Supplemental Information

EVERLAST Study Design and Methods

Methods

EVERLAST is an ongoing, randomized, placebo-controlled, phase 2 clinical trial testing whether mTORC1 inhibition by 24-weeks of once daily (0.5 mg) or once weekly (5 mg) everolimus can improve or maintain physiological and molecular hallmarks of aging (**Figure S5**). We will use a double-dummy design to randomize insulin resistant older adults to one of three groups: 1) once daily everolimus (0.5 mg per day) and once weekly placebo, 2) once daily placebo and once weekly everolimus (5 mg/week) or 3) once daily placebo and once weekly placebo to maintain study blind between everolimus versus placebo and daily versus weekly dosing (**Table S5**). Therefore, all participants will take a capsule once daily and once weekly. All clinical trial activities will take place at the University of Wisconsin-Madison. EVERLAST has received approval by the Health Sciences IRB at UW-Madison (2021-1519) and is registered on clinicaltrials.gov (NCT05835999). Administration of everolimus is conducted under the FDA Investigational New Drug application 161611 according to 21 CFR 312. EVERLAST is monitored by an internal Central Monitoring Services required for FDA regulated research at UW-Madison and by an external Data Safety Monitoring Board that was appointed by the National Institute on Aging.

Dose Selection

Higher doses of mTOR inhibitors are accompanied by greater risks of metabolic disturbances and other adverse events. Our working model indicates that many of the metabolic disturbances by prolonged treatment of high dose rapamycin are mediated by mTORC2 inhibition. In mice, we have found that everolimus and intermittent rapamycin dosing schedules enable more selective mTORC1 inhibition and have less adverse events than daily rapamycin while still extending lifespan[1,2]. We selected the use of daily (0.5 mg/day) or weekly (5mg/week) everolimus because 6-weeks of these doses safely attenuate mTORC1 signaling and improve influenza vaccine efficacy in older adults (n=211; ≥65 yrs, medically stable)[3]. However, the highest weekly everolimus dose tested (20 mg/week) did not improve vaccine efficacy and doubled the number of adverse events compared to the 0.5 mg/day and 5 mg/week dose[3]. Therefore, the 20 mg/week dose was not considered because it did not present an appropriate risk to benefit ratio in otherwise healthy individuals. Collectively, 0.5 mg daily and 5 mg weekly everolimus was well tolerated with mild, transient side effects and improved vaccine efficacy in older adults. To date there are no other studies that have shown improvement in aging-related outcomes in humans after rapamycin treatment and/or higher dosing regimens. Therefore, we chose 0.5 mg/day and 5 mg/week dosing schedules for this Phase 2 clinical trial to test whether longer-term everolimus treatment can improve other clinically relevant outcomes related to healthy aging.

Study Population

Volunteers will be solicited from a recruitment list and databases, email, postal mail, and flyers for the study. We aim to complete the 24-week intervention in 72 participants (n=24/group, 55-80 yrs old) who are either insulin resistant defined by a HOMA-IR of ≥1.5 or prediabetic (fasting glucose: 100-125 mg/dL, 2-hr glucose 140-199 mg/dL, or HbA1c (5.7-6.4%) but free from overt-chronic diseases. Epidemiological studies using diverse subjects without diabetes have defined insulin resistance using HOMA-IR values of 1.5-3.0[4]. Therefore, we chose the lowest HOMA-IR values of ≥1.5 to include those

with mild/early insulin resistance. We also aim to complete all baseline testing but no intervention in 14 adults (18-35 yrs old) to serve as a healthy young reference group. In total we are recruiting 100 participants to account for a ~15% attrition rate to complete the study in 72 insulin resistant, older adults and 14 healthy young adults. All participants will provide written informed consent.

Health and Medical Screening

After documentation of informed consent, we ask participants about their health and physical activity history, including any medical conditions, recent illnesses, hospitalizations, and medications and review electronic medical records. Height and weight, resting blood pressure, and heart rate are also collected. Women of childbearing capacity complete a urine pregnancy test (young group only). Participants complete medical screening, which consists of a fasting blood draw for HbA1c, complete metabolic panel (CMP), complete blood count with differential, lipid panel, and insulin, and a pulmonary function test. On separate visits, participants will complete a resting and exercise ECG and a 75 gram oral glucose tolerance test (detailed below). Supervising study physicians (SP and DM) will review all information and determine subject eligibility, as defined in **Table S6**. As previously described[5], we will use the CDC and WHO guidelines as the objective distinction of chronic disease versus risk factors for chronic disease. 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 everolimus, safety concerns with study procedures, or impact the primary outcomes. We are including subjects that use commonly consumed medications to control cholesterol and some medications to control blood pressure.

