ROAR-DIGAP: A Widely Inclusive, Largely Virtual Pilot Trial Utilizing DIGAP (Deep Integrated Genomics Analysis Platform) To Personalize Treatments

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1 PURPOSE/HYPOTHESES

1.1 Primary Hypotheses

Primary hypotheses are as follows:

a. GENIEUS's Deep Integrated Genomics Analysis Platform (DIGAP) will stratify people with ALS into 4 categories according to the primary pathway driving their disease progression (neuroinflammation, oxidative stress, impaired autophagy & axonal transport, and mitochondrial dysfunction), as proven by a panel of mechanistic biomarkers.
b. Each of 4 individualized treatments based on this stratification (astaxanthin for neuroinflammation, Protandim for oxidative stress, melatonin for impaired autophagy and MitoQ for mitochondrial dysfunction) administered for 6 months will decrease the rate of Revised ALS Functional Rating Scale (ALSFRS-R) progression by 50% relative to a 3-month lead-in period.

1.2 Secondary Hypotheses

Secondary Hypotheses are as follows:

a. Each of the 4 individualized treatments will be safe and tolerable over 6 months of treatment.
b. Each of the 4 individualized treatments will increase the frequency of ALS reversals (defined by an improvement of 4 or more points in the ALSFRS-R over the course of 6 months) from less than 5% observed spontaneously to at least 10%.
c. Each of the 4 individualized treatments will improve serum neurofilament light chain measurements.
d. Each of the 4 individualized treatments will improve at least one of the mechanistic biomarkers.

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 still no significant disease-modifying therapies for this condition (1).

2.2 Failure of Previous ALS Trials

There are several reasons that most 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 the animal models predict human ALS, the vast majority of which is not familial, has been called into question (2). Second, the dosing in previous ALS trials may not have been adequate. Indeed, most ALS trials have not employed pharmacodynamic biomarkers to test their dosing regimen (3). Third, most trials did not attempt to personalize treatment in spite of the fact that the biology of ALS differs between patients, including a variety of genetic and sporadic causes and likely differences in downstream pathologies driving progression (4). Finally, ALS trials are often challenged by slow enrollment (5) and poor retention (6). Some of the enrollment and retention issues may have to do with misconceptions on the part of enrolling clinicians and potential participants (5), and some are due to increasingly narrow trial inclusion criteria, which seem to be necessary when looking for

small degrees of ALSFRS-R slowing (7). Finally, people with ALS do become more disabled over time, and study burdens, including frequent trips to the study site, may eventually become impossible to bear (8).

2.3 Success of Previous Hybrid Virtual/Remote "ROAR" Trials

In order to address some of these issues, we created and have now conducted two "ROAR" (Replication of ALS Reversals) Trials (9, 10). These tested over the counter supplements associated with dramatic motor improvements in people with ALS ("ALS Reversals", 11), with the goal of replicating such large effects. Unusual design features included broad inclusion criteria, use of mostly virtual rather than in-person visits, use of pharmacodynamic biomarkers, and use of historical rather than placebo controls. While both our ROAR trials failed to show benefits from the products they tested, they enrolled more quickly, enrolled a more diverse (and generalizable) sample of patients, and retained patients at least as well as more typical ALS trials (9,10).

2.4 Utilizing DIGAP to Stratify Patients and Individualize Treatment

Despite the remarkable diversity of disease etiology, there are several hallmark pathways commonly affected in individuals with ALS. As a result of these disruptions, motor neurons become dysfunctional and degenerative, but also lose the support of a rigidly protective network of glial cells and neurovascular cells. These molecular pathways include:

- RNA processing
- Synaptic structure
- Mitochondrial function
- Intracellular transport
- Proteostasis
- Oxidative stress
- Cell adhesion
- Retroviral activation
- Neuroinflammation
- Apoptosis/necroptosis
- Blood-brain-barrier integrity

Uncovering high impact genetic variants that occur in genes associated with these pathways may account for missing heritability in ALS and may describe the molecular drivers of ALS in individual patients. Further, we believe that the burden of high impact genetic variants in specific hallmark pathways can be used to stratify ALS cohorts into subpopulations.

