Published Ahead of Print on January 15, 2020 as 10.1212/WNL.0000000000008872

NULL HYPOTHESIS OPEN ACCESS CLASS OF EVIDENCE

Randomized controlled trial of *N*-acetylcysteine therapy for *RYR1*-related myopathies

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Neurology® 2019;00:1-11. doi:10.1212/WNL.0000000000008872

Abstract

Objective

To investigate the efficacy of *N*-acetylcysteine (NAC) for decreasing elevated oxidative stress and increasing physical endurance in individuals with ryanodine receptor 1-related myopathies (*RYR1*-RM).

Methods

In this 6-month natural history assessment (n = 37) followed by a randomized, double-blinded, placebo-controlled trial, 33 eligible participants were block-randomized (1:1) to receive NAC (n = 16) or placebo (n = 17), orally for 6 months (adult dose 2,700 mg/d; pediatric dose 30 mg/kg/d). The primary endpoint was urine 15-F2t isoprostane concentration and the clinically meaningful co-primary endpoint was 6-minute walk test (6MWT) distance.

Results

When compared to the general population, participants had elevated baseline 15-F2t isoprostane concentrations and most had a decreased 6MWT distance (mean ± SD 3.2 ± 1.5 vs 1.1 ± 1.7 ng/mg creatinine and 468 ± 134 vs 600 ± 58 m, respectively, both $p < 0.001$). 15-F2t isoprostane concentration and 6MWT distance did not change over the 6-month natural history assessment ($p = 0.98$ and $p = 0.61$, respectively). NAC treatment did not improve 15-F2t isoprostane concentration (least squares means difference 0.1 [95% confidence interval [CI] −1.4 to 1.6] ng/mg creatinine, $p = 0.88$) or 6MWT distance (least squares means difference 24 [95% CI −5.5 to 53.4] m, $p = 0.11$). NAC was safe and well-tolerated at the doses administered in this study.

Conclusion

In ambulatory *RYR1*-RM–affected individuals, we observed stable disease course, and corroborated preclinical reports of elevated oxidative stress and decreased physical endurance. NAC treatment did not decrease elevated oxidative stress, as measured by 15-F2t isoprostane.

Classification of evidence

This study provides Class I evidence that, for people with *RYR1*-RM, treatment with oral NAC does not decrease oxidative stress as measured by 15-F2t isoprostane.

Clinicaltrials.gov identifier

NCT02362425.

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This Null Hypothesis article is published as part of a collaborative effort between *Neurology* and CBMRT.

Go to Neurology.org/N for full disclosures. Funding information and disclosures deemed relevant by the authors, if any, are provided at the end of the article.

The Article Processing Charge was funded by the NIH.

Glossary

6MWT = 6-minute walk test; **AE** = adverse event; **CI** = confidence interval; **CYS:CYSS** = systemic reduced-to-oxidized ratio for cysteine; **GC/NICI-MS** = gas chromatography/negative ion chemical ionization mass spectrometry; **GSH:GSSG** = systemic reduced-to-oxidized ratio for glutathione; **IMC** = independent monitoring committee; **IRB** = NIH Combined Neuroscience Institutional Review Board; **ITT** = intent-to-treat; **MCID** = minimum clinically important difference; **n-3 PUFA** = omega-3 polyunsaturated fatty acid; **NAC** = *N*-acetylcysteine; **PROMIS** = Patient-Reported Outcomes Measurement Information System; ***RYR1*-RM** = ryanodine receptor 1-related myopathies; **SAE** = serious adverse event.

Ryanodine receptor 1-related myopathies (*RYR1*-RM) are the most frequently encountered nondystrophic neuromuscular disorders, with an estimated pediatric prevalence of >1:90,000.[1] *RYR1*-RM are allelic to malignant hyperthermia susceptibility and affected individuals exhibit diverse clinical manifestations with variable expressivity ranging from hypotonia, proximal muscle weakness, and fatigue to ophthalmoplegia and respiratory insufficiency.[2,3] *RYR1*-RM have an unmet medical need as there is currently no approved treatment.

