S-Equol in Alzheimer's Disease 2 (SEAD2) Trial

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1.0 Introduction

Introductory Statement

This study is called the "S-Equol in Alzheimer's Disease 2" (SEAD2) Study. The SEAD2 study will test whether S-equol can improve a particular energy metabolism deficit that is found in persons with Alzheimer's disease (AD). Specifically, cytochrome oxidase (COX) activity is reduced in both brains and platelets from AD subjects. S-equol, an estrogen receptor β (ERβ) agonist, when administered with other estrogen receptor β (ERβ) agonists, has been shown to increase mouse brain COX activity [1]. Subsequently, a pilot clinical study of S-equol generated data consistent with a possible increase in AD patient platelet mitochondria COX activity. In that study, the original S-equol in Alzheimer's Disease (SEAD) trial, S-equol (10 mg twice daily) was administered to 15 women with AD. The platelet mitochondria COX activity before initiating S-equol (lead-in), after two weeks of S-equol (active treatment), and two weeks after stopping S-equol (wash-out) was determined. Because the intra-individual variation of this enzyme across samples taken at different times was unknown, an anticipated pattern of response design was used as the primary outcome. Participants whose lead-in to active treatment slope exceeded their active treatment to wash-out slope were considered to show a positive response pattern. The primary outcome was reached, as 11 positive responses were required to reject the null hypothesis and 11 positive responses were observed ($p<0.06$).

For the SEAD2 trial, Ausio Pharmaceuticals, LLC will supply S-equol capsules to investigators at the Kansas University Medical Center (KUMC). The KUMC investigators will provide the S-equol capsules to AD patients, and determine whether the S-equol increases platelet COX activity. Because the original SEAD trial addressed issues of intra-individual variation for this enzyme across samples taken at different times, we were able to design SEAD2 so that the intra-individual differences in COX activity observed when subjects are on S-equol versus placebo can serve as continuous values that inform the primary outcome (i.e. does S-equol produce a significant increase in mean platelet mitochondria COX activity over placebo).

As part of an exploratory sub-study, SEAD2 will also evaluate whether platelet mitochondria COX activity is lower in AD participants who possess the APOE4 variant of the APOE gene than it is in AD participants who do not possess the APOE4 variant of the APOE gene. This is an important question to address because during the original SEAD study, a secondary data analysis found platelet mitochondria COX activity was lower in APOE4 carriers than it was in non-carriers. This observation was based on a limited number of participants and therefore we currently consider this a preliminary finding. If validated in a larger number of participants, this finding could have important implications for our understanding of AD etiology, biomarker development, and therapeutic development. In addition, the SEAD2 trial will explore if there are correlations between platelet mitochondria COX activity and cognitive abilities in both APOE4 carriers and non-carriers.

2.0 Objectives

Description of Primary Objective

The primary trial endpoint is the mean intra-individual difference in platelet mitochondria COX activity that is measured when subjects have been treated for one month with S-equol (50 mg twice daily) versus one month with placebo. The study, therefore, uses a crossover design to facilitate a paired statistical analysis for data obtained from each of the treated participants. As the original SEAD study suggested APOE4 non-carriers may experience a more robust response to S-equol than APOE4 carriers, to maximize the power of this study the primary outcome measure will focus on APOE4 non-carrier participants.

Description of Secondary Objectives

Our secondary objectives are as follows:

(1) Determine whether S-equol, in a dose of 50 mg twice daily over one month, is safe and tolerated when taken by AD participants. This secondary objective endpoint is a composite that takes into account vital signs, standard blood-based safety screens, physical and neurological examinations, and ascertainment of participant and caregiver-provided symptom complaints.
(2) Determine whether S-equol, in a dose of 50 mg twice daily over one month, influences participant scores on a cognitive assessment test battery.
(3) Determine whether S-equol, in a dose of 50 mg twice daily over one month, produces evidence of increased platelet mitochondria COX activity within the context of an anticipated pattern of response analysis.

Description of Exploratory Objectives

Exploratory objectives in the SEAD2 trial include:

1) Determine whether platelet mitochondria COX activity is lower in AD participants who possess the APOE4 variant of the APOE gene than it is in AD participants who do not possess the APOE4 variant of the APOE gene.

2) Examine whether there are correlations between platelet mitochondria COX activity and cognitive abilities.

3.0 BACKGROUND

Prevalence

The elderly represent a rapidly growing demographic. This reflects public health initiatives and medical advances that reduce risks and provide treatments for many previously terminal disorders. An increasing population of neurodegenerative disease victims is arguably the biggest downside to this success. For several common neurodegenerative diseases incidence rises with advancing age and prevalence is quite high [2]. AD, the most common neurodegenerative disease, affects 5.4 million Americans and one in every eight Americans over 65 is estimated to

have it [3]. Society is also affected, as families and friends of AD patients provide most day-to-day care and altogether AD now costs our economy $385 billion annually [4].