In this protocol, we will utilize GENIEUS's Deep Integrated Genomics Analysis Platform (12) to classify participants based on their pathway burden. Based on research conducted by GENIEUS and the (unpublished data; https://www.nygenome.org/als-consortium/), we propose focusing on 4 categories:

- CAT1: Neuroinflammation
- CAT2: Oxidative stress
- CAT3: Disrupted intracellular transport and autophagy
- CAT4: Mitochondrial dysfunction

While GENIEUS has already done some validation of DIGAP, we will further this by correlating our participants' DIGAP classification with a panel of mechanistic biomarkers.

3 DESIGN & PROCEDURES

3.1 Design Overview

This will be a widely inclusive, largely remote/virtual, two-center, open-label pilot trial utilizing 50 participants as their own controls. Following informed consent and screening, participants will provide demographics, disease characteristics, co-morbidities, and concomitant medications. They will have a baseline ALSFRS-R score obtained and blood will be drawn for DIGAP classification, PBMCs (which will be used to generate iPSCs from which motor neurons and/or microglia can be generated), baseline mechanistic biomarkers and baseline neurofilament light chain. A urine pregnancy test will be obtained for pre-menopausal females who have not had one by their own doctor in the past 7 days. Each month after that, they will be contacted by phone by study coordinators to review adverse events, new co-morbidities, and concomitant medications, and to generate a new ALSFRS-R score. At month 3, DIGAP classification will be revealed to each participant and based on this, they will receive 1 of 4 treatments (described below). They will take their assigned treatment for 6 months. At months 3, 5 and 9 they will be asked to return for in person blood draws for repeat mechanistic biomarkers and neurofilament light chain measurements. All of the described blood tests and investigational treatments are being performed exclusively for research purposes.

The total time commitment for each of the 50 planned participants is 9 months. A study schematic is shown below.

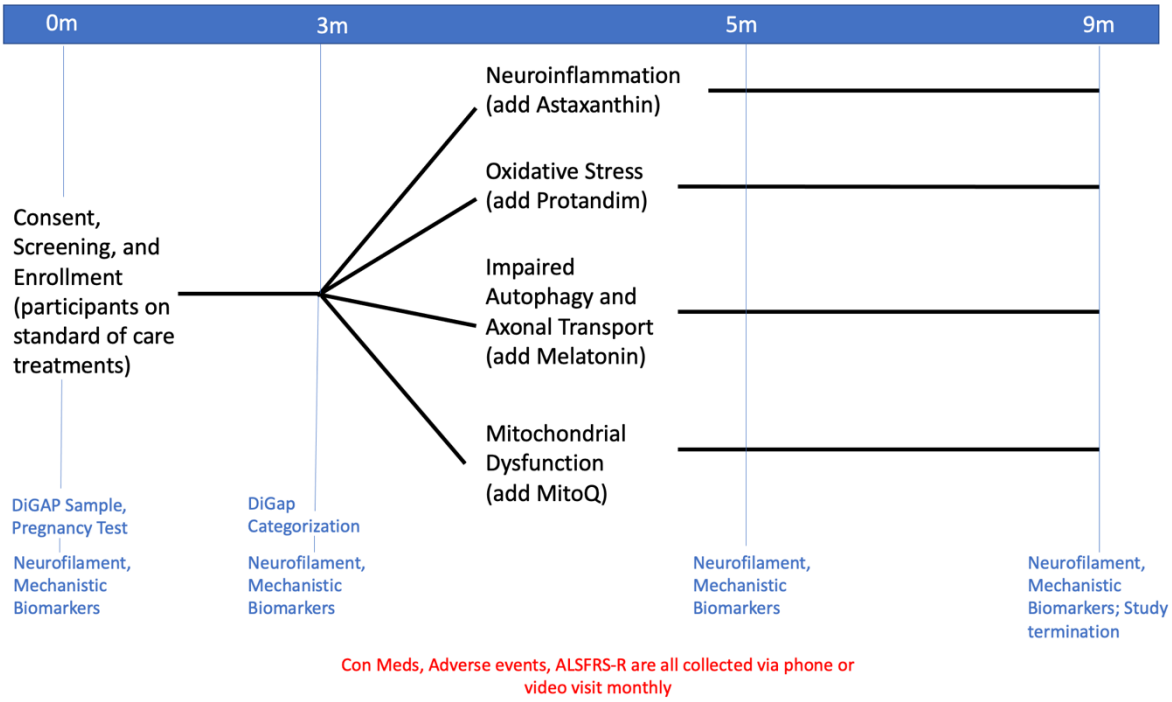

Figure 1: Study Schematic

Table 1: Schedule of Events

	Months									
Event	0	1	2	3	4	5	6	7	8	9
Consent and screening	X									
Demographics and disease characteristics	X									
PBMCs	X									
Mechanistic biomarkers	X			X		X				X
Neurofilament light chain	X			X		X				X
Urine pregnancy test[1]	X									
ALSFRS-R score	X	X	X	X	X	X	X	X	X	X

