Full study protocol and statistical analysis plan

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An open-label, single-center, 12-month trial of a Lunasin regimen for patients with Amyotrophic Lateral Sclerosis (ALS)

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TABLE OF CONTENTS

1. PURPOSE/HYPOTHESES

1.1. Primary Hypothesis

The primary hypothesis is that a supplement regimen containing Lunasin (hereafter referred to as the Lunasin regimen) can decrease the rate of ALSFRS-R progression by 50% relative to matched historical controls.

1.2. Secondary Hypotheses

Secondary hypotheses are as follows:

1. Lunasin regimen can increase the frequency of ALS reversals (defined by an improvement of 4 or more points in the ALSFRS-R over the course of 1 year) from 1% (observed spontaneously) to at least 2%.
2. Lunasin regimen can alter histone acetylation in patients with ALS (PALS).
3. Participants can accurately measure their own ALSFRS-R score and record it on PatientsLikeMe relative to data capture by a healthcare professional.
4. Patients can accurately measure their own weight and record it on PatientsLikeMe relative to data capture by a healthcare professional.
5. The novel features of this pilot trial will be associated with improved participant enrollment compared to prior more traditional ALS trials where this is 2 participants per site per month.
6. The novel features of this pilot trial will be associated with improved participant retention compared to prior more traditional ALS trials where the dropout rate is 22%.

2. BACKGROUND

2.1. Amyotrophic Lateral Sclerosis (ALS) and Its Treatment

ALS is a devastating motor neuron disease that causes rapidly progressive muscle weakness, disability and premature death. In spite of a large number of attempted ALS trials, there are no significant disease-modifying therapies for this condition (1).

2.2. Failure of Previous ALS Trials

There are at least 2 reasons that previous ALS trials may have failed. First, the hypotheses could have been wrong. The hypotheses for many prior trials came from observations made in animal models of familial ALS (1). How well these predict human ALS, the vast majority of which is not familial, has been called into question (2). Second the dosing may not have been adequate. Indeed, most ALS trials have not even employed pharmacodynamic biomarkers to test their dosing regimen (3). In addition to these problems, ALS trials are often challenged by slow enrollment (4) and poor retention (5). While some of the enrollment and retention issues may have to do with misconceptions on the part of enrolling clinicians and potential participants (4), patients with ALS do become more disabled over time, and study burdens including trips to the study site may eventually become impossible to bear.

2.3. ALS Reversal on Lunasin

ALSUntangled is a consortium of ALS clinicians and researchers that reviews alternative and off-label treatments being tried by patients with ALS (6). ALSUntangled recently reported on a patient with a validated diagnosis of ALS, who experienced dramatic objective improvements in speech, swallowing and limb strength while taking a supplement regimen containing Lunasin (7). Several other patients with ALS have also reported improvements on this Lunasin regimen, though records were not obtainable to validate these (7).

2.4. How Lunasin Could Influence ALS

Transcriptional dysregulation has been proposed to be part of the pathophysiology of ALS (8-11). Transcriptional dysregulation perturbs cellular function and compromises protective mechanisms, leading to neuronal death. Many proteins, including histones that affect the actions of RNA polymerase II on individual genes, regulate transcription. Many of these interactions, in turn, are regulated by acetylation, methylation and phosphorylation. Recruitment of histone deacetylases (HDACs) to DNA alters nucleosome structure locally and inhibits transcription. Histone acetylation patterns are known to be abnormal in patients with ALS and have been the target of previous therapeutic trials (12).

Lunasin, as a low potency dietary nutrient, can reportedly affect the expression of a number of genes in multiple regulatory and physiological pathways on different cells in the body. It is able to accomplish this by modifying the epigenome and changing gene expression through modifications to specific lysine residues in histones H3 and H4. Lunasin can turn off disease-causing genes by binding and inhibiting the specific acetylation of Lysine 14 on histone H3 (H3K14ac) by the histone acetylase enzyme, PCAF (13). H3 acetylation, which includes H3K14ac, is an epigenetic mark required to activate genes involved in cholesterol biosynthesis (13), inflammation (14), hormone response (15), metabolic syndrome (16) and stress (17). By impeding the expression of these genes, Lunasin can help prevent formation of age related, degenerative chronic diseases. Lunasin can also turn on and increase the expression of health-promoting genes, such as those involved with cancer prevention, by enhancing the acetylation of Lysine 16 on histone H4 (H4K16ac) (18). H4K16ac is the epigenetic mark associated with the opening of condensed chromatin (DNA + histones) to allow easier access of the cellular transcriptional machinery to activate and increase gene expression (19). Loss of H4K16ac is a common hallmark of human cancer cells (20)

Thus, if Lunasin can restore normal patterns of histone acetylation in patients with ALS, it may help to ameliorate transcriptional dysregulation and importantly slow or reverse ALS progression. The novelty of the outlined proposal is that we will be able to measure alterations in histone acetylation in the treated patients. This will allow us to correlate changes in disease progression with molecular changes. Thus, assessing histone acetylation from patient samples can provide us with a "biomarker" for this study.