Rationale for Use

Associations between aging, AD, and mitochondrial function are well-documented [5]. Deficits that arise with advancing age tend to exaggerate in AD. In AD brains various mitochondria-localized enzymes show reduced activity [6], and in most neurons intact mitochondria are numerically reduced [7]. Investigators increasingly agree AD mitochondrial dysfunction is disease-relevant and a reasonable therapeutic target [8].

A consistently demonstrated AD mitochondrial lesion includes reduced COX activity [6]. Interestingly, COX activity is reduced in both brain and platelet mitochondria obtained from AD subjects. While it is only possible to harvest brain mitochondria from autopsy brains, platelets are easily obtained from living subjects and can be serially acquired. For drugs that may enhance mitochondrial function, platelet COX activity offers a unique opportunity for assessing mitochondrial target engagement.

"Mitochondrial medicine" refers to treating disease by therapeutically targeting mitochondria [9]. More recently, the term "bioenergetic medicine" was introduced to describe interventions that specifically increase cell energy production [10]. For a neurodegenerative disease such as AD, the ideal agent must be systemically safe, cross the blood brain barrier, access neurons, potentially activate mitochondrial biogenesis, and possibly increase mitochondrial respiration.

Description of study agent

S-equol is an ERβ agonist [11, 12]. ERβ is found within mitochondria, and ERβ activation reportedly stimulates mitochondrial function. ERβ has also been implicated in mitochondrial biogenesis, the process through which new mitochondria are generated within cells [13], and which partly determines a cell's mitochondrial mass [14]. For these reasons, we hypothesize S-equol may potentially benefit AD patients.

4.0 PARTICIPANT SELECTION

AD participants will be recruited by the University of Kansas Alzheimer's Disease Center's (KU ADC) Recruitment Division, and will meet current McKhann el al. criteria for probable AD [15]. Those qualifying for the trial intervention must be found at screening to not carry a copy of the APOE 4 gene. Each participant must have a study partner. The study partner will be someone who knows the participant well (typically a spouse, relative, or friend of the participant) and is able to answer questions about the participant's changes in health and/or behaviors over the course of the study. In addition, the study partner will oversee the administration of the study medication at home and agree to accompany the participant to all study visits.

Enrollment Information

SEAD2 will recruit and consent approximately 100 participants and the study will include both men and women. Participants will be recruited from various sources including the KU ADC Clinical Cohort registry and other research recruitment databases such as the KU Heron database, the University of Kansas Medical Center's Memory Disorders Clinic (KU MDC), and persons previously diagnosed with AD who contact the KU ADC Recruitment Division in order to enroll in an AD clinical trial. Our intent is to ultimately identify approximately 40 individuals who meet all study entry criteria, including being non-carriers of the APOE4 variant of the APOE gene, as power calculations performed on data from the first SEAD study indicate the least number of study participants needed to show a mean increase in platelet mitochondria COX activity is observed when just APOE4 non-carrier participants are considered.

Study Recruitment

Based on historical data, approximately 40-60% of AD participants do not carry an APOE4 allele. If we were to consent and screen participants with no *a priori* knowledge of their APOE status, we conservatively estimate we would need to consent approximately 100 participants to identify approximately 40 APOE4 non-carrier participants. Our decision to identify approximately 40 APOE4 non-carriers is based on an analysis of the original SEAD study data; a power calculation performed using those data determined 35 participants should provide an 80% chance of showing an S-equol-associated increase in the mean platelet mitochondria COX activity of the group (as compared to the mean platelet mitochondria COX activity of the group following a period of placebo treatment) if such an effect indeed exists. To account for attrition and ensure that a minimum of 35 subjects complete the intervention, we plan to enroll approximately 40 participants. Additional participants may be enrolled if participants are in screening at the time of enrollment of the 40^{th} participant.

We plan to enrich our recruitment of approximately 40 APOE4 non-carrier study participants by recruiting participants through the KU MDC and KU ADC whose APOE genotype status has already been determined. Some individuals followed by the KU MDC have previously undergone APOE genotype screening as part of their routine clinical diagnostic evaluation and are aware of their APOE genotype. For individuals with a known APOE genotype (i.e. participants for whom the study coordinator, the participant, and the study partner already know an APOE4 allele is not present) that are recruited through the KU MDC, genetic counseling will still be provided during the consent process. Repeat APOE genotyping will also be performed to confirm the accuracy of the commercial genotype test, although we do not anticipate that the commercial determination will be found to be inaccurate.

