supplementation diary to keep track of the daily supplementation record and asked to bring this handout to each intervention visit. Participants will be asked to maintain their physical activity level and body weight during the study period. A Supplement Accountability Log will be used to document dispensing of the dietary supplement or placebo to the participant and the return of unused product. Participants will be instructed to keep a study pill diary and return unused supplement at each scheduled WU study visit and at the end of the study. Compliance will be assessed by a study dietary supplementation diary and pill count.

C.3.5. Accountability of dispensing placebo and NMN capsules. A Supplement Accountability Log will be used to document the dispensing of the dietary supplement or placebo to the participant and the return of unused product by the participant to WU. The Supplement Accountability Log will document the participant ID, the amount of supplement and the date supplement was dispensed to the participant, and the amount of supplement and date that the study participant returned. Participants will be instructed to return unused supplement at every scheduled WU study visit and study end at WU. The study research coordinator will perform a pill count and document in the Supplement Accountability Log at each WU study visit.

C.3.6. Data safety monitoring. All participants will be contacted by the research coordinator and/or research nurse weekly throughout the study. All safety and medical issues will be reviewed by Dr. Klein, and, if needed, will be discussed with the participant's primary physician.

C.3.7. Repeat testing. All studies performed at baseline will be repeated after participants complete at least 16 weeks of treatment. Daily placebo or NMN dosing will continue throughout the repeat testing period, which will take ~2 weeks (weeks 16-18 of treatment).

C.3.8. Calculations.

a. Substrate kinetics and insulin sensitivity. Glucose and palmitate kinetics during the clamp procedure will be used to provide an index of hepatic, skeletal muscle and adipose tissue insulin sensitivity, as we previously described [31, 38, 39]. Glucose Ra in plasma during basal conditions and the clamp procedure will be calculated by dividing the glucose tracer infusion rate by the average plasma glucose TTR during the last 30 min of the basal and clamp periods, respectively. Glucose Ra during basal conditions represents endogenous glucose Ra, an index of hepatic glucose production rate, which equals basal glucose rate of disappearance (Rd). During the clamp procedure, hepatic glucose production rate will be calculated by subtracting the glucose infusion rate (i.e., dextrose solution plus tracer added to it) from total glucose Ra (endogenous plus exogenous); glucose Rd is equal to total glucose Ra (i.e., the sum of endogenous glucose Ra and the rate of infused glucose). Palmitate Ra, an index of adipose tissue lipolysis will be calculated by dividing the palmitate tracer infusion rate by the average plasma palmitate TTR obtained during the final 30 min of the basal period and insulin infusion.

b. β-cell function. Insulin secretion rate (ISR) during the modified 3-h oral glucose tolerance test (MOGTT) will be calculated by stochastic deconvolution analysis of the plasma C-peptide concentration time-course [40]. β-cell response sensitivity to glucose will be assessed as the ratio of the area under the curve (AUC) of ISR (pmol/L) to the AUC of glucose [41-43]. β-cell function in relation to insulin sensitivity will be assessed based on the principle of the disposition index, calculated as the product of the area under the curve (AUC) of ISR (pmol/L) assessed during the MOGTT and the rate of insulin-stimulated glucose disposal normalized for plasma insulin concentration (μmol/kg FFM/min per μIU/mL) assessed during the hyperinsulinemic euglycemic clamp procedure, as we have previously reported [20]. Insulin secretory reserve will be assessed by measuring the AUC of insulin and ISR over 10 min after the intravenous administration of 5 g of arginine [44, 45].

C.3.9. Sample analyses.

All sample analyses are routinely performed by our research group or in our Core laboratories at Washington University School of Medicine.

a. Plasma concentrations of substrates and hormones and markers of inflammation will be measured by our Core Laboratory for Clinical Sciences, as we have previously described [30, 32].

b. Plasma glucose and palmitate tracer-to-tracee ratios will be determined by using gas chromatography-mass spectroscopy, as we have previously described [46].

c. Plasma NMN and nicotinamide, PBMC NAD^+ and tissue NAD^+, nicotinamide and ATP. Plasma NMN and nicotinamide and tissue NAD^+, nicotinamide, and ATP concentrations will be determined by using an HPLC system (Prominence; Shimadzu) with a Supelco LC-18-T column (Sigma) and a Hypercarb column (Thermo Scientific), as we have previously described [2, 5, 7, 14, 47-51].