This study will treat other PALS with the Lunasin regimen used by the above-described patient who experienced ALS reversal and determine if anyone else improves. We will

look for accompanying changes in histone acetylation. We will employ a novel design in hopes of enhancing enrollment and retention rates.

2.5. How Reliv Now and Provantage Might Influence ALS

In addition to Lunarich X capsules (the main source of Lunasin in this study) the other 2 products in the Lunasin regimen are called Reliv Now and Provantage. These are mixtures of more Lunasin as well as various antioxidants and anti-inflammatories. Oxidative stress and neuroinflammation are postulated to be part of ALS pathophysiology and there have been numerous unsuccessful attempts to treat PALS with antioxidants and anti-inflammatories before. This particular combination has never been studied, however.

2.6. PatientsLikeMe

PatientsLikeMe® (PLM; www.patientslikeme.com) is an online platform that has demonstrated an ability to engage patients in learning about their conditions and building a large quantitative and qualitative database about patients' experiences of their conditions. PLM is a patient network with over 300,000 members comprising over 2000 different conditions founded by a family affected by ALS, and to date has conducted over 30 published studies in ALS including studying patient use of off-label treatments. Members who join the site with ALS are asked to complete a number of data fields including demographics, disease condition history, treatments, symptoms, weight, treatment evaluations and lab measurements (e.g. weight, forced vital capacity). In addition, a unique feature of the site is the ability of patients to complete a widely used patient reported outcome, the ALS Functional Rating Scale (Revised, ALSFRS-R) which allows patients to see their disease progression in context in visual form. Data capture is facilitated by a team of PhD scientists, nurses, and pharmacists that incorporate information from various medical databases (e.g. RxNorm, ICD-10, MEDDRA). In addition the site provides means of social support through a message board forum, private messages, and profile commenting. Online methods of data capture have the potential to dramatically accelerate trial recruitment, provide more convenient means of data capture for patients, and minimize trial complexity by using matched historical controls in lieu of a placebo control group. However, as a relatively new technology there are still questions of validity which must be addressed to fully realize the potential of such tools.

3. DESIGN & PROCEDURES:

3.1. Design Overview.

This will be a 12-month, widely inclusive, largely virtual, single-center, open-label pilot trial utilizing a historical control group. Treatment with the Lunasin regimen and all the following study outcome measures and labs are being performed exclusively for research purposes: Vital Signs, ALSFRS-R, biomarker testing, adverse events, and concomitant medications. Participants will be asked to register for an account on PatientsLikeMe ("PLM") (sign-up process can be found in Appendix H) with the help of the study coordinator. User IDs associated with their PatientsLikeMe account will be recorded by the study coordinator and shared with study staff at PatientsLikeMe. When registering, participants will have the opportunity to review PLM's terms and conditions as well as

their privacy policy (Appendix A). After the initial in-clinic visit, participants will be asked to enter the following data: weight (Appendix B), Lunasin treatment evaluation (Appendix C) and their ALSFRS-R score (Appendix D). Participants will be given the following study documents and materials to take home with them:

- A PatientsLikeMe bag (photo Appendix E) including:
  - Welcome letter (Appendix E)
  - Study One-Sheet (Appendix F)
  - Study Checkup Checklist (Appendix G)

3.2. Lunasin Regimen

The intervention is the Lunasin regimen used by the patient with the above-described ALS reversal (7). This consists of LunaRich X Capsules (12 capsules per day), a mixture of 'vitamins, minerals and super-powered antioxidants' called Reliv NOW (three scoops per day) and a mixture of ' soy protein, medium chain triglycerides, creatine, CoQ10 and supercharged amino acids ' called Pro-Vantage (two scoops per day). Patients will taper up to this target regimen as follows: Day 1 (Dosage 1): 3 capsules of Lunarich X, ¼ scoop of Reliv Now and ¼ scoop of Provantage (do this twice a day for 1 day). Day 2 (Dosage 2): 4 capsules of Lunarich X, 1/2 scoop of Reliv Now and 1/2 scoop of Provantage (do this twice a day for 2 days). Day 4 (Dosage 3): 6 capsules of Lunarich X, 1 scoop of Reliv Now and 1 scoop of Provantage (do this twice a day for 2 days). Day 6 (Dosage 4): 6 capsules of Lunarich X, 1 1/2 scoops of Reliv Now and 1 scoop of Provantage (do this twice a day for study duration). It will be suggested that patients open the Lunarich X capsules and mix the contents of these as well as the other 2 ingredients in water to make a shake. If patients do not tolerate advancing to the next Dosage, they will be asked to drop back to the highest Dosage they could tolerate.