Event	Months									
	0	1	2	3	4	5	6	7	8	9
Blood draw[2]	X			X		X				X
Co-morbidities	X	X	X	X	X	X	X	X	X	X
Concomitant medications	X	X	X	X	X	X	X	X	X	X
DIGAP classification				X						
Assigned treatment				X	X	X	X	X	X	
Adverse events		X	X	X	X	X	X	X	X	X

[1]Urine pregnancy test will be obtained for pre-menopausal females who have not had one by their own doctor in the past 7 days

[2]Blood draws will be for research purposes only and will be used for DIGAP classification, PBMCs, mechanistic biomarkers and neurofilament light chain.

3.2 Participants

Participants will be people with ALS who are cared for by Dr. Bedlack's team at Duke or Dr. Heiman-Patterson's team at Temple, or who contact our sites inquiring about this study. 50 participants will be enrolled.

3.2.1 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 per Gold Coast Criteria.
3. Patient is able to understand and express informed consent (in the opinion of the site investigator).
4. Patient is able to read and write English.
5. Patient is expected to survive for the duration of the trial.
6. Able to swallow tablets at enrollment and expected to be able to swallow tablets for the duration of the trial.
7. Women must not be pregnant (will have evidence of a negative pregnancy test obtained by study team at baseline, or by local physician within past 7 days or be post-menopausal)
8. 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.

3.2.2 Exclusion Criteria

Participants will be excluded for any of the following:

1. Actively or recently (within past 30 days) participating in another intervention trial.
2. Currently or recently (within 30 days) taking any of the 4 investigational treatments being used in this trial.
3. Prior side effects from any of the 4 investigational treatments being used in this trial.
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.
6. Life expectancy shorter than the duration of the trial.

3.3 Recruitment & Compensation

Participants will be recruited from Dr. Bedlack's Duke ALS Clinic or Dr. Heiman-Patterson's Temple ALS Clinic or will have called our centers to inquire about the study. Since our clinics 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 Temple ALS clinic website: "ROAR-DIGAP Study is now open. Call name at telephone # for details." No compensation will be provided.

3.4 Consent Process

Consent will be obtained by Dr. Bedlack or Dr. Heiman-Patterson, or by their study coordinators. Potential participants 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.

3.5 Capacity to Give Consent

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

3.6 Costs to Subjects

There is no cost for participating in this study.

3.7 Risk Assessment

The investigational products being used in this trial have all received "GRAS" classification from the FDA and are all available without a prescription. Specific side effect profiles are listed in the description of each treatment 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 or Dr. Heiman-Patterson's multi-disciplinary ALS clinic and access to FDA-approved medications for treating ALS.

3.7.1 Blood draw

Blood will be drawn by a certified phlebotomist, nurse, or physician according to standard hospital techniques. There may be slight pain and bruising or bleeding at the site. There is rare risk for infection.

3.8 Outcome Measures

3.8.1 ALS Functional Rating Scale, Revised (ALSFRS-R)

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 12 functional activities. All 12 activities are relevant to people living with ALS. Initial validity was established by correlating change in ALSFRS-R scores with change in strength over time, and it was closely associated with quality of life measures and predicted survival (14). The test-retest reliability is greater than 0.88 for all items. The ALSFRS-R declines linearly with time over a wide range during the course of ALS and it has been validated for telephone use (15). The ALSFRS-R is our primary efficacy measure, and it will be determined 10 times for each participant (at baseline and at each of the subsequent 9 telephone or in person visits.

3.8.2 Neurofilament Light Chain

Neurofilaments are neuron-specific components of the cytoskeleton (reviewed in 16). They exist in heavy, medium, and light chain forms. Neurofilament light chain levels are elevated in the spinal fluid and the blood of patients with ALS and other neurodegenerative diseases, and higher levels predict more severe disease progression (16). These levels rise dramatically when asymptomatic carriers of ALS-causing genetic mutations begin to convert to an ALS phenotype (16). In a recent trial of an antisense oligonucleotide called Tofersen, a significant reduction in neurofilament light chain levels predicted a much later improvement in ALSFRS-R progression and survival (17, 18). As a secondary efficacy screen, we will measure blood neurofilament light chain levels at baseline, month 3, month 5 (2 months into treatment) and month 9 (6 months into treatment).