Randomization

Eligible participants will be randomized by the study biostatisticians (FO and RC). A sample size of 84 is planned for recruitment. We will therefore randomize 28 participants into each of the three arms of the study 1) once daily everolimus (0.5 mg per day) and once weekly placebo, 2) once daily placebo and once weekly everolimus (5 mg/week) or 3) once daily placebo and once weekly placebo to maintain study blind between everolimus versus placebo and daily versus weekly dosing (**Table S5**). The allocation ratio will be 1:1:1 to allow for equal distribution of participants into each arm. An attrition of 12 subjects is planned to compensate for subject withdrawal from the study due to adverse effects or voluntary withdrawal. We will use a random block size of 6 or 9 to equally assign participants and to avoid chances of selection bias. We will use RedCap for all data collection and randomization The randomization sequence will be created using STATA and uploaded to the RedCap server. Randomization will automatically happen as participants are screened and enrolled into the study. The randomization process will end at the conclusion of the study or when subject recruitment ends. 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, Continuous Glucose Monitoring, and Diet

Eligible subjects complete body composition assessments via dual x-ray absorptiometry (DXA; Lunar iDXA, GE Electric, Boston, MA). After the DXA scan, subjects will wear a continuous glucose monitor (CGM; Dexcom G6 Pro) to evaluate ambulant glucose behavior and variability during 7-10 days

of free-to complement our highly controlled 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). Even in the absence of sustained hyperglycemia, glycemic variability increased oxidative stress which is associated with aging and chronic diseases[6]. Participants will track their diet using a 3-day dietary log and will be analyzed by a registered dietitian (MT) using ESHA Food Processor Nutrition Analysis Software[7]. Participants will repeat the CGM and dietary log at weeks 12 and 24 and repeat the DXA scan during week 25. The CGM wear period will occur during the last 7-10 days of study drug leading up to the post-intervention skeletal muscle biopsy and dual tracer oral glucose tolerance test.

Graded Exercise Testing

To determine VO_2max, eligible participants will complete a graded cardiopulmonary exercise test with 12-lead ECG in the Human Exercise Research Core (HERC). Subjects will cycle on an upright cycle ergometer where the workload increases 20-50 watts per minute until exhaustion. We will measure expired gas using indirect calorimetry and use an RER >1.1 as criteria to determine a true VO_2max as previously performed[8–13].

Skeletal Muscle Biopsy and Dual Tracer Oral Glucose Tolerance Test (OGTT)

All muscle biopsies and dual tracer OGTTs are completed in the Clinical Research Units (CRU) within the Institute of Clinical and Translational Research (ICTR) at University of Wisconsin Hospital. A skeletal muscle biopsy and dual tracer OGTT are performed once before and repeated once after the 24-week intervention. The post muscle biopsy and dual tracer OGTT are completed approximately 2-3 days after the last study drug administration.

Subjects are asked to refrain from exercise for 48 hours before and from alcohol and aspirin for 24 hours before the muscle biopsy and dual tracer OGTT. The night before, participants consume a standardized study meal (~750 kcal) before and after the intervention 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 vitals, subjects will provide a urine and saliva sample. Next, the subjects will rest supine for ~30 min and a retrograde intravenous catheter is placed in a heated hand vein for repeated arterialized-venous blood sampling. The initial blood sample will be used for DNA methylation, RNA sequencing, senescence associated secreted phenotype (SASP) and other proposed biomarkers of aging. A second IV is placed in the contralateral forearm for a primed-continuous infusion of [6,6-^{2}H] glucose (DLM-349-MPT-PK, Cambridge Isotope) for 2.5 hours to measure basal endogenous glucose production.