3.3. Lunasin Supply

The products in the Lunasin regimen are being donated to Duke from the Reliv Corporation (21) and will be given to patients in the study free of charge for 12 months. Products will be stored in Dr. Bedlack's locked office at Duke. At the Screening/Baseline Visit, participants will receive a 1-month supply. At the Month 1 Visit, participants will receive a 2-month supply and thereafter will receive product by mail every 2 months for the duration of the study as long as they are continuing to enter their online data (see below). Once this study ends, participants may elect to continue the Lunasin regimen by purchasing products from Reliv.

3.4. Dose Adjustments and Holidays

The investigator may temporarily reduce or stop administering the Lunasin regimen for adverse events. If the adverse event is mild or moderate, the dose may be reduced to the prior Dosage (see taper above) until the event improves. The investigator may then choose to resume the dosage escalation or maintain the participant at reduced dose. The dosage can be raised to the previous dose once. If a related event is serious or life threatening, the Lunasin should be stopped and will not be re-challenged**.** One drug holiday will be allowed; the holiday can last no more than 7 days. All dose reductions and suspensions must be documented in the Neurobank.

3.5. Compliance

The method to check compliance for study medication will be to record the amount of product dispensed at the Baseline/Screening Visit, and the amount left over at the Month 1 Visit. No further compliance checks will be conducted.

3.6. Concomitant Medications

Throughout the study, investigators may prescribe any other concomitant medications or treatments deemed necessary to provide adequate supportive care, provided that they are not part of a research study.

3.7. Use of Riluzole

The use of riluzole will be permitted during the study. Patients taking riluzole must be on a stable dose for 30 days prior to screening.

3.8. Outcome Measures

3.8.1. ALSFRS-R

The Revised ALS Functional Rating Scale (ALSFRS-R) will be determined at all phone interviews and study visits (Appendix D). ALSFRS-R is a quickly administered (five minute) ordinal rating scale (ratings 0-4) used to determine patients' assessments of their capability and independence in 13 functional activities. All 13 activities are relevant in ALS. Initial validity was established in ALS patients by documenting change in ALSFRS-R scores correlated with change in strength over time, was closely associated with quality of life measures, and predicted survival (22). The test-retest reliability is greater than 0.88 for all 13 items. The ALSFRS-R declines linearly with time over a wide range during the course of ALS. Recent work has shown that the measure can be conducted over the phone (23), and it can even be reliably conducted and recorded online by patients themselves (24). The ALSFRS-R will be administered to participants in-clinic by the study coordinator at Duke on their first visit. Additionally, participants will be asked through email from PLM to fill out the ALSFRS-R test on their PatientsLikeMe profile once every 30 days.

3.8.2. Histone Acetylation

All histone acetylation assays will be carried out in the laboratory of Ghazaleh Sadri-Vakili, PhD at Massachusetts General Hospital. As in a previous study (12) blood will be collected in one 10-ml sodium heparin green top tube and two 5-ml red top tubes (both plastic). The 10-ml green top tube will be inverted 8-10 times following collection and placed on crushed ice. The sample will be centrifuged at 8000-9000x g for 20 min within 1 h of collection. After spinning, the green top tube will be frozen upright on dry ice (-40 C). The 5-ml red top vacutainer will immediately be frozen upright on dry ice. All tubes will be shipped overnight to Dr. Sadri-Vakili. In her lab, peripheral blood mononuclear cells (PBMC) will be isolated using a standardized Ficoll-Hypaque gradient procedure using the Ficoll-Paque Plus (GE Healthcare, Boston, MA) and analyzed for alterations in histone acetylation using the following methods.