3.8.3 Mechanistic Biomarkers

We have selected biomarkers known to measure the mechanistic pathways which define the 4 categories we are using in this study. To measure neuroinflammation we will use C-reactive protein (CRP), monocyte chemoattractant protein-1 (MCP-1), and chitotriosidase (CHIT1). These are known to be abnormal in patients with ALS (19-21), their levels correlate with disease progression (19-22) and they can be altered by drugs in trials (19, 22-24). To measure oxidative stress, we will use the total antioxidant capacity (TAC) as well as uric acid levels. Uric acid is an antioxidant and levels of uric acid have been reported to be lower in ALS subjects and correlated with progression (25, 26). It has also been shown to be responsive to treatments in trials (27). To measure impaired autophagy, we will use Beclin-1. This highly conserved eukaryotic protein has a major regulatory role in autophagy. It is a component of the phosphatidylinositol-3-kinase (PI3K) complex which mediates vesicle-trafficking thereby inducing autophagy (28). Beclin-1 dysfunction has been implicated in many disorders, including cancer and neurodegenerative diseases (29, 30). Finally, to measure mitochondrial dysfunction we will use lactate. Compromised mitochondrial oxidative phosphorylation shifts the cellular bioenergetic system to anaerobic respiration and increases the level of lactate. Lactate has been used as a biomarker for mitochondrial disease in many previous studies (31).

3.9 Treatments

3.9.1 Overview

There will be 4 different treatments evaluated in this pilot trial, one for each of the categories identified by DIGAP. All of the selected treatments have "GRAS" classification by the FDA (32) and are available without a prescription. All have data from human trials suggesting effects on mechanistic biomarkers at dosages that appear safe and tolerable. All of the selected treatments are associated with at least one "ALS Reversal" (11).

3.9.2 Astaxanthin (for CAT1: Neuroinflammation)

Astaxanthin is a red-orange natural pigment belonging to a group of carotenoids called xanthophylls (33-36). In nature, astaxanthin is synthesized by microalgae and phytoplankton and biomagnifies in higher marine animals through the food chain. *Haematococcus pluvialis (H. pluvialis)* produces the largest quantity of natural astaxanthin; however cheaper synthetic production is the predominant source of astaxanthin used for animal feeds. Synthetic astaxanthin comprises a different mixture of isomers as compared to the natural compound and may show only 50% of the biological activity, in addition to potentially containing trace amounts of residual solvents and chemical reagents (37-39).

Astaxanthin has been associated with upregulated expression and downregulated phosphorylation of IκB-α, both of which can decrease NF-κB signaling, ultimately lowering production of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α (40-42). In addition, astaxanthin inhibits pro-inflammatory cyclooxygenase-1 activity and nitric oxide generation (40-42). While there are no trials of astaxanthin specifically addressing immune effects in people living with ALS, in healthy volunteers or patients with gastric inflammation it shifted the inflammatory milieu from CD8 predominant T-lymphocytes to CD4 T-lymphocytes, improving mucosal immunity, and immune-enhancing effects. Astaxanthin dose ranges in these studies ranged from 2 mg/day to 40 mg/day (43-46). It can cross the blood brain barrier (43).

There has never been a trial of astaxanthin in people with ALS. There is one independently verified "ALS Reversal" associated with the use of astaxanthin 4mg daily along with several other products (11, 47). There are also positive trials in patients with cognitive impairment (48-51).

Natural astaxanthin is available as capsules, soft gels, tablets, powders, biomass, creams, energy drinks, oils and extracts and often contains other carotenoids. The compound is available as a United States Pharmacopeia (USP) verified supplement which ensures federally recognized standards for quality and purity (https://www.quality-supplements.org/verified-products/verified-products-listings). No serious adverse events related to natural astaxanthin have been identified at any dose for any duration of time in at least 87 human clinical trials involving over 2,000 participants (reviewed in 48). Adverse events of any kind were very rare (less than 1% of all exposed patients) and were gastrointestinal in nature (ex. stomach/abdominal pain, reddish stool discoloration). While average dose ranges were 8-12mg, short-term doses up to 100mg have also been used. Specific dosing for ALS has not been identified, however, human clinical trials targeting cognitive impairment used doses ranging from 6-12 mg/day (49-51). We plan to source natural astaxanthin from Doublewood (www.doublewoodsupplements.com). The dose will be 12mg daily for the duration of the trial.