After the initiation of the [6,6-^{2}H] glucose and ~1.5 hours before the glucose drink, a skeletal muscle biopsy sample (100-300mg) will be 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[8,14]. 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 X for fresh tissue mitochondrial function analyses or snap frozen in liquid nitrogen and stored at -80°C for subsequent immunoblotting and multi-omics.

Serial blood samples are obtained -20, -10, and 0 min before the glucose drink for the measurement of basal blood glucose concentrations, glucoregulatory hormones, and [6,6-^{2}H] glucose enrichment. Next, participants will orally consume a 75-gram glucose drink with the addition of the stable isotope (1-^{13}C) glucose (297046, Millipore Sigma) to achieve an enrichment of ~4%. Participants have up to 5 minutes to consume the drink. The infusion of [6,6-^{2}H] glucose will be titrated to mimic the anticipated change in endogenous glucose production and maintain steady-state enrichment. Blood will be taken 10, 20, 30, 40, 50, 60, 75, 90, and 120 minutes after the first sip of the glucose drink to measure blood glucose and glucoregulatory hormones. We will measure peripheral (primary endpoint) and hepatic insulin sensitivity by calculating the rate of glucose disposal and suppression of endogenous glucose production during the dual tracer OGTT.

Muscle Function

A minimum of 4 days after the muscle biopsy, knee extensor muscle strength and power will be measured using a dynamometer (Biodex) during two consecutive study visits separated by at least 3 days. Each study visit will begin with 5 minutes of treadmill walking or stationary cycling followed by a series of submaximal contractions. The first visit is to determine muscle strength by completing a 1-reptition maximum as previously performed. We will then use 70% of 1RM to test for maximal muscle power. The first visit will be used to familiarize the participants to the power protocol. The second visit will be to obtain maximal power production which will be the greatest power obtained in 3 consecutive repetitions.

Brain MRI and Cognitive Assessments

Participants will undergo MRI on a 3.0T X750 GE Discovery scanner with an 8-channel array head coil. Scan time is ~45 minutes and will include measures of macro-vascular and micro-vascular cerebral blood flow (CBF) through 4D Flow and arterial spin labeling (ASL), respectively. Additional scans will include a 3D T1-weighted volume, and T2 FLAIR to assess ischemic lesion burden. Macro-vascular CBF through 4D Flow analysis will focus on middle cerebral artery, inferior cerebral, and basilar artery which are impaired with age and IR[15–18]. We will also assess micro-vessel CBF using ASL analysis that will focus on specific regions of interest known to show early changes with IR and responsive to the insulin sensitizing effects of exercise—including the posterior cingulate, medial temporal lobe (hippocampus and parahippocampus) and inferior frontal cortex defined by regions of interest (ROIs) from the AAL atlas implemented in WFU PickAtlas. Exploratory analysis of volumetric and T2FLAIR scans will utilize voxel-wise methods previously used by our group, controlling for multiple comparisons with FWE correction[19,20]. We will also explore the integrity of the blood brain barrier using diffusion weighted ASL scans.

Memory and executive function will be measured using 1) the Montreal Cognitive Assessment (MoCA Test); 2) the California Verbal Learning Test-III: learning slope for trials 1-5 and long delay retention; and 3) Executive function as indexed by: the Delis-Kaplan Executive Function System Trails Test, and Color-Word Interference Test. The MoCA test and CVLT-III will be administered on an electronic application using an iPAD.

Echocardiogram

We will perform a limited trans thoracic echocardiogram to evaluate cardiac function. Based on studies showing that rapamycin rescued the age-related decline in fractional shortening (FS) in both mice

and dogs we will use this as our cardiac function outcome[21,22]. In heart transplant patients, everolimus (0.5mg twice daily) added to low dose tacrolimus improved cardiac function as evident from increased left ventricular (LV) strain and decreased LV mass[23]. Therefore, we will also explore the impact of everolimus on LV global peak systolic strain, ejection fraction (EF), E/A ratio, early filling velocity to early diastolic mitral annular velocity (E/E'), left atrial size, and tricuspid regurgitant velocity. Additionally, each of these metrics decrease with age and increases cardiac event risk[24,25].