RYR1-RM are caused by pathogenic variants in the *RYR1* gene (19q 13.2), which is highly intolerant to change and encodes the major calcium (Ca^{2+}) channel in skeletal muscle (RyR1).[1,4] *RYR1* variations can result in dysregulation of Ca^{2+} release from the sarcoplasmic reticulum, hypersensitivity or hyposensitivity to channel agonists, and decreased RyR1 protein expression.[5] Data from *RYR1*-RM cell culture and animal models have consistently demonstrated elevation of oxidative stress owing to intracellular Ca^{2+} dysregulation.[6–8] *N*-acetylcysteine (NAC), a direct precursor to the ubiquitous antioxidant glutathione, was subsequently shown to protect *RYR1*-RM patient myotubes against a hydrogen peroxide challenge and, when tested in *ryr1b* mutant zebrafish, improved measures of physical endurance and skeletal muscle histopathology.[6] An additional study, in the Y522S mouse model of *RYR1*-RM, reported elevated lipid peroxidation, which compromised the structural integrity of mitochondria and decreased force production. Histopathology and force production were rescued upon treatment with NAC.[7]

Given the potential therapeutic efficacy of NAC for *RYR1*-RM, we conducted a double-blind, randomized, placebo-controlled trial to determine whether NAC treatment decreases oxidative stress in this rare disease population.

Methods

Standard protocol approvals, registrations, and patient consents

The study protocol was approved by the NIH Combined Neuroscience Institutional Review Board (IRB), Bethesda, MD, and all participants and parents of participants <18 years of age provided written informed consent, according to the Declaration of Helsinki, before enrollment. Assent was obtained for those <18 years of age. The clinical trial was prospectively registered at clinicaltrials.gov (NCT02362425) and an independent monitoring committee (IMC) was established to oversee trial safety.

Primary research question

The primary research question for this study was to determine if NAC treatment decreases oxidative stress in *RYR1*-RM–affected individuals. This study provides Class I evidence that, for people with *RYR1*-RM, treatment with oral NAC does not decrease oxidative stress as measured by 15-F2t isoprostane.

Participants

Inclusion criteria

Ambulatory adult (≥18 years) and pediatric (7–17 years) individuals with a confirmed genetic diagnosis of *RYR1*-RM, or individuals with a clinical *RYR1*-RM diagnosis and a family member with a confirmed genetic diagnosis of *RYR1*-RM, were included. If available, a muscle biopsy report detailing *RYR1*-RM histopathology, such as central or multi-minicores, was considered supporting evidence for inclusion in this study.

Exclusion criteria

The exclusion criteria were history of any of the following: liver disease, peptic ulcers, gag reflex depression, severe pulmonary dysfunction (forced expiratory volume in 1 second <40% predicted), pulmonary exacerbation, unable to provide consent or did not have parent to provide assent, pregnant or breastfeeding, reported consumption of antioxidants within 4 weeks of recruitment, reported daily use of acetaminophen, nitroglycerine, or carbamazepine during the last 7 days, β2-adrenergic agonist use, for the purpose of increasing muscle mass, and intending to participate in trials for other therapeutic investigational drugs simultaneously or 4 weeks before recruitment; and if opting in for muscle biopsy, use of aspirin, ibuprofen, Advil, Motrin, or Aleve within 3 days prior to the muscle biopsy procedure, or consumption of Plavix, fresh garlic, gingko, or ginseng 5 days prior to the muscle biopsy.

Study design

The study had 2 components: a prospective natural history assessment and a parallel-group, randomized, double-blind, placebo-controlled trial. The study was conducted at the NIH

Clinical Center, Bethesda, MD, between 2015 and 2017. The total study duration was 12 months and consisted of a 6-month natural history assessment followed by a 6-month intervention phase. Participants attended 3 study visits: baseline (month 0), preintervention (month 6), and postintervention (month 12).

The allocation scheme was computer-generated using random permuted blocks (block size of 4) to maintain balance of the 2 study arms between children and adults. Participants were randomized (1:1) to receive NAC or placebo by a pharmacist who was independent of the study team. All participants and study investigators were blinded to the allocations until study completion (i.e., after the study was closed and the database locked).