3.9.3 Protandim (for CAT2: Oxidative Stress)

Protandim is an oral tablet derived from five different plants: Silybum marianum (milk thistle), Withania somnifera (Ashwagandha), Camellia sinensis (green tea), Curcuma longa (turmeric) and Bacopa monniera, (52). It can reportedly activate an intracellular molecule called Nrf2 (nuclear factor erythroid 2-related factor) (53). Once activated, Nrf2 can bind to another molecule called ARE (antioxidant response element) and increase the expression of more than 200 antioxidant and anti-inflammatory genes (54). One study showed that Protandim can upregulate antioxidant biomarkers and decrease markers of lipid peroxidation in humans (53). There has never been a trial of Protandim in people with ALS. One verified "ALS Reversal" is associated with the use of Protandim (11, 55).

From our literature review and discussions with a doctor who has treated 18,000 patients with Protandim, it appears reasonably safe, with only rare loose stools and rash having been noted (55,56). It is most commonly taken at a dose of 1 tablet (675mg) per day, though some studies use more (55). Since the above-described human biomarker study (52) and the one verified ALS reversal (55) used the same dose (675mg daily), we will also use this dose throughout our trial. We will source our Protandim from LifeVantage (www.lifevantage.com/us-en/).

3.9.4 Melatonin (for CAT3: Impaired Autophagy and Axonal Transport)

Melatonin is a hormone that has long been known to play a role in regulating sleep (57). Melatonin supplements are commonly used to treat insomnia (57), but in recent years, melatonin has been found to play a wider role in human physiology (57) including the potential regulation of autophagy (58-61). Given these effects, the observation that endogenous melatonin levels decline with aging, and the fact that that exogenous melatonin can cross the blood brain barrier (62), the possibility of using melatonin supplements to treat age-related neurodegenerative diseases (63) including ALS (64,65) has arisen.

We found no controlled trials of melatonin in people with ALS, but we did find a retrospective study utilizing the PRO-ACT database (66). The eighteen PALS in this database that reported taking melatonin had significantly slower ALSFRS-R and FVC progression, and significantly longer survival, compared to 1604 PALS who were not on melatonin. The authors point out several significant flaws in their study, including the small number of melatonin users, the lack of information on melatonin dosing and adherence, and the fact that the 2 groups were somewhat imbalanced at baseline, with melatonin users being younger and having better breathing measurements. We also validated 2 "ALS Reversals" occurring in association with multiple supplements including melatonin (67).

Optimal melatonin dosing for people with ALS has not been established and will be challenging due to its short half-life of around 30 minutes (57). There is a slow release form which has a half-life of 6 hours (64). In one small case series using this slow release form, three PALS tolerated daily doses of 30-60mg orally for over one year (64). No adverse events were reported, and a panel of safety labs including CBC, electrolytes, lipid panels, and liver functions were unchanged. Melatonin can also be administered by suppository. This route of administration reduces the burden of swallowing pills or capsules and mitigates the "first pass" metabolism of melatonin by the liver (65). In a series using this route of administration, 31 people with ALS were given daily doses of 300mg for one year (65). Thirteen of these patients died (all due to ALS progression), eight patients dropped out (five reportedly due to disease progression and the

desire to stop all medications). The authors note that "mean routine laboratory data remained essentially unchanged." Initially elevated levels of an oxidative stress marker called "serum protein carbonyls" declined coincident with melatonin treatment to the levels of healthy controls (65).

Reviews of melatonin trials in adults with a variety of different conditions conclude that it is safe, even at fairly high doses of up to 1200mg daily (57,68-70). Adverse events included sedation, dizziness, headache, and nausea; these were generally mild and occurred in less than 10% of patients (57, 68-70). We plan to use the extended release 4mg melatonin tablets nightly. We will source this from Pharmavite (NatureMade) (www.pharmavite.com).