Study Drug

Doyles Pharmacy over encapsulates everolimus (NDC 00054-0471-21; NDC 0054-0481-13) and placebo (Microcrystalline Cellulose NF PH-105). Everolimus capsules are backfilled with the same excipient as placebo. Study drug is shipped to the Pharmaceutical Research Center in the ICTR. The study biostatistician (FO) communicates randomization directly with Doyles Pharmacy to maintain study blind. Study drugs are equal in size and shape, with no distinguishing marks. If a subject experiences known common side effects associated with everolimus; participants can take the 0.5 mg everolimus every other day or skip a weekly dose (5 mg/week). Based on our previous work with other proposed geroprotector drugs, subjects in both active and placebo report symptoms commonly associated with study drug side effects and therefore reporting these symptoms does not jeopardize the study blind[8,26].

Pharmacokinetics

After 4 weeks of study drug, participants will come to the CRU to evaluate everolimus pharmacokinetics. Participants will receive a fasted blood sample (pre-dose) and additional blood samples 0.5, 1.5 and 4 hrs after taking daily and weekly capsule. Whole blood will be collected in EDTA vacutainers and everolimus will be evaluated through the CILA certified UW-Health Clinical Laboratory via Quantitative Liquid Chromatography-Tandem Mass Spectrometry.

Adverse Event Monitoring

Numerous side effects, with some being serious, have been reported in patients taking rapamycin and rapamycin analogs at doses consistent with the FDA label. Everolimus has immunosuppressive properties which increases the risk of infection from bacteria, viruses and fungi; decreased immune response to vaccinations while actively on treatment; and increased risk of cancer, particularly skin cancer. Additionally, the most common side effects may include mouth, tongue, gum blisters, sores or ulcers; weakness or fatigue; fever, cough, headache; nausea, loss of appetite, diarrhea, indigestion; edema; increase in cholesterol and triglycerides; or increase in blood sugar or HbA1c and less likely new-onset diabetes. These side effects are largely based off patient populations being treated for cancer or organ transplant who receive 1.5 to 10 mg of everolimus per day. This study will use 0.5 mg per day or 5 mg per week of everolimus in relatively healthy people to help minimize these risks.

A minimum of every 4 weeks, participants will receive a fasted blood draw to determine any changes to blood chemistries, blood cell counts, lipids, and insulin. At 12 and 24-weeks we will also measure HbA1c. Blood samples will be taken ~24hrs after the last study drug dose to perform therapeutic drug monitoring for everolimus. We will also have participants complete a 20-point questionnaire to query about potential adverse events, review diaries with participants and record any self-reported adverse events using Common Terminology Criteria for Adverse Events (CTCAE) V6.0.

Surveys on health and geriatric conditions

Participants will also complete a series of validated questionnaires and surveys to understand whether mTOR inhibitors can improve participants perception of their overall health and wellness and select conditions, such as joint pain, risk of obstructive sleep apnea, sleep duration and quality, and lower urinary tract symptoms as outlined in the RAP PAC supplement.

Whole Genome Methylation

To evaluate blood DNA methylation as a potential biomarker of human aging and in response to geroprotective therapies, we will perform whole genome sequencing with bisulfite pre-treatment[27–30]. This unbiased approach uses bisulfite exposure and deamination chemistry to convert unmethylated cytosines to uracil, while leaving methylated cytosines unmodified. Subsequent sequencing of the treated DNAs provides single base-pair resolution of all methylated sites in the human genome, and will expose novel genes and alleles of interest if present. A 10 mL whole blood sample will be collected from each participant before the muscle biopsy and OGTT, anticoagulated in EDTA, and stored at -20°C. Genomic DNA will be extracted using the Gentra Puregene Blood kit (Qiagen). DNA-sequencing libraries will be constructed using ~50 nanograms of high molecular weight genomic DNA and the Pico Methyl-Seq Library Prep Kit (ZymoResearch). Whole genome sequencing will be performed on Illumina NovaSeq600 to yield ~150 billion 150 bp-sequence reads for each library, thereby providing the methylation status of ~25 million CpG positions in the linear DNA sequence of bases along its 5' → 3' direction when a cytosine nucleotide is followed by a guanine nucleotide (*i.e.*, CpG sites), with a coverage depth of ≥50 sequence reads. Image processing and sequence extraction use the Illumina Pipeline.