Participants randomized to receive NAC were provided with a 6-month supply of 900 mg effervescent tablets (PharmaNAC; BioAdvantex Pharma Inc., Toronto, Canada). The NAC content of tablets was verified by an independent laboratory using high-performance liquid chromatography (Hermes Arzneimittel GmbH, Pullach, Germany) and found to be compliant with the stated dose. For the first week, all participants received approximately half of the final dose (15 mg/kg/d divided 3 times daily; no more than 1,800 mg/d). Participants who weighed <50 kg continued with a weight-based dose from the second week (30 mg/kg/d, divided 3 times daily) for the duration of the study (not to exceed 2,700 mg/d). For the 30 mg/kg/d dose, participants were provided with a marked syringe and trained how to take the appropriate liquid dose. Participants who weighed >50 kg received a 2,700 mg total daily dose, divided 3 times daily. The 2,700 mg total daily dose was deemed suitable based upon (1) evidence of safety and tolerability in pediatric and adult populations and (2) evidence that 2,700 mg/d is sufficient to elicit a beneficial effect on oxidative stress.[9,10] Participants randomized to receive placebo were provided with a 6-month supply of 900 mg effervescent tablets that were identical but did not contain NAC. Placebo tablets were also manufactured by BioAdvantex Pharma and dosages were prescribed identically to the NAC group. All participants were instructed to self-administer 3 effervescent tablets per day with water or clear liquid as recommended in the package insert. Both NAC and placebo were produced in accordance with good manufacturing practice. Participants were asked to return all remaining tablets at the final study visit. Percentage compliance with intervention was then assessed by a postintervention pill count at month 12 ([pills returned at final study visit, n ÷ expected number of pills to be returned at final study visit, n] × 100). At each study visit, blood, urine, and saliva samples were obtained following an overnight fast. Participants also completed a range of physical performance tests, underwent clinical assessments, participated in a qualitative interview, and answered several self-report questionnaires. Adult participants who opted to have skeletal muscle biopsy underwent this procedure immediately preintervention and postintervention (months 6 and 12). Safety and adverse events (AEs) were assessed on-site for 24 hours following each participant's first dose and at monthly intervals thereafter.

Primary endpoints

The primary endpoint was urine 15-F2t isoprostane concentration (corrected for creatinine), a widely reported biomarker of in vivo oxidative stress and byproduct of lipid peroxidation. This was assessed at the Eicosanoid Core Laboratory (Vanderbilt University Medical Center, Nashville, TN) using a validated gas chromatography/negative ion chemical ionization mass spectrometry (GC/NICI-MS) method with stable isotope dilution and (2H4)-15-F2t-IsoP as the internal standard. The GC/NICI-MS assay methodology used in this study has been described in detail by Milne et al.[11] with a precision and accuracy of ±6% and 96%, respectively. Urine free 15-F2t isoprostane concentrations were corrected for urinary creatinine (15-F2t isoprostane ng/mg creatinine). Creatinine was quantified using a colorimetric assay based on the Jaffe reaction (Roche COBAS Integra 800; F. Hoffmann-La Roche AG, Basel, Switzerland).

The clinically meaningful co–primary endpoint was 6-minute walk test (6MWT) total distance (meters), as a measure of physical endurance. At each study visit, participants completed a 6MWT at the NIH Clinical Center, Rehabilitation Medicine Department. Participants were asked to walk along a corridor for 6 minutes with total distance walked recorded to the nearest meter. The 6MWT was administered by physical therapists according to American Thoracic Society recommendations.

Secondary endpoints

The following were secondary endpoints of oxidative stress: systemic reduced-to-oxidized ratios for glutathione and cysteine (GSH:GSSG and CYS:CYSS, respectively) and 2′,7′–dichlorofluorescin fluorescence intensity (AU), assessed from skeletal muscle homogenates. Additional secondary endpoints included Motor Function Measure–32 (percentage of maximum scores for each domain); peak torque (nM); time to ascend/descend 4 steps, supine to stand, and 10-meter walk/run (seconds); grip/pinch strength (kg); Patient-Reported Outcomes Measurement Information System (PROMIS) and quality of life in neurologic disorders quality of life scale (*t*) scores; and multidimensional fatigue inventory–20 and functional assessment of chronic illness therapy–fatigue questionnaires (total/subscale scores).