3.8.2.1. Histone extractions and Western blots

Histones will be extracted from PBMCs as described previously (25). Briefly, cells will be washed with ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 mM Na33VO4, 1 mM NaF) supplemented with a protease inhibitor mixture (Roche Applied Sciences, Indianapolis, IN, USA) for 10 min. The pellets will be collected by centrifugation at 10,000 × *g* for 10 min. The pellets will be washed once in 10 mM Tris-HCl and 37 mM EDTA (pH 7.4), and resuspended in 200 μL 0.4 N H2SO4. After overnight incubation on ice, the supernatant will be collected by centrifugation at 14,000 × *g* for 15 min and mixed with 2 mL cold acetone and kept at −20°C overnight. The histones will be collected by centrifugation at 14,000 × *g* for 15 min. After one wash with acetone, the histones will be air dried and suspended in sterile deionized water. Total histone concentration in each sample will be measured using the Bradford assay according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA). Western blot analysis will be used to analyze alteration in histone acetylation following treatment as described previously (26, 27). Briefly, 1-5 μg of extracted histone proteins from samples will be resuspended in sample buffer. The resuspended samples will be boiled at 95°C for 5 min, and fractionated on a 10-20% tricine gel (Invitrogen, Carlsbad, CA) for 90 min at 120V. Proteins were transferred to PVDF membranes in transfer buffer (3% Tris base, 14.4% glycine, 20% MeOH) at 400 mA x 1 h, and the PVDF will be blocked with 3% milk in phosphate-buffered saline before immunodetection with a number of acetylated histone antibodies including: anti-di acetyl lysine 9 and lysine 14 histone H3 (H3K9K14ac2) 1:1500 dilution (Millipore, Billerica, MA) anti-histone H3 1:500 dilution (Millipore, Billerica, MA) anti-pan acetylated histone H4 1:1000 (Millipore, Billerica, MA) overnight (4°C). Primary antibody incubation will be followed by 4 washes (10 min, RT) in diH_2O before incubation with the secondary antibody (HRP-conjugated goat anti-rabbit IgG; Jackson ImmunoResearch Laboratories, West Grove, PA), 4 washes, and visualized using the ECL detection system (NEN, Boston, MA). Coomassie gels will be used to ensure equal protein loading for western blots.

3.8.2.2. Chromatin immunoprecipitation (ChIP) assay

ChIP studies will be used to measure alterations of acetylated histones at specific gene promoters as described previously (26-29). The same acetylated histone antibodies as outlined in the Western Blot methods above will be used for ChIP studies. We will also include negative controls such as a no antibody mock and IgG (Jackson, West Grove, PA). Input and IP samples will be interrogated with gene promoter-specific primers in triplicate reactions using real-time PCR analysis as previously described. Threshold amplification cycle numbers (T_c) using iCycler software will be used to calculate IP DNA quantities as percentage of corresponding inputs.

3.8.2.3. RNA extraction and reverse transcription

RNA will be extracted from samples using RNeasy kit (Qiagen, Valencia, CA) according to manufacturer's instructions and as described previously (26,28) to measure changes in gene expression. Reverse transcription reactions will be performed using the Superscript

First Strand Synthesis System for RT-PCR reactions (Invitrogen, Carlsbad, CA) using specific primers to quantitate the amount of gene expression as compared to a standard curve. Quantitative real time-PCR will be performed in an iCycler (Bio-Rad) with the use of SYBR-green PCR Master Mix (Applied Biosystems, Foster City, CA) through 50 PCR cycles (95°C for 30 sec, 57°C for 60 sec, 72°C for 90 sec). The threshold cycle for each sample will be chosen from the linear range and converted to a starting quantity by interpolation from a standard curve run on the same plate for each set of primers. The mRNA levels will be normalized for each well to the GAPDH mRNA levels.

3.8.3. Weight

The study coordinator will measure and record the weight of all participants at the Screening/Baseline, Month 1 and Month 12 Visits. Patients will measure and record their own weights at Month 2-11 Visits on their PLM profile (See Appendix B).

3.8.4. Enrollment Rate

Enrollment rate will be calculated as the number of participants enrolled per month. A previous meta-analysis showed the mean ALS trial enrollment rate to be 2.2 participants per site per month (4).

3.8.5. Retention

Retention will be assessed by looking at the dropout rate (the percentage of surviving enrolled patients who complete the Month 12 Visit). A previous meta-analysis showed the mean ALS participant dropout rate to be 22% (5).

3.9. Study Schedule

Participants will be required to make 3 visits to Duke (Screening/Baseline Visit, Month 1 and Month 12 Visit). At months 2-11, participants will make "virtual visits" by measuring their own ALSFRS-R score and weight, and logging onto the website PatientsLikeMe (31) to record it as well as perceived efficacy, compliance, adverse events and changes in concomitant medications. Participants will be reminded to log into their PLM profile and record the above-mentioned data points via email reminders from PLM (Appendix E). The study coordinator will call participants at weeks 1, 2 and 3 for training and any time when participants have not entered data for 6 consecutive weeks. The call script for such communications can be found in Appendix E.

3.9.1. Screening/Baseline Visit

During the Screening/Baseline Visit, (Time 0) participants will be consented. Inclusion and Exclusion Criteria will be reviewed to ensure the participant qualifies. Vital signs, weight and concomitant medications will be recorded. Participants will be taught to self-administer the ALSFRS-R (22) on PLM. Participants will be taught to register for an account on the website PatientsLikeMe (31) (Appendix H) and taught to enter their ALSFRS-R, perceived efficacy, compliance, adverse events and concomitant medications. The ALSFRS-R has previously been validated for similar online use by patients (24). Blood will be drawn for baseline biomarker assessments. Urine will be collected for pregnancy testing in at-risk females.