We have already determined the APOE status for members of the KU ADC Clinical Cohort. However, KU ADC Cohort members were APOE genotyped under a clinical protocol that does not permit us to disclose APOE status to these individuals. Therefore, when recruiting from the KU ADC Clinical Cohort, for every 4 APOE4 non-carriers we invite to participate we will also invite 1 APOE4 carrier. During the consenting process we will provide genetic counseling about the implications of APOE genotyping and explain to the invited individuals and their care

providers that following informed consent APOE status will be determined and they will be told whether the individual does or does not possess an APOE4 allele. All participants recruited through the KU ADC Clinical Cohort will, therefore, undergo repeat APOE genotyping as part of the SEAD2 study.

Additional participants will be recruited from outside the KU MDC or the KU ADC Clinical Cohort (i.e. through word of mouth, media appeals, and from individuals who for other reasons contact the KU ADC Recruitment Division specifically to learn about potential clinical trial options). For these individuals, we will likely have no *a priori* knowledge of their APOE genotype. During the consenting process we will provide genetic counseling about the implications of APOE genotyping and explain to these individuals and their care providers that, following informed consent, their APOE status will be determined and they will be told whether the participant does or does not possess an APOE4 allele.

Intervention Inclusion Criteria

Individuals are eligible if they meet the following criteria:

1. Have a diagnosis of AD, per McKhann et al. criteria [15].

2. Have a study partner. The study partner must have a close relationship with the participant and have sufficient contact, such that observations of any changes in health or behaviors can be made. The study partner must be willing to attend study visits with the participant, be willing to answer questions regarding any changes in the participant's health/well-being and/or changes in medications, and help to ensure the study medication is taken as directed.

3. Do not possess an APOE4 variant of the APOE gene.

4. Speak English as their primary language.

5. Are age 50 to 90.

6. Have not had any medication changes within the past 30 days.

Intervention Exclusion Criteria

Individuals are ineligible if they meet any of the following criteria:

1. Reside in a nursing home or dementia special care unit.

2. Have a potentially confounding, serious medical risk such as insulin-requiring diabetes, any history of cancer that required a systemic chemotherapy or radiation therapy intervention within the past 5 years (basal or squamous cell cancer and localized prostate cancer that has been treated successfully within the past 5 years is allowed), or a recent cardiac event (i.e. heart attack, angioplasty, etc.).

3. Have any clinically significant abnormal safety laboratory values at the SEAD2 screening visit.

4. Have any clinically significant abnormal findings on vital signs measurements, or on physical or neurological examination at the SEAD2 screening visit.

5. Use any type of systemic estrogen or testosterone replacement therapy.

6. Has received any investigational drug or investigational therapy within 30 days before the Screening Visit.

5.0 TREATMENT PLAN

Overall Study Design

SEAD2 is a randomized, double-blind, crossover study whose primary endpoint is platelet mitochondria COX activity. We will test for differences in platelet mitochondria COX activity through a paired statistical analysis of the difference in COX activity means observed when participants are maintained for 1 month on S-equol versus 1 month on placebo. It will specifically focus on how S-equol affects the primary endpoint in AD participants who do not possess an APOE4 allele, since SEAD study data suggest APOE4 non-carriers may have a more robust platelet COX activity response to S-equol treatment.

Study Procedures

Visit 1 (also referred to as the Screening Visit): Informed Consent, Study Introduction, Genetic Counseling, Demographics, Medical History, Medication Review, Vital Signs, Physical and Neurological Examination, Blood Samples for Safety Laboratory Testing, APOE Status, Baseline COX Activity.

For the evening that precedes this visit, participants will be informed to take nothing per oral (NPO), other than water or coffee or medications, after midnight. The visit will occur in the morning, and will be conducted at the KU Clinical Translational Science Unit (CTSU). Prior to any study procedures taking place, informed consent will be obtained. As part of the informed consent process, genetic counseling about the implications of APOE genotyping will be provided (the implications of APOE genotyping include learning whether one had an increased genetic risk of developing AD; family members may also gain insight into their chances of having inherited an APOE4 allele). Participants will be told that only those who do not carry an APOE4 allele will be able to proceed to the intervention part of the study, and that if they proceed to the intervention part of the study at different points they will receive either S-equol or an inert placebo.

Following consent, the participant's demographic information, full medical history, and current medications will be reviewed/recorded with both the participant and study partner. A diagnosis of Alzheimer's disease will be confirmed by obtaining copies of the participant's medical

records. Participants will undergo a measurement of vital signs and a physical and neurological examination to ensure he/she meets study entry criteria and does not have any findings of concern regarding health. Participants will then undergo a 48 ml phlebotomy, for the following testing:

- Approximately 8 ml of the blood will be used for safety lab testing, to include chemistry, liver function, renal function, and complete blood cell counts
- Approximately 1 mL of the blood will be used for APOE genotyping
- Approximately 39 ml of the blood will be used to determine platelet mitochondria COX activity

Visit 2: APOE Genotype Disclosure, Cognitive Testing, and Randomization. Visit 2 will occur at the KU CTSU. The outcome of the APOE genotype test will be shared. For the APOE4 carrier participants we will not disclose whether they are APOE4 homozygotes or heterozygotes, only that they are APOE4 carriers.