Multi-Omics

We will perform a broad and unbiased multi-omics approach to identify metabolic pathways and biological mechanisms engaged by mTORC1 inhibition by everolimus and sirolimus treatment. We will collect 3mL of whole blood in a RNA-stabilizing tube, and isolate whole blood RNA. RNA will also be isolated from a portion of the skeletal muscle biopsy samples. RNA samples will be sequenced via Illumina sequencing at the UW-Biotechnology Center, which we have previously utilized for transcriptional profiling studies[31–33]. Analysis of significantly differentially expressed genes (DEGs) will be completed in R using *edge* [34] and *limma*[35]*.* To reduce the impact of external factors not of biological interest that may affect expression, data will be normalized to ensure the expression distributions of each sample are within a similar range, using the trimmed mean of M-values (TMM), which scales to library size. Heteroscedasticity will be accounted for using the voom function, DEGs were identified using an empirical Bayes moderated linear model, and log coefficients and Benjamini-Hochberg (BH) adjusted p-values will be generated for each comparison of interest[36]. DEGs will be used to identify enriched pathways, with both Gene Ontology (for Biological Processes) and KEGG enriched pathways determined for each contrast.

Blood plasma will be collected in a separate tube, and along with a portion of the skeletal muscle biopsy will be processed using a methyl-tert-butyl ether extraction. The organic phase will be used for untargeted lipidomics by quadrupole time of flight liquid chromatography mass spectrometry (QTOF-LC/MS) while the aqueous phase will be processed for untargeted metabolomics using GCMS. Annotation of the lipids and metabolites will be performed with LipidAnnotator and with a curated personal compound database library, respectively[33,37,38]. Metabolomics and lipidomics data will be normalized and

analyzed using the *metabolomics* package in R. Pathway enrichment of significantly altered metabolites and lipids will be conducted using Metaboanalyst[39] and Lipid Ontology (LION) respectively[40].

To identify clusters of physiological and molecular changes that respond similarly to everolimus treatment, significantly differentially expressed molecules found between everolimus vs control groups will be identified and integrated. Metabolomics, transcriptomics, and lipidomics data will be log2 transformed, z-scale normalized across molecules and samples for each data type individually. Phenotypic data will be similarly z-scale normalized across phenotypes. We will concatenate all four data types for comparison. Correlations will be performed using Spearman's rank, hierarchical clustering will be used and number of clusters will be determined using silhouette scores. For each cluster, the over representation of KEGG pathways from genes will be determined using kegga and the gene ontology terms will be determined using goana from limma.

Skeletal Muscle Mitochondrial Bioenergetics

We prepare permeabilized fibers for the assessment of skeletal muscle mitochondrial bioenergetics as previously performed[8,9,41,42]. We will provide complex I and II linked substrates (succinate, glutamate, maleate, pyruvate) to stimulate leak respiration and explore maximal mitochondrial hydrogen peroxide (H_2O_2) emissions. We will then perform an ADP titration to 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[43]. We will also evaluate maximal oxidative phosphorylation (OXPHOS) and electron transfer system (ETS) capacity.

Statistical approach

- Intention-to-Treat (ITT): Our primary and secondary outcomes will include ITT for all randomized subjects.
- Safety Analysis Dataset: clinical blood work and adverse events reporting will be conducted on all randomized participants.
- Protocol-compliant Population: Our exploratory analysis will include all randomized subjects who received the required study intervention and complied with the protocol sufficiently (85%) to ensure that the data would be likely to represent the effects of the study intervention according to the underlying scientific model.

Demographics

For patient baseline data, we will summarize the categorical variables using counts and percentages. Categorical group comparisons will be conducted using a chi-squared test or Fisher's exact test. All continuous data will be summarized as mean plus standard deviation (SD) or median plus inter-quartile range (IQR) and compared between groups using the analysis of variance (ANOVA). Continuous data that is not normally distributed will be compared using the non-parametric Kruskal-Wallis test. We will use histograms and Bartlett's test to determine the skewness and normality of continuous variables. P-values less than or equal to 0.05 will be considered significant. We will use both STATA 16 and SAS 9.4

for all analyses (StataCorp. 2021. Stata Statistical Software: Release 17. College Station, TX: StataCorp LLC).

