Supplemental Information

Methods

The University of Oklahoma Health Sciences Center serves as the single IRB of record and approved all study procedures. The study is monitored by an external Data Safety Monitoring Board that was appointed by the National Institute on Aging. To ensure fidelity between OKC and UWM study sites, we have performed multiple site visits, exchanged reagents, and have monthly study-specific virtual meetings to address recruitment, enrollment, study protocol deviations, amendments and troubleshooting.

Study Population

We are recruiting 148 participants (40-75yrs old) who are either insulin sensitive or insulin resistant defined by a HOMA-IR of $\leq$2.2 or $\geq$2.5. Epidemiological studies using diverse subjects without diabetes have defined insulin resistance using HOMA-IR values of 1.5-3.0[1]. Therefore, we chose the midpoint of these HOMA-IR values $\leq$2.2 to determine insulin sensitivity and $\geq$2.5 to determine insulin resistance. Since some studies have found that HOMA-IR values of $\leq$2.0 define insulin resistance, an alternative exploratory approach will be to assess how alternative thresholds for insulin resistance based on HOMA-IR impact metformin treatment on primary and secondary outcomes. Importantly, HOMA-IR has been successfully used to stratify participants when confirmed using a hyperinsulinemic-euglycemic clamp[2] as performed in the current study.

Pre-Screening

Individuals expressing interest in participating in the study are contacted by the study team to determine potential eligibility using general inclusion/exclusion criteria questions and self-reported medical history.

If subjects are eligible via telephone screening, they are scheduled for health and medical screening and are sent an electronic copy of the informed consent document to review.

Informed Consent, Health and Medical Screening

After documentation of informed consent, we query subjects for their health and physical activity history, including any medical conditions, recent illnesses, COVID-19 symptoms, and medications. Height and weight, resting blood pressure, and heart rate are also collected. Women of childbearing capacity complete a urine pregnancy test. Participants complete medical screening, which consists of a fasting blood draw for HbA1c, glucose and insulin, complete metabolic panel (CMP), Vitamin B12, resting 12-lead ECG, and a pulmonary function test. Supervising study physicians at each site (RK and RHS) will review all information and determine subject eligibility, as defined in **Table 1**. We use the objective distinction of chronic disease versus risk factors for chronic disease as defined by both the CDC and WHO. We will screen subjects to eliminate those with a chronic disease (e.g. cardiovascular disease, type 2 diabetes), but will include people with the following risk factors for cardiometabolic disease: family history, physical inactivity, obesity, hyperlipidemia, hypertension, and impaired fasting glucose (<126 mg/dL). Table 1 also includes a list of drugs or drug classes that have been reported to be contraindicated with metformin, safety concerns with study procedures, or impact the primary outcomes. We are including subjects that use commonly consumed medications to control cholesterol and blood pressure. For example, we have previously found that subjects (40%) are on statins but they have not responded differently to metformin than non-statin users[3,4] .

Randomization

The study biostatistician (KGK) randomizes eligible, consenting participants to metformin and placebo (1:1). The randomization sequence is stratified by study center (OKC or UWM) and baseline insulin

resistance status (HOMA-IR ≤2.2 versus ≥2.5) and created using randomly chosen block sizes of four or six (STATA 16). Only the biostatistician and pharmacy have access to the randomization and stratification scheme. Therefore, all members of the investigative team will remain blinded throughout data collection and analysis.

Body Composition and Continuous Glucose Monitoring

Eligible subjects complete body composition assessments via dual x-ray absorptiometry (DXA; Lunar iDXA, GE Electric, Boston, MA). After the DXA scan, all subjects have the choice to opt in to wearing the continuous glucose monitor (CGM; Dexcom G6 Pro) to evaluate glucose behavior and variability during 7-10 days of free-living conditions that complements our gold-standard laboratory assessments of glucose control and insulin sensitivity. The CGM provides a high-resolution assessment of glucose behavior by measuring interstitial glucose values every 5 minutes (288 data points per 24 hours). The Dexcom G6 CGM has a mean absolute relative difference (MARD) to clinical reference values of 9% across a 10 day wear period[5]. Glucose variability, even in the absence of sustained hyperglycemia, has been shown to have deleterious effects, such as increased oxidative stress, that are associated with aging and chronic diseases[6]. Participants track their physical activity using a 7-day physical activity recall, which for a subset of participants overlaps the CGM wear period. Participants repeat the DXA scan and CGM wear period during the last 7-10 days of study drug leading up to the post-intervention skeletal muscle biopsy and hyperinsulinemic-euglycemic clamp.