All participants will undergo a cognitive screening battery. For APOE4 non-carriers, this cognitive screen will determine the cognitive baseline performance. For APOE4 carriers, the cognitive performance data will be analyzed to test for correlations between platelet mitochondria COX activity and cognitive abilities. For APOE4 carriers, this will complete their participation in the study.

APOE4 non-carrier participants will be randomized (assignments will be established prior to the visit by the study statistician) to receive S-equol between Visit 2 and Visit 3 with placebo between Visit 3 and Visit 4, or to receive placebo between Visit 2 and Visit 3 with S-equol between Visit 3 and Visit 4. Participants and other study staff will remain blinded to the assignment, allowing this to proceed as a double-blind study. Randomized participants will also:

1) Undergo vital sign measurements.
2) Receive two bottles (each bottle contains 35 capsules) of either S-equol 50 mg or placebo capsules (the placebo capsules cannot be distinguished from the active treatment), as determined by the randomization process.
3) Commence their initial one-month treatment intervention; the study medication will be taken twice daily for approximately one month.

Visit 3: Completion of First Intervention: Review of Adverse Events & Concurrent Medications, Vital Signs, Blood Samples for Safety Laboratory Testing and COX Activity, Cognitive Battery, Assessment of Study Drug Compliance, Physical and Neurological Examination, and Intervention Crossover. At the end of the first one-month treatment period, participants and study partners will return to the KU CTSU for Visit 3. Visit 3 will occur in the morning, and participants will be informed to remain NPO, other than water or coffee or medications, after midnight of the preceding evening. At this visit they will:

1) Undergo a review of any changes in health/adverse events since the last visit.
2) Undergo a review of any changes in concurrent medications since the last visit.
3) Undergo vital signs measurement.
4) Undergo a 40 ml phlebotomy (under fasting conditions), which will be used to measure platelet mitochondria COX activity and safety labs (CBC, electrolytes, liver function, renal function).
5) Undergo a cognitive battery (Participants will be given a snack/break before proceeding to cognitive testing).
6) Return all used and unused study medication containers.
7) Undergo determination of study medication compliance.
8) Be provided two bottles of either S-equol or placebo capsules; participants originally randomized to S-equol capsules at Visit 2 will be provided placebo capsules, while participants originally randomized to placebo capsules will be provided S-equol capsules. Participants and staff (aside from the study statistician) will remain blinded to the treatment assignment.
9) Commence their second one-month treatment intervention.

Visit 4: Completion of Second Intervention: Review of Adverse Events & Concurrent Medications, Vital Signs, Blood Samples for Safety Laboratory Testing and COX Activity, Cognitive Battery, Physical and Neurological Examination, Assessment of Study Drug Compliance. At the end of the second one-month treatment period, participants and study partners will return to the KU CTSU for Visit 4. Visit 4 will occur in the morning, and participants will be informed to remain NPO, other than water or coffee or medications, after midnight of the preceding evening. At this point they will:

1) Undergo a review of any changes in health/adverse events since the last visit.
2) Undergo a review of any changes in concurrent medications since the last visit.
3) Undergo vital signs measurement. (Participants will be given a snack/break before proceeding to cognitive testing).
4) Undergo a 48 ml phlebotomy (under fasting conditions), which will be used to measure platelet mitochondria COX activity and safety labs (CBC, electrolytes, liver function, renal function).
5) Undergo a cognitive battery.
6) Return all used and unused study medication containers.
7) Undergo determination of study medication compliance.

Post-Interventions Phone Call. Approximately one month after completing Visit 4, participants and their study partners will be contacted by phone. At this time a review of any changes in health/adverse events and changes in concurrent medications will be conducted. At the time of the post-intervention telephone call, all previously reported, as well as newly reported adverse events, will be assessed. Open adverse events that are determined not to be related to the study

treatment will not be further followed, while those that are determined to be related to the study treatment will be followed until such time as the adverse event has resolved or been declared stable, or the participant is lost to follow up, or it is determined by the PI (or his designee) that the adverse event no longer needs to be followed.