d. Adipose tissue gene expression of inflammatory markers (*IL6, TNF, MCP-1, IL1β, EMR1, CD68*) and adiponectin (a key downstream target of adipose tissue NAD^+ [7] will be determined by using Real time-PCR. Total RNA will be isolated from frozen adipose tissue biopsy samples in RNeasy kit (Qiagen, Valencia, CA) and reverse transcribed (High-Capacity cDNA Reverse Transcription Kit; Invitrogen, Carlsbad, CA) [30, 32,

52-56]. Tissue gene expression was determined using an ABI 7500 real-time PCR system (Invitrogen) and SYBR Green Master Mix (Invitrogen), as we have previously described [30, 32, 53]. The expression of each gene will be normalized to the housekeeping control gene, ribosomal protein (*RPLP0*).

e. Skeletal muscle transcriptome analysis by using RNA-seq. Total RNA will be isolated from frozen skeletal muscle biopsy samples in Trizol reagent (Invitrogen), as we have previously described [53-55, 57]. We will first evaluate the effects of NMN supplementation on muscle gene expression of specific NAD^+ downstream targets (mitochondrially encoded genes, *ND1-ND6, Cytb, COX1, COX2, COX3, ATP6, ATP8*) [16] and inflammatory markers (*IL6, TNF, MCP-1, IL1β, EMR1, CD68*) by using Real time-PCR as described above.

f. Assessment of insulin signaling and NAD^+ downstream targets. Western blot analysis will be performed to evaluate the muscle content and phosphorylation of intracellular proteins involved in the insulin signaling cascade (AKT and AMPK) as we previously described [52, 54, 58]. We will also evaluate muscle content of proteins involved in the electron transport complex [14, 59] and global protein acetylation status, and a key NAD^+-SIRT1 downstream target, acetyl-NF-κB p65 [5, 7, 48]. Blots will be probed with polyclonal and monoclonal antibodies including anti-phospho-AKT (Ser473) (#4060; Cell Signaling Technology), anti-total-AKT (#4691; Cell Signaling Technology), anti-phospho-AMPKα (Thr172) (#2535; Cell Signaling Technology), anti-total-AMPKα (#2532; Cell Signaling Technology), anti-aceylated lysine (#9441; Cell Signaling Technology), anti-acetyl-NF-κB p65 (Lys310**)** (#3045; Cell Signaling Technology), and anti-total OXPHOS cocktail (#ab110413; Abcam).

D. Statistical plan and Data analysis

Statistical evaluation of the study data will be carried out in collaboration with Dr. Kenneth Schechtman, who is an applied biostatistician with expertise in the design and analysis of clinical trials. Our primary analysis will be intention-to-treat in people with prediabetes. Preliminary analyses involving t-tests or Wilcoxon's test for continuous variables and chi square tests for dichotomous variables will be performed to confirm that baseline characteristics of subjects are similar in the placebo and NMN treatment groups. ANCOVA with the post-treatment value of the metabolic outcome measure (e.g., our primary outcome variable is insulin-mediated glucose uptake) as the dependent variable will be used to determine whether there are between group differences in the outcome measure after adjusting for the pre-intervention value and any measures that were significantly different before or during the intervention. We will explore the distributional properties of the metabolites; if the variables are skewed, we will analyze the data following a data transformation (e.g., a log transformation). If an appropriate transformation cannot be found, we will analyze the data semi-parametrically by using the ranks of the data. An exploratory goal of this study is motivated by preliminary data from rodents suggesting that the efficacy of NMN on the primary outcome may be greater in female than male animals. Therefore, we will evaluate the interaction between sex and treatment group by using analyses of covariance. These analyses should provide evidence regarding the possibility that efficacy differs according to the sex of the individual. We emphasize the exploratory nature of these analyses. This study is not powered to evaluate the relevant interaction term, and our purpose is to generate preliminary data to determine whether a subsequent larger study to assess the response to NMN based on sex is worthwhile.

Sample size considerations. The single primary endpoint in this study is insulin-mediated glucose Rd. All other endpoints are secondary. Because there is only one primary endpoint, the power computations for that endpoint are not adjusted for multiple testing. We also present power computations for important secondary endpoints below, but emphasize that the target sample size is based on considerations related to the primary outcome only.