Primary Outcome Analysis (Placebo vs. each treatment)

Our primary endpoint is the change in peripheral insulin sensitivity as determined by the rate of glucose disposal relative to circulating insulin during a 75g oral glucose tolerance test (OGTT) at baseline (pre-intervention) and 24 weeks after the baseline (post-intervention). The primary analysis will compare intervention groups (daily everolimus, weekly everolimus) versus the placebo group using the mean change in peripheral insulin sensitivity from the pre-and post-intervention, in a linear mixed model. Our goal is to detect significant differences between treatment groups compared to the placebo subjects. Both random (study participants) and fixed effects (treatment groups) will be considered in the model. We will report estimates from the linear mixed models using beta coefficients, 95% confidence intervals, and p-values. We will obtain predicted means and graphics using predicted margins and marginsplots.

Secondary Outcome Analysis

Our secondary outcomes include cardiac fractional shortening, cerebral macro and micro vessel blood flow, number and frequency of adverse events, and phosphorylation status of mTOR and downstream substrates in blood and muscle as a readout for mTOR signaling. Secondary outcomes are collected at baseline and 24 weeks. We will analyze secondary outcomes using the same methods specified for the primary outcome with an emphasis on non-parametric alternatives for skewed or non-normal data.

Exploratory Analysis

Our exploratory analysis includes cardiorespiratory fitness, knee extensor muscle function, hepatic insulin sensitivity, glucose variability and glucose tolerance. We will examine changes in exploratory phenotypic endpoints using mean changes from the pre- and post-measures. We will employ the methods for our secondary analysis as part of this exploration as deemed fit with the sample size and the group comparisons. Comparisons between the placebo and treatment groups will be made using univariate and adjusted-multivariate logistic regression models. Estimates from this model will be reported as odds ratios with 95% confidence limits.

We will also perform a multi-omics approach (epigenomics, transcriptomics, proteomics, metabolomics, and lipidomics) as well as assessments of skeletal muscle mitochondrial bioenergetics and markers of the senescence-associated secretory phenotype (SASP) to understand the basis for how mTOR inhibition by everolimus impacts fundamental biological mechanisms of aging.

Subgroup analysis

Treatment vs. treatment (Daily vs weekly dosing groups)

Sub-group analysis of both the primary and secondary outcomes will be compared between subjects in the daily treatment to those in the weekly treatment group. Our goal in making this comparison is to determine whether certain predictors in outcomes will differ just between the treated subjects. We will make comparisons using the Wilcoxon sign rank test for numerical data and the Fisher's exact test for categorical variables. Further, we will use a Bayesian linear mixed model with fixed and random effects for all continuous outcome comparison and Bayesian logistic mixed models for binary outcomes. We will report coefficients and variance estimates of the random.

Placebo vs. combined treatment groups (both daily and weekly)

While there could be differences between the treatment groups in our current study, the literature does not show that these treatment dosing groups statistically differ from one another. Therefore, we aim to conduct a supplementary analysis that combines both treatment groups compared to the placebo. This has the potential to increase the overall power of the comparison and aid in detecting in subtle but clinically meaningful differences in our pre-specified outcomes. We will use similar statistical tests proposed in the "treatment vs. treatment" comparison above.

Age-related conditions

Sub-group analysis of patients with risk factors for age-related chronic conditions such as hypertension and hypercholesterolemia will be examined where sample size permits. We will use the same methods for the primary and secondary outcomes as fit. We will examine the incidence of acute conditions within sub-groups with greater chances of reporting these conditions such as the incidence of mouth ulcers and/or survival outcomes using cox proportional hazard models and Poisson regression with incidence rates. Further, the incidence of acute conditions between the treatment and placebo will be graphically examined using Kaplan-Meier curves or Nelson-Aalen cumulative hazard functions.

Adherence Analysis

Adherence is defined as compliance of 85% and greater. This is calculated from a percent of the total number of pills taken versus the number of pills administered. We will call the compliance rate based on the logs collected at 4, 8, 12, 16, 20, and 24 weeks. Adherence can serve as a valuable confounder for the primary and second endpoints. We will compare compliance rates between treated groups and the placebo. Additionally, we will adjust for compliance in all regression models in order to capture the true effect of the intervention.