Study Procedure Details

Mitochondrial Assays. Approximately forty ml of blood will be collected in tubes containing acid-citrate-dextrose as an anticoagulant and kept at room temperature. Within 24 hours of phlebotomy this blood is transferred to the KU ADC Mitochondrial Genomics and Metabolism Core for processing. Upon receipt platelets are isolated by centrifugation and enriched mitochondrial fractions are prepared using previously described methods [16]. The protein concentrations of the enriched mitochondrial fractions are measured using a DC protein assay kit (BioRad, Hercules, CA). COX Vmax activity is determined as a pseudo first order-rate constant (sec^{-1}/mg protein) by measuring the oxidation of reduced cytochrome *c* at 550 nm [16, 17]. In addition to measuring COX activity, we will also measure citrate synthase (CS) Vmax activity (nmol/min/mg protein). The COX activity will be referenced to its corresponding CS activity to correct for potential inter-sample differences in mitochondrial mass. This assay is performed spectrophotometrically by following the formation of 5-thio-2-nitrobenzoate (412 nm) following the addition of 100 μM oxaloacetate at 30°C [16]. Therefore, in addition to referencing COX activities to total protein, we will also determine the CS activity so that we can correct the COX activity for inter-sample differences in mitochondrial mass that arise during the platelet mitochondria isolation procedure. The COX/CS activity will be the activity used in our primary analysis and in all secondary analyses except for the secondary analysis comparison of platelet mitochondria COX activity between APOE4 carriers and non-carriers. For that analysis we will reference COX activity to mg of protein in the assay sample (COX/mg protein), as the original SEAD study suggested CS activity may also vary by APOE genotype. Regarding our analysis of potential COX activity-cognitive test correlations we will consider both the COX/CS and COX/mg protein values.

Future Use of Samples: If any portion of the blood samples collected for mitochondrial assays is left over, it will be placed into a storage freezer in the KU ADC Mitochondrial Genomics and Metabolism Core Lab for future use. The leftover blood sample may be stored for up to 3 years and may be used for further studies of metabolism, in order to help better understand results found in the main part of the study .

For all participants, the mitochondrial enzyme measurements will be performed before APOE genotype determination. This will ensure that the study personnel performing the Visit 1 enzyme measurements are blinded to the participant's APOE genotype status.

Cognitive Battery. The SEAD2 cognitive battery will consist of the following tests:

(1) Montreal Cognitive Assessment (MoCA);

(2) Alzheimer's Disease Assessment Scale-Cognitive Portion (ADASCog), 11 item test;
(3) Logical memory test (LMT) immediate and delayed recall;
(4) Stroop testing.

Vital Signs. Vital signs measurements will include blood pressure, pulse, temperature, respiration rate, and body weight.

Genetic Counseling. We will counsel the participant and their study partner that participation in this study will not further inform other family members about their risk of developing AD. The lifetime risk of an individual developing AD when they have a first degree relative (a parent or a sibling) who has or had sporadic AD (by far the most common type of AD) is about 30%. If an individual has one APOE4 allele, their lifetime risk is similarly about 30%. Therefore, determining whether the APOE genotype of the participant is or is not an APOE4 carrier will not further refine the AD risk estimate of the first degree relatives.

If a participant is found to carry an APOE4 allele, then the chances that a first degree relative will also have that same APOE4 allele is 50%. Therefore, although this study will not further inform first degree relatives about their risk of developing AD, they could learn that they have at least a 50% chance of inheriting an APOE4 allele.

If a participant is found to carry two APOE4 alleles (homozygous APOE4 carriers), then children of the participant will have a 100% chance of having at least one APOE4 allele. In order to minimize the chances of generating anxiety amongst the children of our genotyped participants, we will not disclose whether a participant was an APOE4 homozygote or heterozygote, but rather only that the participant "carries an APOE4 allele." By participating in this study participants, care partners, or other family members will not be able to infer whether or not the participant is an APOE4 homozygote or heterozygote.

It is important to note that the same genetic information provided during our genetic counseling process is provided in the KU MDC setting when APOE genotyping is pursued as part of a clinical evaluation. MDC experience reveals providing this information does not cause patient or family stress. Also, we wish to emphasize that the genetic counseling process for SEAD2 will differ from the KU MDC process because SEAD2 participants will already carry a diagnosis of AD, whereas patients undergoing APOE genotyping in the KU MDC as part of a clinical evaluation typically have not already been diagnosed with AD. For patients genotyped as part of a KU MDC clinical evaluation, APOE determination therefore has a far greater diagnostic implication than it will in the SEAD2 trial. As problems with APOE genotype disclosure are not observed in KU MDC patients who do not already carry a diagnosis of AD, we do not anticipate any problems with APOE genotype disclosure amongst SEAD2 participants who already carry a diagnosis of AD.

Safety Labs. A complete blood cell count, serum electrolytes, liver function tests, and a screen of renal function will be obtained at the start of the study and for those completing each of the two

intervention stages of the study. These analyses will be performed through the University of Kansas Hospital's clinical laboratory. These laboratory assessments will allow us to screen for adverse effects on electrolyte balance, bone marrow, liver function, and renal function.

6.0 EXPECTED TOXICITIES

There are no expected safety issues. SEAD2 will expose participants to S-equol at a dose of 50 mg, twice daily, over a one-month time period. Clinical and safety trials performed to date do not indicate appreciable safety issues arise when S-equol is taken at 50 mg BID for periods of up to one month.