Exploratory endpoints

Exploratory endpoints were salivary fatigue biomarker index (GGHPPPP/ESPSLIA ratio), VO_2 peak (L/min), anaerobic threshold (L/min), electrical impedance myography (phase angle), and tissue oxygenation index.

Statistical analysis

Sample size was estimated based on oxidative stress data from a study that tested the effects of NAC therapy on glutathione concentrations in an HIV-positive population. This approach was taken because the current study was the first clinical trial in the *RYR1*-RM population.[12] An a priori power calculation, with power at 80% and a 2-sided α of 0.05, determined that n = 76 participants (n = 38 per group) would be required to detect

a statistically significant postintervention difference in plasma glutathione concentration between NAC and placebo groups. An α of 0.05 was used to determine statistical significance for all subsequent tests. To refine the initial sample size estimate, based on the *RYR1*-RM population, a sample size reevaluation was conducted once 30 participants completed the study. This consisted of a formal comparison between treatment and placebo groups and was inclusive of cases subject to intent-to-treat (ITT). Due to technical difficulties with the GSH:GSSG analysis, the primary endpoint for oxidative stress for the randomized controlled trial was changed to urine 15-F2t isoprostane, an alternative, well-established biomarker of oxidative stress. Data for urine 15-F2t isoprostane had been successfully obtained for all study participants as a secondary endpoint.[11,13] This decision was made before unblinding and final statistical analyses. Changing of the primary endpoint was approved by an IMC, an independent scientific review committee, and the IRB, all of which were blind to the treatment allocation scheme. All members came to this conclusion independent of study data.[14] As such, the above-mentioned sample size reevaluation was based on urine 15-F2t isoprostane data collected on the first 30 study completers. The reevaluation determined that a larger sample (total n = 182) would be required to detect an effect of NAC treatment on urine 15-F2t isoprostane concentration. The study was closed at this point because the new sample size was unlikely to be feasible given the rarity of the disease.

Once 30 participants completed the 6-month preintervention study visit, disease progression was assessed by using paired *t* tests to determine change over time between 0- and 6-month visits for each outcome measure.[15] In addition, the baseline means (±SD) of 15-F2t isoprostane concentration and GSH:GSSG ratio of participants were compared to previously reported general population values using summary independent *t* tests.[13,16] The normal distribution assumption was tested prior to running parametric analyses. In the case of 15-F2t isoprostane, the standardized mean difference in concentration between *RYR1*-RM affected and otherwise healthy individuals was also calculated using Hedges *g*.

For 6MWT distance, the baseline mean (±SD) for *RYR1*–RM–affected individuals was compared to the mean (±SD) of general population predicted values,[17–19] using a summary independent *t* test, accounting for age, height, and sex of the individual. A disease-specific minimum clinically important difference (MCID) for 6MWT distance was also determined, using preintervention data, by a combined distribution and anchor-based cross-sectional approach. This provided an MCID range (in meters) derived from the standard error of measurement, 1/3 SD at baseline, and difference in 6MWT distance between participants who achieved a *t* score ± 60 (moderate fatigue) on the PROMIS fatigue subscale.

To determine the effect of the intervention on primary and secondary outcome measures, statistical analyses included all randomized participants ITT and therefore conformed to the Consolidated Standards for Reporting Trials guidelines. Missing data, considered unrelated to the intervention (i.e., categorized as missing at random), were imputed based on the average of 40 imputed datasets. Imputed datasets were subject to minimum and maximum value constraints, based on per protocol data. Following assessment of data distribution, generalized linear modeling was employed to compare postintervention oxidative stress measure concentration between NAC and placebo groups with preintervention value included as a covariate. Generalized linear modeling was also used to compare postintervention 6MWT distance between NAC and placebo groups with preintervention 6MWT distance and participant height included as covariates. Statistical analyses were performed using SAS version 9 (SAS Institute Inc., Cary, NC).