Skeletal Muscle Biopsy and Hyperinsulinemic-Euglycemic Clamp

All muscle biopsies and hyperinsulinemic-euglycemic clamps are completed in the Clinical Research Units (CRU) at the Oklahoma Clinical and Translational Science Institute (OCTSI) at OKC and the

Institute of Clinical and Translational Research (ICTR) at UWM. A skeletal muscle biopsy and hyperinsulinemic euglycemic clamp are performed once before and repeated once after the 12-week intervention. The post muscle biopsy and insulin clamp are completed approximately 36 hours after the last study drug administration to understand the long-term effects of metformin and not the acute response.

Subjects are asked to refrain from exercise, alcohol, and aspirin for 24 hours prior to the muscle biopsy and insulin clamp. The night before, participants consume a standardized study meal provided by the study team. The meals (~750 kcal) before and after the intervention are matched for macronutrient composition (40% carbohydrates) to minimize inter and intra-subject variability. Subjects will arrive to the CRU the next morning (~0700) after an overnight fast where body weight and vitals (temperature, heart rate, and blood pressure) are recorded. After resting for ~30 minutes, muscle samples (100-300mg) are obtained from the *vastus lateralis* after administration of local anesthetic (1-2% lidocaine without epinephrine) using a 5mm UCH needle (Millennium Surgical) with manual suction[3,7]. Pressure is held for 10 minutes before the incision is closed with steri-strips and a pressure bandage is applied and worn the remainder of the day. Muscle samples are placed on a culture dish on ice and cleared of visible adipose and connective tissue. Samples are subsequently incubated in ice-cold Buffer Z for fresh tissue mitochondrial function analyses or snap frozen in liquid nitrogen and stored at -80°C for subsequent quantitative and kinetic proteomics.

Approximately 30-45 minutes after the muscle biopsy is performed, an intravenous catheter is placed in a heated hand vein for repeated arterialized-venous blood sampling and in the contralateral forearm for infusion of insulin and dextrose. Blood is obtained before the start of insulin and dextrose infusions for the measurement of glucose, glucoregulatory hormones and proposed biomarkers of aging. Next, peripheral insulin sensitivity is measured during a 180 min hyperinsulinemic-euglycemic clamp similar to previously described procedures[8–10]. Insulin (2.3 $mU \cdot kgFFM^{-1} \cdot min^{-1}$) is infused to mimic post-

prandial insulin values and dextrose (25%) is infused at a variable rate to maintain euglycemia, defined here as a target range of 85-95 mg/dL. Our study will measure insulin sensitivity based on the glucose infusion rate to maintain euglycemia during the last 30 minutes as well as exploratory analysis of total or incremental area under the curve (AUC_{180}, AUC_{120}, AUC_{60}).

Study Drug

Belmar Pharmacy has compounded both metformin (NDC: 23155-102-01) and placebo (PROSOLV® SMCC, Sodium Starch Glycolate, Magnesium Stearate), which is then shipped to each study site pharmacy to dispense to enrolled subjects. The study biostatistician (KK) communicates randomization directly with Belmar Pharmacy to maintain study blind. Metformin and placebo are provided in tablet form and dosed as 500 mg tablets. Study drugs are equal in size and shape, with no distinguishing marks. Study drug is administered using a ramped protocol, starting at 500 mg per day in week 1, increasing to 1000 mg per day in week 2, and then to 1500 mg per day in week 3, as tolerated. The dose is maintained at 1500 mg/day for the remaining 9 weeks. Two 500 mg tablets are taken in the morning, and one 500 mg tablet taken in the evening. After 5 weeks of study drug, participants will complete an additional fasting blood draw for CMP and Vitamin B_{12} to ensure maintenance of normal reference values. If a subject experiences gastrointestinal discomfort or other common side effects associated with metformin while taking 1500 mg per day, the dose is reduced to 1000 mg per day. The minimal dose required to remain enrolled in the study is 1000 mg/day. We have previously found that subjects in both placebo and metformin report symptoms commonly associated with metformin side effects and therefore reporting these symptoms do not jeopardize the study blind[3,4].