3.9.2. Week 1, 2 and 3 Calls

During the Week 1, 2 and 3 Calls the study coordinator will confirm that participants are logging into the PatientsLikeMe site and entering data without difficulty. The coordinator will measure an ALSFRS-R by phone and compare it to the participant-generated score. Differences will be reconciled as part of the teaching process.

3.9.3. Month 1 and 12 Visits

During the Month 1 and 12 Visit, vital signs and weight will be measured, and concomitant medications will be reviewed. Participants will be asked to bring in any leftover product as a measure of compliance. ALSFRS-R's will be independently obtained by the study coordinator and the participant and recorded in the Neurobank. Comparison of the coordinator and participant generated ALSFRS-R scores will be used to confirm the accuracy of participant scoring at this time point. Blood will be drawn for Month 1 biomarker assessments.

3.9.4. Week 1, 2, 3 and Month 2-11 Virtual Visits

During the Week 1, 2, 3 and Month 2-11 Virtual Visits, participants measure their own ALSFRS-R and weight and will log into the PatientsLikeMe site to record these as well as perceived efficacy, adverse events and concomitant medications. If participants have not entered data within 7 days of a visit, they will receive a reminder email from PatientsLikeMe (Appendix E). If they still have not entered data after another 7 days, they will receive a reminder call from the study coordinator.

3.9.5. Schedule of Events Table

The following Table shows the schedule of study visits and calls (C=coordinator initiated task, P=participant initiated task):

	Screening/Baseline Visit	Weeks 1-3 and Month 2-11 Virtual Visits	Month 1 and 12 Visits	Weeks 1,2,3 Calls
Consent	C			
Inclusion and Exclusion Criteria Review	C			
Enrolled Participants Entered into Neurobank	C			
Vitals, Weight, ALSFRS-R, Con Meds, AE Recorded in Neurobank	C		C	
Participant Registers Account on PatientsLikeMe	P			
Teach Participant to Measure and Record Con Meds and AE in PatientsLikeMe Database	C			C
Participant Measures and Records ALSFRS-R, Weight,	P	P	P	

Compliance, Perceived Efficacy, Con Meds and AE in PatientsLikeMe Database				
Blood Draw for Biomarkers	C		C	
Urine for Pregnancy Testing	C			
Confirm that patients are logging into PatientsLikeMe and entering ALSFRS-R, Weight, Perceived Efficacy, Compliance, Con Meds and AE				C
Objectively check compliance with Lunasin regimen			C	

3.10. Participants

Participants will be patients with ALS (PALS) who are cared for by Dr. Bedlack, or who call our site inquiring about this study. Approximately 50 participants will be enrolled at Duke.

3.10.1. Inclusion Criteria

Each participant must meet all of the following criteria at screening and baseline (unless otherwise specified) to participate in the study:

1. Male or female, aged at least 18 years.
2. Sporadic or familial ALS diagnosed as possible, laboratory-supported probable, probable, or definite as defined by revised El Escorial criteria.
3. Patient is able to understand and express informed consent (in the opinion of the site investigator).
4. Patient has access to the Internet on a desktop computer, laptop, or tablet and has a working email address.
5. Patient or caregiver is willing and able to use a computer and enter data on a secure website.
6. Patient is able to read and write English.
7. Women must not be able to become pregnant (e.g., post-menopausal, surgically sterile, or using adequate birth control methods) for the duration of the study and three months after study completion. Adequate contraception includes: abstinence, hormonal contraception (oral contraception, implanted contraception, injected contraception or other hormonal contraception, for example patch or contraceptive ring), intrauterine device (IUD) in place for $\geq$ 3 months, barrier method in conjunction with spermicide, or another adequate method.

3.10.2. Exclusion Criteria

Participants will be excluded for any of the following:

1. Patient is taking other experimental treatments for ALS.
2. Prior side effects from Lunasin.
3. Known soy allergy.
4. Patient has a medical or psychiatric illness that could in the investigator's opinion interfere with the patient's ability to participate in this study.
5. Pregnant women or women currently breastfeeding.

3.11. Recruitment & Compensation

Patients will be mainly recruited from Dr. Bedlack's Duke ALS Clinic or will have called our center to inquire about the study. Since Dr. Bedlack's clinic and our phone lines are open to all relevant demographic groups, all groups will have access to this study. In addition we will post the following on the Duke ALS Clinic website and Twitter feed: "Duke Lunasin Study is now open. Call Karen Grace at 919 668-2844 for details." No compensation will be provided.