Using G*Power 3.1.9.2 and day-to-day variance we have previously reported in similar intervention studies [38, 60], we estimated that 22 participants/group will be needed to detect *realistic and physiologically and clinically meaningful* between-group differences in insulin-mediated glucose Rd [61, 62] with >90% power using a two-sided test and an α value of 0.05 in all primary outcomes listed below. Therefore, we will enroll 28 participants

in each group to ensure that an adequate number of participants complete the study (allowing for an estimated 6 drop-outs/group). All of the power computations below are based on a power of 0.9.

Insulin-mediated glucose Rd. We have reported day-to-day variability in insulin-mediated glucose Rd during high-dose insulin infusion (means ± SD) to be 3.4 ± 6.1 μmol/kg FFM/min in people with prediabetes. Using these SDs and assuming similar variance in the placebo and NMN groups, we estimate we will be able to detect a ~6.2 μmol/kg FFM/min difference between groups in subjects with prediabetes (~14% difference based on an expected mean glucose Rd at baseline of 44.5 μmol/kg FFM/min) [61].

Tissue gene expression. It is difficult to make robust estimates of our study power to detect changes in the molecular outcomes in adipose tissue and muscle, because of limited data evaluating these variables and the unknown effects of altering sleep. However, in our own experience, the day-to-day variance in muscle and adipose tissue gene expression is ~30% [52]. Accordingly, we estimate that we have ≥90% power to detect statistically significant effect of NMN therapy in both people with prediabetes, if the differences from placebo are >30.5%. Our previously published data [61] indicate that differences in adipose tissue gene expression of this magnitude are *realistic* in response to dietary and pharmacological intervention. Our study also has other (secondary) outcomes listed in Section C.3.1.b. These outcomes are important and we believe they will generate important information. However, it is difficult to estimate statistical power for these outcomes because of inadequate available information.

E. Retention and Recidivism plan.

Participants will be provided with a monthly pill organizer coded by days of the week after completing baseline testing, to help improve adherence to take individual doses of dietary supplementation each day. In addition, participants will be provided with a dietary supplementation diary with dates between study visits to keep track of the daily dietary supplementation record. Participants will be asked to bring this handout to each intervention visit. Several strategies will be used to enhance adherence with the study protocols and reduce drop-outs: i) a philosophy of partnership and collaboration will be encouraged between research site personnel and study participants; ii) reimbursement for time required for study visits will be provided; iii) participants will be contacted weekly by the research coordinator and/or research nurse; and iv) participants who are not adhering to the study protocol will receive additional support by the study team with an intervention plan established after reviewing the participant's specific barriers for compliance.

F. Timeline.

A total of 56 subjects will be enrolled in this study. We estimate that 6 subjects from each treatment group (placebo and NMN) will drop out before completing the intervention, so that 44 subjects will complete the studies after the intervention. Baseline and post-treatment assessments each require two CRU admissions. We plan to complete all studies within 3.5 years and conduct final sample processing and data analyses in the subsequent 6 months.

G. Foreseeable Risks or Discomforts

Arterial and Venous Catheter insertion, Blood drawing and Intravenous infusions

Likely

- It is likely that the intravenous catheter insertion will cause discomfort, bruising, and/or bleeding.

Less Likely

- Occasionally some people experience dizziness or feel faint during catheter insertion and/or blood drawing.

Rare

- The total amount of blood that will be collected for this entire research study is about 490 ml (~33 tablespoons) over about 18-20 week period. The risks associated with giving this amount of blood include headache, nausea and lightheadedness; however, this amount is spread out over the entire study period so it is very unlikely to cause a problem.

- When an arterial line is placed, a blood clot can occur at the site of the catheter insertion and could decrease blood flow to the hand and cause tissue damage requiring corrective surgery. However, the risk of this is extremely small because a small size catheter is used and it is kept in for a short period of time. Furthermore, before catheter placement, you are examined by using ultrasound to make sure that you have adequate blood flow to your hand from the other major artery that supplies the hand. If you are judged to have insufficient blood flow, a catheter will not be inserted in your radial artery.
- An infection can occur at the catheter insertion sites. Careful techniques are used when inserting the catheters, and when obtaining blood samples to decrease the risk of infection.
- If the IV catheter slips out of the vein, fluid could collect in your arm and cause swelling and discomfort.
- Infusion of insulin can cause an allergic reaction (including rash, swelling of the tongue or throat, and/or difficulty breathing).
- Rarely, an infection can occur from the infusions. However, careful sterile technique will be used at all times in preparing the solutions to decrease the risk of this complication.