Adverse Reaction Monitoring

Although metformin is commonly used to treat diabetes and is safe, there are some notable side effects. Lactic acidosis is the most serious known adverse event and occurs most frequently in patients with impaired liver, kidney or heart function. Those with liver, kidney or heart conditions will be screened out from our study and minimize the risk of lactic acidosis. The most common and less serious side effects of metformin include gastrointestinal distress including loss of appetite, nausea, vomiting, diarrhea, flatulence, or stomach discomfort. There is also a risk of Vitamin B12 deficiency. Participants will be screened for Vitamin B12 levels at baseline and after 5 weeks of study drug.

Deuterium Oxide Labeling and Mitochondrial Protein Turnover

To understand long-term mitochondrial protein remodeling, we use deuterium oxide (D_2O) labeling to measure protein turnover as we have previously published[3,7,11,12]. The principle of the method is the same as other approaches that rely on precursor and product labeling over a period of time to measure a rate of synthesis. In this case, provision of D_2O daily can maintain a steady state body water enrichment where the heavy hydrogens equilibrate with nonessential amino acids through pathways of intermediary metabolism. In our study, the subjects consume 70% D_2O (Millipore Sigma Cat# 756822) 3 x 50 ml/day for one week followed by 2 x 50 ml/day for 3 weeks to maintain a body water enrichment of 1-2%. At the completion of the labeling period, the precursor enrichment is determined from body water enrichment of plasma from each subject, while the product enrichment is determined from proteins of the skeletal muscle biopsy. For this study, we will be using a targeted proteomics approach that allows assessment of the change in concentration and synthesis rates of individual mitochondrial proteins. By measuring both the change in protein concentrations of individual mitochondrial proteins and the rates of synthesis of those individual proteins, we can also calculate protein breakdown. To accomplish these measurements,

approximately 20 mg of the muscle biopsy will be analyzed using targeted proteomics of mitochondrial proteins by LC-MS/MS (ThermoScientific Q-Exactive Plus). These analyses will allow us to determine differences in mitochondrial remodeling in skeletal muscle mitochondria with metformin treatment.

Skeletal Muscle Mitochondrial Bioenergetics

We prepare permeabilized fibers for the assessment of skeletal muscle mitochondrial bioenergetics as previously performed[3,8,11,13]. Since complex I activity is one purported mechanism by which metformin regulates the biology and metabolism of aging, our primary objective is to evaluate complex I-linked submaximal and maximal mitochondrial respiration at both study sites using a similar ADP titration as described previously[3,11,13]. At OKC, there will be an additional protocol using complex I and II substrates (pyruvate-glutamate-malate and succinate) to explore maximal mitochondrial hydrogen peroxide (H_2O_2) emissions. We will determine the sensitivity of mitochondria to ADP to stimulate 50% maximal respiration and to suppress maximal H_2O_2 emissions by 50% from Michaelis-Menten kinetics and one-phase exponential decay analysis to calculate the apparent K_m and half maximal inhibitory concentration (IC_{50}) for ADP[14]. At UWM, we will use a creatine kinase energetic clamp technique described in detail by Fisher-Wellman et al.[15] to model in vivo demands for ATP. Most approaches that assess maximal respiration through an ADP bolus create an ATP/ADP ratio that is not physiologically relevant. The creatine kinase clamp exploits the enzymatic activity of creatine kinase to titrate the extra-mitochondrial ATP/ADP ratio to evaluate whether metformin is changing the mitochondrial respiratory control and conductance across a range of physiological ATP free energy states.

Oxidative Stress Measures

Metformin can alter redox status in the liver[16], while the impact on skeletal muscle is less clear. To determine the redox status of skeletal muscle, we will assess the redox pairs GSH/GSSG, NADPH/NADP+, NADH/NAD+ by ion pairing reverse-phase HPLC quantified using electrochemical, fluorescence, or UV/VIS detection[17]. For a sensitive measure of oxidative damage, we will assess lipid peroxidation by measuring F_2-isoprostanes using GC/MS[17].

Biomarkers of Aging

IL-6, CRP, Tumor Necrosis Factor-RII, GDF15, IGF-1, Cystatin-C, B-type natriuretic peptides (NT-proBNP), and HbA1C were recently suggested as blood-based biomarkers for the use in geroscience clinical trials[18]. To determine whether the proposed biomarkers of aging predict changes in disease-free individuals by metformin treatment, we will assess these outcomes pre and post intervention. These analyses will be performed using the Bioplex 200 bead array system for xMAP in a 4-plex format in a 96 well plate. Some biomarkers will be assessed in plasma samples using analyte-specific direct ELISAs that are commercially available. These analyses will be compared to control (matched age, sex, race) plasma samples. All samples will be randomized across plates in order to control for bias. ELISAs will be read on standard plate readers and will be assessed using the manufacturer-recommended analytical parameters.