3.12. Consent Process:

Consent will be obtained by Dr. Bedlack, or by his study coordinator. Patients will be given all the time they need to review the written consent and ask questions about it. No study procedures will occur prior to the consent form being signed.

3.13. Capacity to Give Consent

Only patients with appropriate capacity to provide informed consent will be offered a consent form.

3.14. Risk Assessment

The above-described Lunasin regimen appears reasonably safe. A recent review found only rare patients reporting any side effects from it (7). These included swelling, lightheadedness, rash and stomachache. One website cautions patients with soy allergies against taking Lunasin (31). While Reliv says this is safe (32), we will exclude patients with known soy allergies from this study.

The only measures associated with any risk are the blood draws (pain, bleeding, bruising, infection).

Risk associated with participation in the Duke or PatientsLikeMe database include loss of confidentiality. Risks of logging into Internet for any website including PatientsLikeMe include inadvertently downloading malicious software such as viruses that could put personal information or computer integrity at risk. These risks are minimized by the properties of the Neurobank and the PatientsLikeMe website (see below).

Participants will be provided with contact numbers for the research staff. All sites will have an appropriate staff member available 24 hours a day, 7 days a week in the event of an emergency. Participation in this research study is purely for research purposes, and is not intended to provide any direct benefit to the volunteers other than altruism (knowledge that they are helping investigators develop better treatments, and potentially helping other patients down the road). No at risk populations such as minors, prisoners,

pregnant women, or cognitively impaired adults will be included in this study. Patients who elect not to participate will have standard of care options for their ALS including participation in Dr. Bedlack's multi-disciplinary ALS clinic and the drug riluzole.

3.15. Costs to Subjects

There is no cost to subjects for participating in this study.

3.16. Data Analysis & Statistical Considerations

3.16.1. Historical Controls

For each enrolled participant, matched historical controls will be identified from the PatientsLikeMe database. Participants will be matched according to their ALSFRS-R progression rate before they start on the Lunasin regimen (estimated by assuming their score was normal at 48 on the date of symptom onset). Full details of the matching process are described online (33).

3.16.2. Primary Hypothesis and Sample Size

For the primary hypothesis, we will run a t-test comparing the ALSFRS-R progression rate in our participants to a group of matched historical controls identified from the PatientsLikeMe community (30). This type of analysis has previously been used to look for treatment effects in patients with ALS taking lithium (33), as well as dexpramipexole and various forms of sodium chlorite (34). We made the following assumptions to calculate a sample size requirement for this analysis:

1. The mean ALS-FRS progression in 12 months is 11 points (rate of ~0.9 points/month) and the standard deviation at 12 months is ~8 ALS-FRS points (estimates based on unpublished data from PatientsLikeMe.
2. The effect size we are looking for is to halve the mean progression rate
3. The treatment is not having a harmful effect (allows us to use a one-tailed comparison)
4. There is a 5% chance of seeing an effect this big by chance alone
5. We desire a 90% chance of finding an effect this big, assuming it is real
6. There will be a dropout rate of 15% (less than the 22% rate seen in more traditional ALS trials (5).

Based on these assumptions we will again need to enroll 50 participants (and compare them to 50 matched historical controls).

3.16.3. ALS Reversals

To look for an increase in the frequency of ALS reversals, we will count the number of participants who have an ALSFRS-R score that improves by 4 points or more over 12 months. The observed frequency of spontaneous ALS reversals defined in this way is 1% (35). We will look for an increase in this frequency to at least 2% (1 in 50).

3.16.4. ALSFRS-R Accuracy

To confirm that our participants can accurately measure their own ALSFRS-R, we will compare the ALSFRS-R obtained by the coordinator with that obtained by the participants themselves at the Month 1 Visit. Correlational analysis between these 2 scores will be performed with Spearman's rho, as was done in a previous study (24), because of the ordinal nature of the ALSFRS-R.

3.16.5. Weight Accuracy

To confirm that our participants can accurately measure their own weight, even as they become more disabled by ALS, we will compare the participant-generated weight with the weight obtained by the study coordinator at the Month 1 and Month 12 visits. A simple description of the accuracy (percent agreement between the weights) will be used.

3.16.6. Enrollment

To determine whether the participant enrollment rate in this study is greater than in studies with more traditional designs, we will compare our enrollment rate (participants enrolled per month) to the mean ALS trial enrollment rate of 2 (4) using a chi-squared test.

3.16.7. Retention

To determine whether participant retention in this study is greater than in studies with more traditional designs we will compare our dropout rate (percentage of living enrolled participants that complete the Month 12 Visit) to the mean ALS participant dropout rate of 22% (28) using a chi-squared test.