3.9.5 MitoQ (for CAT4: Mitochondrial Dysfunction)

The active ingredient in MitoQ is ubiquinone (71), the same as found in coenzyme Q10 and idebenone. However, the ubiquinone in MitoQ is attached to a positively charged, lipophilic molecule called TPP (triphenyl phosphonium), which allows it to selectively accumulate in mitochondria (72). This makes it more potent than untargeted ubiquinone analogs at protecting mitochondria in cultured cells (73, 74). It can be administered orally (74) and, at least in animals, can cross the blood brain barrier and accumulate in brain mitochondria (75). In an animal model of ALS, oral MitoQ improved markers of mitochondrial health and overall survival (76). In healthy humans, MitoQ at a dose of 20mg daily reduced serum biomarkers of mitochondrial damage (77) and improved exercise performance (78).

While CoQ10 failed in a human ALS trial (79), we found no trials of the potentially more potent MitoQ in people with ALS. We have validated 3 "ALS reversals" on various forms of ubiquinone.

MitoQ safety data was gathered systematically in 2 randomized non-ALS human trials (80, 81): a year-long trial of 130 patients with Parkinson's disease (89 received MitoQ), and a 28-day trial of 30 patients with hepatitis C (20 received MitoQ). No MitoQ-related serious adverse events were noted in the patients randomized to this treatment (80, 81). Nausea and vomiting were the only adverse events that were more common in MitoQ-treated patients than in placebo-treated controls (80, 81). Withdrawal due to nausea was more common in MitoQ-treated patients than placebo-treated patients (80, 81). It should be noted that these trials were using higher than typical MitoQ doses of 40mg or 80mg daily. An email from Greg Macpherson, CEO of MitoQ, stated that his company is aware of more than 50,000 patient months' worth of use, with the percentage of side effects being "virtually nil" (82). The problem with this data is that adverse events have not been systematically gathered during these exposures.
We will use MitoQ from MitoQ (https://www.mitoq.com) at a dose of 20mg daily for the duration of our trial.

4 DATA ANALYSES AND STATISTICAL CONSIDERATIONS

4.1 ALSFRS-R Efficacy Analyses

Within each treatment arm, we will compare on-treatment ALSFRS-R slope (month 3 to month 9) to the measured pre-treatment slope (enrollment to month 3), and also to the slope of matched historical controls from a natural history database. Since we do not know how many patients DIGAP will put into each category, we are unable to calculate statistical the power of each arm

ahead of time. This calculation is not critical since this is meant to be a hypothesis-generating pilot trial rather than an efficacy trial.

4.2 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 6 months of treatment. The observed frequency of spontaneous ALS reversals defined in this way is less than 5% (83). We will look for an increase in this frequency to at least 10% (1 in 10).

4.3 Biomarker Analyses

As described above, we also plan to examine serum/plasma biomarkers to assess neurodegeneration as well the mechanisms that underlie each of our disease categories (neuroinflammation, oxidative stress, impaired autophagy and axonal transport, and mitochondrial dysfunction). Plasma and serum samples will be obtained from all individuals in the study at baseline, month 3, month 5 (2 months into treatment) and month 9 (6 months into treatment). Sampling will be done between 8 am to 12 pm in a fasting condition to minimize the influence of circadian and diet related variations. The blood will be collected into plain red top tubes (with separator gel) for serum or EDTA-coated (purple top) vacutainers for plasma. The serum is separated after centrifugation (3000g for 15 minutes at 4°C) and stored at -70°C until assayed.

4.3.1 Neurodegeneration Biomarker: Neurofilament Light Chain

Serum Neurofilament light chain will be measured utilizing available ELISA kits along with standard concentration curves. All spectrophotometric assays will be performed with a Molecular Devices (Sunnyvale, CA) Spectramax 190 spectrophotometer. All assays will be performed in duplicate according to the manufacturers' instructions.

4.3.2 Inflammation Biomarkers: CRP, MCP-1 and CHIT1

For hsCRP we will utilize an ELISA kit (Creative Diagnostics) based on competitive enzyme immunoassay technique utilizing a monoclonal anti-hsCRP antibody and an hsCRP-HRP conjugate. The assay sample and buffer are incubated together with hsCRP-HRP conjugate in pre-coated plate for one hour. After the incubation period, the wells are washed, incubated with HRP substrate forming a blue colored complex that can be measured spectrophotometrically at 450nm. A standard curve is plotted relating the intensity of the color (O.D.) to the concentration of standards. The hsCRP concentration in each sample is interpolated from this standard curve.