Muscle and fat biopsies

Likely

- Possible side effects of the biopsy procedure are pain during and for some time after the procedure. The biopsy site may also be tender or sore for two to three days after the biopsy.
- Swelling and/or bruising may occur at the biopsy sites.
- Lidocaine injection may be painful during the injection followed by numbness in the area injected (see lidocaine risks below).

Less Likely

- Muscle biopsies can cause temporary numbness or loss of sensation in the region of the biopsy site.

Rare

- Muscle biopsies can cause long-term numbness, loss of sensation and/or burning pain in the region of the biopsy site.
- An infection can occur at the biopsy sites. Careful techniques are used when obtaining biopsy samples to decrease the risk of infection.

Lidocaine: <u>Please inform the doctor or nurse if you have a known allergy to Lidocaine</u>

Likely

- It is common to feel some mild discomfort when the Lidocaine is first administered.

Rare

- Rarely, an allergic reaction may occur that would result in minor swelling or irritation at the injection site.

Very Rare

- People may feel lightheaded and/or nauseated due to the pain at the injection site.
- An allergic reaction may be severe and cause itching, swelling of the face or extremely low blood pressure or difficulty breathing may occur. All of these symptoms will be treated should they develop.

Urinary Catheterization

Likely

- Pressure, pain or discomfort during insertion
- Burning with urination

Less Likely

- Bladder spasm or cramping
- Leakage

Rare

- Urinary tract infection
- Injury to urethra caused by insertion
- Narrowing of urethra due to scar tissue caused by insertion
- Injury to bladder caused by incorrect insertion
- Bladder stones

Dual energy x-ray absorptiometry

Likely

- This research involves exposure to radiation from the dual-energy X-ray absorptiometry (DXA) test used to measure body composition. The amount of radiation from these procedures, when averaged over the entire human body, is equivalent to a uniform whole-body dose of less than 1 mrem. This is equivalent to 1/300 of the amount of the natural background radiation exposure people living in St. Louis receive each year. If you want to know more about radiation exposure, please see the "Radiation Fact sheet" on the Guidelines page of the Human Research Protection Office website at http://hrpo.wustl.edu or ask the study staff for a copy.
- Because certain research studies are subject to specific radiation exposure limits, it is important that you inform us if you have been in any other research studies or medical procedures in the last 12 months that involve exposure to radiation (from x-rays, CT scans, PET scans or other nuclear medicine procedures). It is also important that you tell future investigators about your participation in this research study if you are asked to participate in another research study.

Magnetic resonance imaging (MRI) / Magnetic resonance elastography (MRE)

Common risks:

- discomfort inside the MRI scanner if you do not like to be in closed spaces ("claustrophobia")
- muscle stiffness from lying still
- muscle cramping caused by nerve stimulation
- tissue heating which may cause you to feel very warm

Rare risks:

- hearing loss due to the loud hammering noise from the MRI scanner
- sensation of flashing lights while in the MRI scanner
- burns that could be serious

During the procedure, you will be able to talk with the MRI staff through a speaker system. You will be given earplugs to reduce the risk of hearing loss. If you experience any of these symptoms and do not wish to continue, you can ask that the scan be stopped immediately.

Devices

If you have a device such as a pacemaker, bone hardware, cardiac stent, or device placed in your uterus there may be additional risks. We will review what device you have and inform you of these risks. In general, these risks could be:

- heating or movement of the device
- device malfunction
- damage to the tissue that surrounds the device.

If you have claustrophobia, you will be given the option of taking a medication to help relax during the procedure (Ativan or Xanax). If you choose to take the option, Ativan or Xanax can be prescribed by a study physician after careful medical evaluation. If you choose to take Ativan or Xanax during MRI scan, you will be required to have someone drive home after you complete the visit.

Ativan or Xanax

Likely

- If Ativan or Xanax is taken before the MR scans, you may experience dizziness, lightheadedness, unsteadiness and weakness.