3.17. Adverse Events Monitoring

All adverse events (AEs), whether observed by the Investigator, elicited from the participant or volunteered by the participant, and whether ascribed to the drug or not, will be recorded. This will include the following: a brief description of the event, the date of onset, the date of resolution, the duration and type of the event, the severity, contributing factors and any action taken with respect to the study drug. This recording will commence with the institution of protocol-specific procedures (including any pretreatment procedures) and continue at each study visit or telephone contact until 4 weeks following the last study related visit.

For each adverse event, the relationship to the study drug will be recorded as one of the choices on the following scale:

> **DEFINITE** Causal relationship is certain (i.e., the temporal relationship between drug exposure and the adverse event onset/course is reasonable, there is a clinically compatible response to de-challenge, other causes have been eliminated and the event must be definitive pharmacologically or phenomenologically using a satisfactory re-challenge procedure if necessary).
>
> **PROBABLE** High degree of certainty for causal relationship (i.e., the temporal relationship between drug exposure and the adverse event onset/course is

reasonable, there is a clinically compatible response to de-challenge [re-challenge is not required] and other causes have been eliminated or are unlikely).

POSSIBLE Causal relationship is uncertain (i.e., the temporal relationship between drug exposure and the adverse event onset/course is reasonable or unknown, de-challenge/re-challenge information is either unknown or equivocal and while other potential causes may or may not exist, a causal relationship to the study drug does not appear probable).

UNLIKELY Not reasonably related, although a causal relationship cannot be ruled out (i.e., while the temporal relationship between drug exposure and the adverse event onset/course does not preclude causality, there is a clear alternate cause that is more likely to have caused the adverse event than the study drug).

NOT RELATED No possible relationship (i.e., the temporal relationship between drug exposure and the adverse event onset/course is unreasonable or incompatible, or a causal relationship to study drug is implausible).

The severity of each adverse event must be recorded as one of the choices on the following scale:

MILD	No limitation of usual activities
MODERATE	Some limitation of usual activities
SEVERE	Inability to carry out usual activities

The expectedness of an AE must be indicated when reporting adverse events. An unexpected adverse event is any adverse experience for which the specificity or severity of the event is not consistent with the current investigator brochure.
A serious adverse drug event (SAE) is defined as any adverse event that occurs during the study that results in any of the following outcomes: death, a life-threatening adverse event (i.e., the participant was at immediate risk of death from the event as it occurred; does not include an event, that had it occurred in a more severe form, might have caused death), inpatient hospitalization or prolongation of existing hospitalization (hospitalizations scheduled before enrollment for an elective procedure or treatment of a pre-existing condition which has not worsened during participation in the study will not be considered a serious adverse event), a persistent or significant disability/incapacity (substantial disruption of one's ability to conduct normal life functions), a congenital anomaly/birth defect, a medically important event or required medical intervention to avoid one of the above outcomes. In addition to the above procedures for AEs, all SAEs will be reported to the IRB within 24 hours of recording. All serious adverse event information will be followed until resolution or an appropriate end point is reached. This may involve contacting other clinicians responsible for the participant's care to obtain information on diagnoses, investigations performed and treatment given

Fatal or life-threatening, unexpected adverse events will be reported to the FDA by telephone, facsimile, or in writing as soon as possible, but no later than 7 calendar days after first knowledge by the Sponsor-investigator. Serious,

unexpected adverse events that are not fatal or life-threatening will be reported to the FDA by telephone, facsimile, or in writing as soon as possible, but no later than 15 calendar days after first knowledge by the sponsor-investigator.

3.18. Data & Safety monitoring

Adverse events will be tracked throughout the study as described above and below. There is no formal safety monitoring plan or DSMB, nor are there any formal stopping rules. PI will review and sign off on all adverse events and promptly report these to the IRB.

3.19. Data Collection and Management

3.19.1. NeuroBANK™

NeuroBANK is a collaboration and data repository platform maintained by the Massachusetts General Hospital (MGH) Neurological Clinical Research Institute (NCRI). This platform facilitates:

1. Capture of clinical and research data from neurologic patients for individual projects in a structured and secure system;
2. Aggregating and sharing uniform, deidentified and/or anonymized datasets for secondary analyses.

3.19.2. NeuroBANK™ Data Management (DM)

DM is responsible for the development, execution and supervision of plans, policies, programs, and practices that control, protect, deliver, and enhance the value of data and information assets.

All data will be managed in compliance with applicable Sponsor and regulatory requirements. Site personnel will collect, transcribe, correct, and transmit the data onto source documents, Case Report Forms (CRFs), and/or other forms used to report, track and record clinical research data. DM is responsible for developing, testing, and managing clinical data management activities.