For MCP-1, we will use a commercial ELISA kit (ELAB Sciences) using the Sandwich-ELISA principle. The micro ELISA plate is pre-coated with an antibody specific to Human MCP-1. Standards or samples are added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Human MCP-1 and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Following rinse, substrate solution is added and in wells that contain Human MCP-1, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. Following termination with stop solution the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of Human MCP-1. The concentration of Human MCP-1 in the samples is calculated by comparing the OD of the samples to the standard curve.

For CHIT1, we will measure of enzymatic CHIT1 activity in plasma.5 mL of undiluted plasma will be incubated with 100 mL of a solution containing 22 mmol/L of the fluorogenic substrate 4-methylumbelliferyl-beta- D-N,N′,N″-triacetylchitotriose (Sigma) in 0.5 M citrate-phosphate buffer (pH 5.2), for 15 min at 37◦C, as originally described (https://doi.org/10.1172/jci117084). The reaction is stopped by using 2 mL of 0.5 mol/L Na_2CO_3-$NaHCO_3$ buffer, pH 10.7. The fluorescence of formed 4-methylumbelliferone is read on a fluorimeter, on 365 nm excitation and 450 nm emissions. CHIT1 activity is expressed as nanomoles of substrate hydrolyzed per milliliter per hour (nmol/mL per h).

4.3.3 Oxidative Stress Biomarkers: TAC and Uric Acid

The presence of oxidative stress may be tested in one of three ways: (1) direct measurement of the reactive oxygen species (ROS, e.g., H_20_2, NO, and universal kits for Reactive oxygen species); (2) measurement of the resulting damage to biomolecules (DNA/RNA measured by 8-OHdG, lipids measured by 8-isoprostane, malondialdehyde, 4-HNE, and TBARS; proteins measured by 3-nitrotyrosine, advanced glycation end products, and protein carbonyls); and (3) detection of antioxidant levels (catalase and superoxide dismutase (SOD)). Directly measuring ROS might seem the preferred method, but many reactive oxygen species are extremely unstable and difficult to measure directly. Because of this, it may be preferable to measure the damage on DNA/RNA, lipids, proteins, or other biomolecules. While this is an indirect approach, many markers of damage are extremely stable and therefore provide a more reliable method to measure oxidative stress. An additional approach is to measure the levels of antioxidant enzymes and other redox molecules which serve to counterbalance ROS generated in the cell. Although assays are available to measure the activity of specific antioxidant enzymes, including catalase and SOD, in this proof of concept study, we will measure the total antioxidant capacity (TAC) since specific effects on catalase and superoxide dismutase (SOD) are not expected. Additionally, uric acid is an antioxidant and levels of uric acid have been reported to be lower in ALS subjects and correlated with progression (23, 24).

We will measure 8-OHdG, 3-NT and TBARS as markers of oxidative damage to DNA, proteins, and lipid membranes while antioxidant capacity will be determined by measuring TAC at the specified time points. The levels of TBARS in tissue or biological fluids is a well-established method for monitoring lipid peroxidation. Serum levels of TBARS will be determined with the colorimetric TBARS assay kit (Cayman Chemicals, Ann Arbor, Michigan). Oxidative damage to nucleic acids will be determined by ELISA using the DNA/RNA oxidative damage ELISA kit (Cayman Chemicals, Ann Arbor, Michigan). Levels of 3-NT will be determined with the Nitrotyrosine ELISA Kit (Cell Biolabs, San Diego, CA). Colorimetric determination of the TAC of biological fluid will be determined with an assay kit from BioAssay Systems (BioAssay Systems, Hayward, CA). All spectrophotometric assays will be performed with a Molecular Devices (Sunnyvale, CA) Spectramax 190 spectrophotometer. All assays will be performed in duplicate according to the manufacturers' instructions.

4.3.4 Autophagy Biomarker: Beclin-1

Serum will be collected and allowed to clot at room temperature overnight at 2–8 °C. It will be centrifuged at 1000*g* for 15 min. Serum is removed and stored at −80 °C. An ELISA kit (MyBioSource, Inc, California, United States) will be used to quantify serum Beclin-1 level in ng/ml. This test will be performed according to manufacturers' instructions.