Covariates and regression diagnostics

All adjusted models will include key confounders such as baseline metabolic health (HOMA-IR, fasting glucose, glycemic variability, insulin sensitivity, etc.), body mass index, physical activity status, and dietary caloric content, macronutrient composition, pharmacokinetics and trough everolimus blood concentrations. We will create interaction terms between known covariates and the primary/secondary outcomes. We will assess for multicollinearity between all independent variables and selected covariates using the variance inflation factor (VIF) to determine the strength and correlation between these factors. A VIF value below 1 will be considered non-threatening to the model while a VIF value above 5 will be considered as a significant collinear value. We will simply exclude all collinear variables from the specific regression model of interest.

Stratification

From our crude analysis, we will identify strata of subjects with outcome differences such as age or sex. We will examine these differences using similar methods as previously specified for our primary and secondary outcomes.

Multiple comparisons

All multiple mean comparisons will be adjusted for significance testing using the Sidak post-hoc test. The Sidak test provides more power for difference testing. The Sidak equation is denoted as, $\alpha S{-}B = 1 - (1 -$

αFWE)1/K .Where: αS−B = Sidak : k = number of comparisons (for example, 21 variables) and: αFWE = Familywise error.

Table S3 “Double Dummy” Design	Bottle A (Once Daily)	Bottle B (Once Weekly)
Placebo (n=28)	Placebo	Placebo
0.5 mg/day everolimus (n=28)	0.5 mg everolimus	Placebo
5.0 mg/week everolimus (n=28)	Placebo	5 mg everolimus

TABLE S4 Inclusion and Exclusion Criteria
Inclusion Criteria
1. Young (18-35 years) or older (55-80 years) adults of age free of overt chronic disease
2. Willing to provide informed consent.
3. Willing to comply with all study procedures and be available for the duration of the study.
4. Able to use and be contacted by the telephone
5. Ability to take oral medication. (N/A to young).
6. Insulin resistant based on a HOMA-IR of ≥1.5 or prediabetic defined here as impaired fasting glucose (100-125 mg/dL), HbA1c (5.7-6.4%), glucose 2 hours after a 75 gram oral glucose tolerance test (140-199 mg/dL), previous diagnosis of prediabetes in the past year. (N/A to young).
7. Not planning to change diet or physical activity status
8. Adequate organ function as indicated by standard laboratory tests: hematology (complete blood count), and clinical chemistry.
9. Women of childbearing potential must have a negative urine pregnancy test before DEXA and before the OGTT.
10. Women of childbearing potential in sexual relationships with men must use an acceptable method of contraception from 30 days prior to enrollment until 4 weeks after completing study visits. Males must agree to avoid impregnation of women during and for four weeks after completing study visits through use of an acceptable method of contraception.
Exclusion Criteria
1. Pregnancy or breastfeeding
2. Heart disease (history, abnormal ECG, abnormal stress ECG)
3. Cerebrovascular disease (history)
4. Cancer or less than 5 years in remission (history)
5. Chronic respiratory disease (history, if both FEV1/FVC < 70 and FEV1 < 80% predicted)
6. Chronic liver disease (history, abnormal blood liver panel, ALT >104 IU/L, AST >80 IU/L)
7. Diabetes (history, HbA1C ≥ 6.5, fasting blood glucose≥126 mg/dl, OGTT ≥ 200 mg/dl at 2 hrs)
8. Alzheimer's (history)
9. Chronic kidney disease (history, abnormal blood kidney panel including serum creatinine > 1.4, eGFR <60 ml/min/1.73m^2, and urine protein creatinine ratio >0.3 mg/mg)
10. Problems with bleeding, on medication that prolongs bleeding time (if subject cannot safely stop prior to biopsy)
11. Taking azathioprine (Imuran), cyclosporine (Gengraf, neoral, Sandimmune), dexamethasone (Decadron, Dexpak), methotrexate (Rhumatrex, Trexall), prednisolone (Orapred, Pediapred, Prelone), prednisone (Sterapred, sirolimus (Rapamune) and tacrolimus (prograf) or other medications proposed to lower immune system. Daily use of high potency topical corticosteroids used on greater than or equal to 10% of body surface area will not be eligible. Once daily nasal sprays or inhaled corticosteroids will be reviewed on a case-by-case basis.
12. Taking strong or moderate CYP3A4 and/or P-glycoprotein (PgP) inhibitors such as ketoconazole, itraconazole, clarithromycin, atazanavir, nefazodone, saquinavir, telithromycin, ritonavir, indinavir, nelfinavir, voriconazole, amprenavir, fosamprenavir, aprepitant, erythromycin, fluconazole, verapamil, diltiazem
13. Taking strong CYP3A4 activators such as phenytoin, carbamazepine, rifampin, rifabutin, rifapentine, phenobarbital.
14. Subjects who are not willing to restrict the use of grapefruit, grapefruit juice, cannabidiol, and other foods/substances that are known to inhibit cytochrome P450 and PgP activity and may increase everolimus exposures and should be avoided during treatment