Rare

- If Ativan or Xanax is taken before the MR scans on rare occasions you may experience agitation, depression, eye function disorders, headache, memory impairment, mental disorientation, sleep disturbance, low blood pressure, confusion, nervousness, increased salivation, muscle cramps, nasal congestion, and/or heavy breathing. However, the odds of experiencing these side effects are extremely low because you will only take one dose of the medication during each visit.

Oral glucose tolerance test, insulin sensitivity test, and Arginine stimulation test

- Blood sugar levels could change (become low or high) during these tests, causing you to feel sweaty, shaky and/or nauseated. However, blood sugar is carefully monitored throughout these tests to decrease the risk of these problems.
- Infusion of insulin may cause an allergic reaction (including rash, swelling of the tongue or throat and difficulty breathing).
- Risk of infusing stable isotope tracers include fever or infection. However, all solutions will be tested before infusion and will be administered under strict sterile conditions.

Sleep monitor

Less Likely

- Wearing the activity monitor may irritate the skin.

NMN

There are no known risks associated with NMN supplementation given at this dose. However, NMN is made from Vitamin B3, so it is possible that you could experience the following side effects that are known to be associated with oral Vitamin B3 supplementation.

Rare

- Flushing reaction, with burning, tingling, itching, and redness of the face, arm, and chest.

Confidentiality

Rare

- One risk of participating in this study is that confidential information about you may be accidentally disclosed. We will use our best efforts to keep the information about you secure. Please see the section in this consent form titled *"How will you keep my information confidential"* for more information.

H. Protection Against Risk

Medical screening will include laboratory safety evaluation, including Complete Blood Cell count [CBC] (which include red blood cell count), Complete Metabolic Panel [CMP] (which include electrolytes, renal function tests and liver biochemistries), HbA1c (which indicates plasma glucose control), and PT-INR (which indicates coagulation disorders), and history and physical examination to screened out those who have increased potential risks. Participants will be questioned in detail about medical history including metal placement in the body and claustrophobia.

Only skilled nursing staff (Nurse practitioners and RNs) will be used to placement of intravenous and arterial catheters. Only skilled nurse practitioners and physicians will perform abdominal fat and thigh muscle biopsy.

Safety monitoring during intervention period will include measurement of blood pressure and evaluation for any adverse effects with a study team member. Intravenous tracer solutions will be prepared in a designated, sterile

mixing room, by a certified pharmacy technician who completes annual recertification through BJH Pharmacy and is also GMP certified. Careful aseptic techniques will be used when inserting the catheters and when obtaining blood samples to decrease the risk of infection. In addition, continuous safety monitoring will be completed by Dr. Klein (PI) on a bi-monthly basis in person or remotely. All laboratory data and adverse events will be collected by the research nurse practitioner and the research investigator and will be reviewed and discussed by Dr. Klein (PI).

At time of informed consent each participant will be assigned a unique participant identifying number (study ID) and the link between ID and PHI will be kept in the REDCap. Consent forms and any other paper forms for the study will be stored in a locked filing cabinet in the office

The study ID is not related information about the individual and is not capable of being translated so as to identify the individual. Data and biological samples collected from study participants during the study participation will be labeled with the study ID and will *not* be used in combination with any personal identifiable information (PII).

All of the requested information as part of this study including PHI will be treated confidentially, protected from improper use and disclosure and will not be reused or disclosed to any other person or entity, except as required by law, for which the use or disclosure of the requested information would be permitted by the HIPAA Privacy Rule.

The use of 38 U.S.C. 7332 Information is to conduct scientific research and that no personnel involved may identify, directly or indirectly, any individual patient or subject in any report of such research or otherwise disclose patient or subjects in any manner.

The PII obtained from human subjects will only be available to individuals approved by WUSM IRB (only after participant signed WU informed consent form). Subject research records will be secured in areas under the 2-key lock concept (e.g., in a locked cabinet with access limited to the study team members in a locked room) at WUSM (Suite 301 at IWJ building). Electronic records (e.g., computer files and database) that contain PHI will be stored on firewall-protected secured servers at WUSM facilities that require approved access authorization.

Data Transfer/Sharing Plan

Data transferred/shared after DUA is approved will be stored at REDCap electronic data capture tools hosted in the Biostatistics Division of Washington University School of Medicine. Any computer security events will be reported to the Research Office and the Facility Information Security Officer (FISO). When study personnel position terminates, access to study data will be terminated. REDCap allows the PI to easily allow and disallow access.

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