7.0 DRUG FORMULATION AND ADMINISTRATION

Drug Formulation

S-equol will be provided by Ausio Pharmaceuticals, LLC. The study drug and matching placebo will be provided as 50 mg capsules, which will be taken twice per day approximately 12 hours apart. Bottles will be coded to protect the blinded nature of the study.

Drug Administration

Study drug or matching placebo will be dispensed at Visits 2 and 3. The study drug/placebo is taken orally, twice daily, by the study participant. The study partner serves the role of overseeing the administration of the study medication in the home as needed, to ensure it is taken as directed.

Drug Production Site

Study drug or matching placebo will be produced by Patheon (Toronto Development Centre) for Ausio Pharmaceuticals, LLC.

Drug Transportation

The study drug and matching placebo will be shipped to the study site by express mail.

8.0 CORRELATIVE STUDIES

Prior studies show after a single oral dose of ^{14}C S-equol (2 mg/kg in rats, 1 mg/kg in monkeys) the radiolabel is rapidly absorbed and is present in the brain [18]. For this reason, we expect drug-induced changes in platelet mitochondria will reasonably reflect changes in brain platelet mitochondria. The dose and dosing schedule are based on previous work performed by Ausio Pharmaceuticals, LLC, which suggests 50 mg BID affects human physiology.

S-equol was tested in AD women as part of the SEAD study. The exposure in that study was 10 mg twice daily for 2 weeks, and no serious adverse events occurred. S-equol has further undergone rigorous phase I testing in 101 subjects, Phase 2a testing in 169 menopausal women with vasomotor symptoms, and Phase 2a testing in 124 men with benign prostatic hypertrophy. In all cases, S-equol has shown an excellent safety profile, and doses up to 150 mg BID for one month are well-tolerated. We do not anticipate any safety issues with the 50 mg BID, one-month course the SEAD2 study will use.

9.0 STUDY CALENDAR

Visit	**Visit 1**	**Visit 2**	**Visit 3**	**Visit 4**	**Post-Interventions Phone Call**
Timeframe	**Day 0**	**Month 1 (+ 7 days/ - 14 days)**	**Month 2 (+/- 7 days)**	**Month 3 (+/- 7 days)**	**Month 4 (+/- 7 days)**
Informed Consent	X				
Review medical history	X				
Review concurrent medications	X	X	X	X	X
Review health changes/adverse events		X	X	X	X
Genetic Counseling	X				
Physical and Neurological Exam	X		X	X	
Phlebotomy (Fasting Conditions) For APOE (at V1 only) For COX Activity For Safety Labs	 X X X		 X X	 X X	
Cognitive Battery		X	X	X	
Notification of APOE Status and Eligibility to Proceed to Intervention		X			
Randomization of APOE4 Non-Carriers		X			
Two bottles of S-equol 50 mg or Placebo Capsules (35 capsules per bottle) Dispensed*		X*	X		
Vital Signs	X	X	X	X	
Pill Count For Compliance			X	X	
*During Visit 2, APOE4 carrier participants who are not randomized to the intervention will not undergo these procedures.					

10.0 MEASUREMENT OF EFFECT

The primary outcome measure will consider the mean intra-individual difference in COX/CS activity for participants concluding one month of placebo treatment versus one month of S-equol treatment. If the change in mean activity shows a statistically significant increase following S-equol treatment, it will be concluded that S-equol increases platelet mitochondria COX/CS activity in APOE4 non-carrier individuals.

Secondary outcomes from the intervention part of the study will include safety data, cognitive scores, and categorical response patterns defined by platelet mitochondria COX/CS activity measurements.. The safety endpoint will consist of a composite of adverse events reported at Visit 2, Visit 3, Visit 4, and the post-Interventions Phone Call; any clinically significant abnormal vital signs as determined at Visit 2, Visit 3, and Visit 4; any clinically significant findings on the physical and neurological examinations performed at Visit 3 and Visit 4; and any clinically significant abnormal safety lab values as determined at Visit 3 and Visit 4.

The cognitive endpoint will include analyses of scores on the MoCA, ADASCog-11, LMT, and Stroop tests.

For the final secondary outcome, we will categorically define participants as responders or non-responders depending on the slope of COX/CS activity change across their V1, V3, and V4 measurements. This anticipated pattern of response analysis will be performed according to the rules defined in the original SEAD study.

As part of our exploratory analysis we will examine whether platelet mitochondria COX activity is lower in AD participants who possess the APOE4 variant of the APOE gene than it is in AD participants who do not possess the APOE4 variant of the APOE gene. For this analysis we will reference COX to mg protein, in addition to referencing it to CS activity, because data from the first SEAD trial suggested platelet mitochondria CS activity may also be influenced by APOE genotype. We will also examine whether there are correlations between platelet mitochondria COX activity and cognitive abilities in both APOE4 carriers and non-carriers.