4.3.5 Mitochondrial Dysfunction Biomarker: Lactate

Concentration of *l*-lactate will be measured using Abcam's L-Lactate Kit (Cambridge, UK) as per manufacturer's protocol for serum samples. The colorimetric assay kit is based on the oxidation of *l*-lactate to pyruvate mediated by lactate dehydrogenase where it detects a color formation at a wavelength of 450 nm produced by the reduced electron mediator. 5 µL of serum per well will be assayed in technical duplicates and the concentrations will be calculated by comparing against a standard curve, in nmol/uL.

4.4 Whole genome sequencing and analyses

DNA extracted from PBMCs will be transported to an appropriate sequencing provider (Duke Sequencing and Genomic Technologies, Durham, NC; or equivalent). It is currently planned for sequencing activities to take place at Duke, but GenieUS may need to utilize other vendors or academic laboratories for this purpose as costs or logistics dictate. Alternative laboratories used by GenieUS will have met the same privacy stipulations as Sequencing and Genomic Technologies. Data will not remain at the sequencing provider but will be deleted after transfer back to GenieUS. Sequencing is not being carried out on an FDA-approved platform and no sequencing information will be returned to participants.

Four 8 mL Vacutainer CPT tubes will be obtained by standard phlebotomy for the separation into PBMCs and plasma. Within 2 hours of blood collection, a PBMC cell pellet will be taken from the Vacutainer CPT tubes according to manufacturer's instructions. Following extraction of the phase containing PBMCs, plasma will also be taken by aspirating the remaining volume of liquid above the gel phase. Plasma will be aliquoted and frozen at -80C. After washing, the PBMC fraction will be processed for DNA extraction using Qiagen DNeasy kit.

Sequencing data will be analyzed using the DiGAP™ pipeline to produce a Deep Insight Report™ for each patient, which includes Pathway Mutation Burden analyses and Variant Prioritization. All data will be shared with Dr. Bedlack and Dr. Heiman-Patterson. No data from GenieUs will be shared with participants.

4.5 Statistical Analyses

The statistical analyses will be performed at Temple. This study is designed to evaluate the ALSFRS-R data in two different ways. The first evaluates each subject at different time points (before, during and after treatment). This design (differences on the same subject at various time points) reduces variability and allows for greater statistical power. The second compares ALSFRS-R progression during treatment in each arm to a propensity matched group of controls derived from the Natural History database. For parametric variables (Biologic fluid levels of TBARS, TAC), statistical significance will be determined by repeated measures ANOVA. For non-parametric variables (ALS FRS score, ALSSQOL-R), we will use the Friedman test. For parametric variables, statistical differences between groups will be determined by analysis of variance (ANOVA) and the Kruskall–Wallis test will be used for non-parametric variables. Determinations will be considered significant if $p < 0.05$. Statistical calculations will be accomplished with the aid of data analysis software, Systat 13.1 (Systat Software, Chicago, IL).

5 SAFETY AND ADVERSE EVENTS

5.1 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 study or its treatments 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 known side effects of one of the study treatments, as described in section 3.9 above.

5.2 Reporting of Serious Adverse Events

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. 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.

5.3 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.

6 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.

6.1 Data handling procedures at GenieUS

6.1.1 Biosample storage

For iPSC generation, PBMCs will be transported to our collaborators and stored there in their lab. Once they provide us with the further details on the methodology for this work GenieUS will pass this on for inclusion here. Storage will be in a locked, access-controlled laboratory. If samples are sent for analysis elsewhere, no identifiable information will be included.

6.1.2 Data storage

All clinical data will be stored at GenieUS labelled with the GUID and no identifiable information. Storage will be in password protected spreadsheets. If clinical information needs to be shared with samples for analysis, no identifiable information will be included. All sequencing data will be transferred from contract sequencing companies to the GenieUS Genomics secure servers and deleted from the contract companies' or labs' databases.

7 GLOBAL UNIQUE IDENTIFIER (GUID)

A patient Global Unique Identifier (GUID) will be used as an identifier. 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). 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. Participants will be given the option to opt out of sharing their de-identified information across studies.

8 SECURITY INFORMATION

Paper electronic case report forms as well as the computers containing study Redcap databases will be stored in locked offices at Duke and Temple University. These paper forms and all Redcap data will be de-identified. No identifiable data will leave the site at which the participant was enrolled.

9 GOOD CLINICAL PRACTICES AND HUMAN SUBJECTS PROTECTION TRAINING

The investigators and coordinators 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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