Statistical approach

Sample size and power calculations. The primary outcomes from this study are insulin sensitivity and mitochondrial respiration. Based on preliminary data from a 12-week treatment period with metformin, the mean (SD) change in insulin sensitivity (post- minus pre-treatment) was 0.67 (2.86) among insulin

sensitive (HOMA-IR≤2.5) and 1.36 (1.85) among insulin resistant individuals[3]. Assuming no change on average among the placebo-treated participants, a total sample size of 148 (37 per insulin sensitivity group at each site) will result in 80% power to detect an interaction between metformin treatment and baseline insulin sensitivity where the root mean square term (standard deviation) for the interaction is 0.65 or greater (an effect size of 0.25) (PASS13, NCSS, Utah, USA). This calculation is based on a 2x2 factorial ANOVA model and assumes a within-group root mean square error of 2.64, a two-sided 0.05 alpha level, and a withdraw rate of no more than 10% over the 12-week treatment period.

Based on preliminary data from a 12-week treatment period with metformin, the mean (SD) change in the complex I activity (post- minus pre-treatment; $pmol \bullet s^{-1} \bullet mg\ tissue^{-1}$) was 5.86 (16.91) among insulin resistant (HOMA-IR ≥ 2.5) and -0.28 (21.97) among insulin sensitive individuals[3]. Among participants receiving placebo, the estimates were 9.87 (11.93) and 12.01 (15.87), respectively. A total sample size of 148 (37 per insulin sensitivity group at each center) will result in 80% power to detect an interaction between metformin treatment and baseline insulin sensitivity where the root mean square term (standard deviation) for the interaction is 4.0 or greater (an effect size of 0.25) (PASS13, NCSS, Utah, USA). This calculation is based on a 2x2 factorial ANOVA model and assumes a within-group root mean square error of 16.35, a two-sided 0.05 alpha level, and a withdraw rate of no more than 10% over the 12-week treatment period. Similarly, the targeted sample size will result in 80% power to detect an interaction between metformin treatment and baseline insulin sensitivity where the root mean square term (standard deviation) for the interaction is 5.25 or greater (an effect size of 0.25) and the within-group root mean square error is 20.73 based on the ADP titration protocol with mean (SD) among metformin-treated participants of 7.65 (22.85) and -10.19 (17.40) for IR and IS participants, and among placebo-treated participants of 19.43 (23.14) and 6.68 (32.69), respectively.

Statistical analysis plans. The mean change in insulin sensitivity (calculated as the 12-week minus baseline measure) will be compared between groups defined by baseline insulin sensitivity (IR or IS) and metformin/placebo assignment using a 2-factor ANOVA model that includes the treatment by insulin sensitivity interaction. A significant interaction will be followed by analyses stratified by baseline insulin sensitivity. Transformations will be used as appropriate to satisfy modeling assumptions. Analyses will be based on the intention-to-treat principle. A separate per-protocol analysis, analyzing data from all participants who took at least 80% of the pills, will also be performed. Sex will be investigated as a biologic variable by including sex, and interactions with sex, in the ANOVA model. Modification by sex will be explored, however, interpreted cautiously given that the study is not powered to detect modification by sex. A similar approach will be used to explore age, BMI, and physical activity as biologic factors. A mixed effects ANOVA model will be used for the other outcome measures (utilizing day-level average measures) where the model includes fixed effects for baseline insulin sensitivity category, metformin/placebo assignment, time (baseline or 12-week), and all possible interaction effects. A random participant effect also will be included. The primary term of interest is the time by insulin sensitivity by treatment interaction (indicating that the effect of metformin on the outcome following the 12-week treatment period differs depending on baseline insulin sensitivity). The mixed effects ANOVA modeling for repeated measures described for insulin sensitivity will be used to analyze mitochondrial function. Statistical significance will be declared at $p<0.05$. For quantitative proteomics, we will use our previously published methods using a Benjamini-Hochberg false discovery rate correction ($q = 5\%$)[19,20]. Heatmap and principal component analysis (PCA) plot will be constructed with ClustVis with default settings (Row scaling = unit variance scaling, PCA method = SVD with imputation, clustering distance for rows = correlation, clustering method for rows = average, tree ordering for rows = tightest cluster first).

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