11.0 ADVERSE EVENT REPORTING REQUIREMENTS

11.1 Notifying Local Regulatory Authorities

Adverse event data (injuries/illness) will be presented to the principal investigator (PI) throughout the trial as they occur. The PI (or his designee) will assess adverse events for severity, serious criteria, and relatedness to the study medication or study procedures. Adverse events will be reported to the KUMC Human Subjects Committee (HSC) per their guidelines. Upon conclusion of the study, all adverse events will be further analyzed and summarized.

11.2 Notifying the FDA

The PI/Sponsor will report serious and unexpected adverse events in an expedited fashion, in compliance with FDA regulations. These written notifications of adverse events (IND safety reports) will follow the following guidelines:

- ***Within 7 calendar days***
  Any study event that is:
  - associated with the use of the study drug;
  - unexpected;
  - fatal or life-threatening, and

- ***Within 15 calendar days***
  Any study event that is:

  - associated with the use of the study drug;
  - unexpected, and
  - serious, but not fatal or life-threatening

    -or-
  - a previous adverse event that was not initially deemed reportable but is later found to fit the criteria for reporting (reporting within 15 calendar days from when the event was deemed reportable).

12.0 DATA AND SAFETY MONITORING

Data Collection

Data Collection will be overseen by the PI. The PI will routinely review secondary endpoint safety data as it accrues. The study coordinator will ensure transfer of the secondary endpoint safety data to the PI in a timely manner. The PI will also review primary endpoint (biochemical assay) data as it is generated, but in a blinded fashion. The unblinded statistical analyses will be conducted following study completion.

Data Monitoring

The Data Monitoring plan for this trial focuses on monitoring by the PI and the trial coordinator. Review of the rate of participant accrual and adherence to inclusion/exclusion criteria will occur during the recruitment phase to assure that participants meet eligibility criteria.

Safety Monitoring

The Safety Monitoring plan for this trial focuses on monitoring by the PI and the trial coordinator.

Data will be collected regarding participant and caregiver reports of any changes in health and/or behaviors at study visit 3, study visit 4, and the post-interventions phone call (which each occur at approximate 1 month intervals). The frequency and severity of adverse events will be examined and their potential causal relationship to the study drug (as determined by the PI or his designee) will be assessed at the time of data collection.

Any emergent medical condition or safety finding that, in the opinion of the PI, may jeopardize the participant's safety if he/she continues in the study will be sufficient reason for participant discontinuation from the study. The participant will be followed for the adverse event until it is resolved, declared stable, judged to be unrelated to the study drug, or the participant is lost to follow up.

13.0 REGULATORY CONSIDERATION

Protection of Human Participants and Informed Consent

Regulations in regard to Human Participants Protection and Informed Consent will be followed in accordance with 21 CFR 50. All institutional, FDA, state and federal regulations concerning informed consent will be followed.

Institutional Review Board (IRB)

Regulations regarding initial and continuing review of the study by an Institutional Review Board will be followed. The University of Kansas Medical Center has a federally approved Institutional Review Board, the KUMC Human Subjects Committee (HSC), which will be involved in all aspects of study planning and considerations as per 21 CFR 56. The KUMC HSC FWA# is FWA00003411.

Financial Disclosure

There are no financial disclosures to be made. No marketing approval for the study drug is going to be pursued by the PI/Sponsor.

Obligations of Investigators

The PI/Sponsor, Dr. Russell Swerdlow, will be responsible for all elements of 21 CFR 312 subpart D. Dr. Swerdlow will maintain oversight of the clinical trial ensuring relevant regulatory requirements are met and necessary guidelines are followed. The following are co-investigators:

- Jeffrey Burns, MD,
- Jonathan Mahnken, PhD.

The PI has confirmed all co-investigator credentials, experience, and training. He has also ensured additional personnel will follow the appropriate investigational plans and guidelines as submitted in the IND. All changes to the study, including modifications to the IND, will be communicated by the PI and he will be the primary liaison to the IRB.

All detected and newly discovered adverse effects and risks will be reported by the PI at the time of the event, per institutional guidelines. Record-keeping and data is strictly confidential, only to be shared between investigators in the study and only those who are directly involved in the care of the patient.

14.0 STATISTICAL CONSIDERATION

The primary outcome measure will compare the mean COX activity of participants concluding their one-month placebo treatment to that of participants concluding their one-month S-equol treatment by calculating the difference for each participant. We will use the intent-to-treat principal (with respect to the randomized treatment order) for primary analysis, but if there are protocol deviations with respect to treatment order then we will also conduct a per protocol analysis as a secondary assessment. As the outcome measures (difference scores) are continuous data values, we will summarize by means and standard deviations, and compare S-equol versus placebo using the paired, one-sample t-test with a two-sided p-value of <0.05 considered statistically significant. We will assess normality assumptions through visual assessments of

residual plots, and also a histogram and q-q plots for further residual analyses. In the event this test's assumptions appear violated we will conduct the nonparametric Wilcoxon rank sum test of the difference scores for the primary assessment.