15. Subjects who are not willing to restrict the use of St. John's Wort (Hypericum perforatum) because it may decrease everolimus exposure unpredictably.
16. Subjects taking daily NSAIDs with the exception of baby aspirin (81mg)
17. Subjects who are not willing to avoid blood donations 8 weeks prior to the first visit and 8 weeks after the last visit.
18. Contraindications with MRI which could include metal on your body.
19. Low white-blood cell count (<4,000 cell/µL)
20. History of stomatitis or ulcers in the mouth
21. Those on glucose lowering drugs
22. Participating in intensive exercise training program (high to moderate intensity exercise greater than 150 minutes per week) or planning to start new exercise program during study period.
23. Tobacco use
24. Allergies to lidocaine or everolimus
25. Subjects currently enrolled in other clinical trials. Subjects may be eligible after a washout period that will be reviewed on a case-by-case basis. 26. Individuals with limited English proficiency
27. Subjects who are planning to have elective surgery 12 weeks prior to or during the intervention

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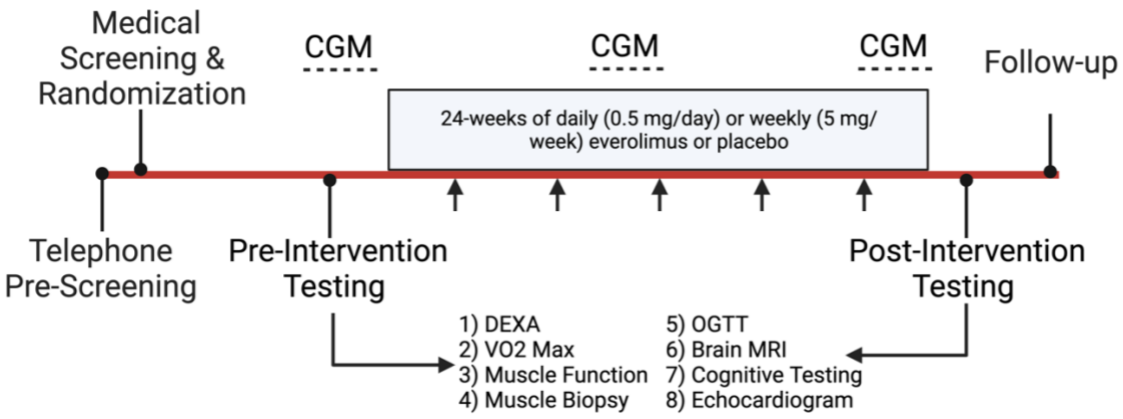
Figure Legends

Figure S5. Representative overview of the study timeline. After medical screening, participants will be randomized and complete several visits to assess metabolic, cardiac, cognitive and physical function before and after 24-weeks of daily (0.5 mg/day) or weekly (5mg/week) everolimus or placebo. Continuous glucose monitoring (CGM) for 7-10 days will occur on three occasions, once before, during and at the end of the 24-weeks. Every four weeks (as shown by the ↑), participants will come to the clinical research unit for adverse event review, concomitant medication reporting, clinical blood work, and exchange of study drug. After the first four weeks, participants will complete serial blood draws to evaluate pharmacokinetics.

Figure S5


Medical Screening & Randomization
CGM
CGM
CGM
Follow-up
24-weeks of daily (0.5 mg/day) or weekly (5 mg/ week) everolimus or placebo
Telephone Pre-Screening
Pre-Intervention Testing
Post-Intervention Testing
1) DEXA
2) VO2 Max
3) Muscle Function
4) Muscle Biopsy
5) OGTT
6) Brain MRI
7) Cognitive Testing
8) Echocardiogram