3.19.3. NeuroBANK Data Entry and Checks

Site personnel are instructed to enter information into the NeuroBANK™ Electronic Data Capture (EDC) System. Data capture is the responsibility of the staff at the site under the supervision of the Site Investigator (SI). During the study, the Site investigator must maintain complete and accurate documentation for the study.

The NeuroBANK™ platform provides password protection. An edit checking and data clarification process will be put in place to ensure accuracy of the data. Logic and range checks as well as more sophisticated rules may be built into the eCRFs to provide immediate error checking of the data entered. The system has the capability to automatically create electronic queries for forms that contain data that are out of range, out of window, missing or not calculated correctly. The sites will only have access to the queries concerning their subjects.

3.19.4. Data Lock Process

The platform will have the ability to lock the project-specific visits to prevent any modification of data once the project is closed. Once this option is activated, every user will have Read-Only access to the data.

3.19.5. Data Handling and Record Keeping

The Site Investigator (SI) is responsible to ensure the accuracy, completeness, legibility, and timeliness of the data reported. Data reported in the eCRF derived from source documents should be consistent with the source documents and discrepancies should be explained.

3.19.6. Confidentiality

The NeuroBANK™ software and patient data reside on servers located in the Partners Healthcare Systems (Partners) server farm. Physical and software access to the servers and security is provided by the Partners IT department. Members of the NeuroBANK™ management team will do everything, within reason, to keep a participant's identity protected.

3.19.7. Global Unique Identifier (GUID)

A patient Global Unique Identifier (GUID) will be used as the identifier for individuals participating in the study in NeuroBANK™. The GUID is an 11-character string that is generated using encryption technology and algorithms licensed by the NCRI from the National Institutes of Health (NIH).

The GUID is generated on a secure website that utilizes 128-bit Secure Socket Layer (SSL). Of note, this website is not linked to NeuroBANK™. The GUID is generated using an irreversible encryption algorithm – it accepts twelve identifying data elements, (e.g. last name at birth, first name at birth, gender at birth, day, month and year of birth, city and country of birth, etc.), and produces a unique random-generated character string, or GUID. No identifying information is stored in the system; it is simply used to generate the GUID. If the same information is entered again, the same GUID will be returned.

The GUID is entered into NeuroBANK™ when the patient is being created in the system. As the same patient may participate in multiple studies, NeuroBANK™ will also allow capturing a study-specific ID for the patient. Participants will be given the option to opt out of sharing their de-dentified information across studies in Neurobank. For more information about NeuroBANK™ or the GUID, please go to: *www.neurobank.org*.

3.20. PatientsLikeMe

3.20.1. General Technical Information on the PatientsLikeMe Website

The PatientsLikeMe website is one of the oldest and most complex (in terms of lines of code) Ruby on Rails commercial website in the world. We manage over 300,000 users, 2,300 conditions, and over 20 million medical datapoints of information. Our published uptime target is 99.97% uptime; we have beaten this benchmark for the past three years.

For 2014, our uptime number was 99.998%. We count unplanned downtime against our uptime number, but actual planned downtime is very small. We push out releases almost on a daily basis, only a handful of which require any downtime at all, and the average downtime is under 2 minutes.

3.20.2. Security Information

We require that patient data be stored on physical servers with clearly documented physical and network security protocols. We minimize accounts and access to servers with patient data on them. All individual machines that handle patient or industry partner data are encrypted and subject to audit according to our SOP's. In addition, all PatientsLikeMe employees undergo background checks as a condition of employment. On the topic of physical security, our servers are hosted by RackSpace, a recognized leader in data center security. Physical access to the servers is tightly controlled and monitored. At PatientsLikeMe's offices, all means of entry to the building are locked and controlled. Team members must use a key, key card, or input a door code to enter the building. To date, we are unaware of any unauthorized access to our building and have not experienced any issues with break-in, theft, and so forth. Our building is monitored by security personnel 24 hours a day, 7 days a week.

On the topic of network security, we maintain strict firewall rules. Virtual private network access is required to access any part of our server infrastructure. We maintain separate virtual networks for production, testing, and client access environments to further enhance our network security.

Please refer to the whitepaper covering Security and Privacy Policies included as Appendix J within this document.

3.21. Good Clinical Practices and Human Subjects Protection Training

The investigator and coordinator involved with the conduct of this study will be certified in Good Clinical Practices (GCP) and Human Subjects Protection training. Human Subjects Protection training certification will be obtained by completing approved training, such as the online computer based training offered by the NIH Office of Human Subjects Research (*http://ohsr.od.nih.gov*).

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