We will also conduct secondary analyses of COX activity using linear mixed models. This will incorporate all three COX activity measures on each APOE4 non-carrier participant, and will also allow inclusion of data for the APOE4 allele carriers with appropriate model structure/ specification. We will use this model as the basis for various contrasts to test our hypotheses statistically with respect to secondary objective. We will assess this model by residual analyses (observed versus residual [conditional and unconditional] plots, residual [conditional and unconditional] histograms, and q-q plots [conditional and unconditional]). We will similarly assess other study outcomes using this mixed modeling approach, and as needed for non- or semi-continuous outcomes we will utilize other exponential family distributions under the generalized linear mixed models framework to incorporate dependence structures appropriately. Lastly, we will assess for a pattern of response with respect to secondary objective. Specifically, this simplifies to a nonparametric runs test for the difference between the active drug versus placebo COX activity measures.

15.0 PUBLICATION PLAN

Publications resulting from this study will be prepared by the PI. Co-authors will include personnel from the study site and from Ausio, LLC who have meaningfully contributed to study execution or development. Only co-authors who agree with the final manuscript will be listed as authors on the final manuscript.

16.0 REFERENCES

1. Brinton, R.D., *The healthy cell bias of estrogen action: mitochondrial bioenergetics and neurological implications.* Trends Neurosci, 2008. **31**(10): p. 529-37.
2. Swerdlow, R.H., *The neurodegenerative mitochondriopathies.* J Alzheimers Dis, 2009. **17**(4): p. 737-51.
3. Hebert, L.E., et al., *Alzheimer disease in the US population: prevalence estimates using the 2000 census.* Arch Neurol, 2003. **60**(8): p. 1119-22.
4. *Alzheimer's Association. 2011 Alzheimer's disease facts and figures.* Alzheimer's and Dementia, 2011. **7**: p. 208-244.
5. Swerdlow, R.H., *Brain aging, Alzheimer's disease, and mitochondria.* Biochim Biophys Acta, 2011. **1812**(12): p. 1630-1639.
6. Swerdlow, R.H., *Mitochondria and cell bioenergetics: increasingly recognized components and a possible etiologic cause of Alzheimer's disease.* Antioxid Redox Signal, 2012. **16**(12): p. 1434-55.
7. Hirai, K., et al., *Mitochondrial abnormalities in Alzheimer's disease.* J Neurosci, 2001. **21**(9): p. 3017-23.
8. Swerdlow, R.H., *Role and treatment of mitochondrial DNA-related mitochondrial dysfunction in sporadic neurodegenerative diseases.* Curr Pharm Des, 2011. **17**(31): p. 3356-73.

9. Swerdlow, R.H., *Mitochondrial medicine and the neurodegenerative mitochondriopathies.* Pharmaceuticals, 2009. **2**: p. 150-167.
10. Swerdlow, R.H., *Bioenergetic Medicine.* Br J Pharmacol, 2013.
11. Jackson, R.L., J.S. Greiwe, and R.J. Schwen, *Emerging evidence of the health benefits of S-equol, an estrogen receptor beta agonist.* Nutr Rev, 2011. **69**(8): p. 432-48.
12. Zhao, L., Z. Mao, and R.D. Brinton, *A select combination of clinically relevant phytoestrogens enhances estrogen receptor beta-binding selectivity and neuroprotective activities in vitro and in vivo.* Endocrinology, 2009. **150**(2): p. 770-83.
13. Klinge, C.M., *Estrogenic control of mitochondrial function and biogenesis.* J Cell Biochem, 2008. **105**(6): p. 1342-51.
14. Onyango, I.G., et al., *Regulation of neuron mitochondrial biogenesis and relevance to brain health.* Biochim Biophys Acta, 2010. **1802**(1): p. 228-34.
15. McKhann, G.M., et al., *The diagnosis of dementia due to Alzheimer's disease: Recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease.* Alzheimers Dement, 2011. **7**(3): p. 263-9.
16. Mosconi, L., et al., *Reduced mitochondria cytochrome oxidase activity in adult children of mothers with Alzheimer's disease.* J Alzheimers Dis, 2011. **27**(3): p. 483-90.
17. Parker, W.D., Jr., C.M. Filley, and J.K. Parks, *Cytochrome oxidase deficiency in Alzheimer's disease.* Neurology, 1990. **40**(8): p. 1302-3.
18. Schwen, R.J., K.T. Henry, and R.L. Jackson, *Absorption, distribution and excretion of [14C]-S-equol in the rat and cynomolgus monkey.* International Journal of Pharmacognosy and Phytochemical Research 2013. **5**: p. 155-163.