Data availability

The study sponsor, NINR, is committed to sharing trial data with qualified external researchers. This includes providing access to deidentified (unlinked) individual patient-level data from study participants who consented to data sharing for additional research. Data will be available beginning 3 months and ending 5 years following article publication. Requests for access to data must be accompanied by a methodologically sound proposal. Requests can be addressed to meilleurk@mail.nih.gov. A signed data sharing agreement is required before access can be provided.

Results

Recruitment and study flow

Baseline characteristics are shown in table 1. Overall, 150 individuals were screened for participation in this study, of whom 53 were eligible (figure) and were enrolled between March 23, 2015, and November 26, 2017. A total of 37 participants completed the 6-month natural history assessment, and 33 were randomized to NAC or placebo groups (n = 16 and n = 17, respectively), as 4 were excluded due to screening failure. During the randomized controlled trial, a total of 4 participants were lost to follow-up and 29 completed the study per protocol. Compliance with intervention was determined to be 96% based on the postintervention pill count. Details regarding loss to follow-up are provided in the figure. Use of ITT did not change the outcome of the study when compared with per protocol analyses.

Baseline characteristics

The study population comprised mild to moderately affected individuals, as ambulation was a required eligibility criterion for 6MWT performance. Participants' clinical manifestations were consistent with previous reports, in which recessive cases were typically more severe than dominant and de novo cases. More severe clinical manifestations identified in recessive cases included greater difficulty ambulating, neonatal hypotonia, ptosis, and ophthalmoplegia. We have reported elsewhere a comprehensive assessment of participant clinical manifestations and histopathology, by mode of inheritance and affected protein structural domain.[2]

Table 1 Baseline characteristics

Measure	Total cohort (natural history)[a] (n = 53)	Total cohort (RCT) (n = 33)	NAC (n = 16)	Placebo (n = 17)
Age at enrollment, y	29.2 ± 17.5	27.4 ± 17.7	28.1 ± 17.4	26.7 ± 18.5
Sex, male	24 (45)	14 (42)	7 (44)	7 (41)
BMI, kg/m^2	22.1 ± 8.7	21.9 ± 7.5	22.8 ± 8.2	21.5 ± 6.5
Mode of inheritance, dominant/de novo	40 (76)	25 (76)	12 (75)	13 (77)

Abbreviations: BMI = body mass index; NAC = *N*-acetylcysteine; RCT = randomized controlled trial.
Data are n (%) or mean ± SD.
[a] Only individuals with confirmatory genetic testing included in analyses.

At baseline, participants had a significantly greater mean 15-F2t isoprostane concentration compared to the general population (n = 44, mean ± SD 3.2 ± 1.49 vs n = 1881, 1.1 ± 1.70 ng/mg creatinine, $p < 0.001$).[13] In fact, all participants demonstrated baseline 15-F2t isoprostane concentrations greater than the 1.1 ng/mg creatinine general population mean reference value. Moreover, the standardized mean difference in 15-F2t isoprostane concentration for *RYR1*–RM vs the general population exceeded the small effect Hedges *g* criterion of 0.8 and ranked among the highest of 50 other diseases associated with oxidative stress (Hedges *g* in *RYR1*-RM: 1.24 vs other diseases: range −0.2 to 2.3).[13] *RYR1*-RM–affected individuals also had a lower mean GSH:GSSG ratio compared to the general population (n = 38, mean ± SD 10.8 ± 7.2 vs n = 24, 16.4 ± 6.3, $p < 0.01$).[16] On average, *RYR1*-RM–affected individuals performed at 79% (range 32%–119%) predicted distance for 6MWT (n = 45, 468 ± 134 vs n = 45, 600 ± 58 m, $p < 0.001$).

Natural history assessment

Overall, in this cohort, 15-F2t isoprostane concentration was stable during the 6-month natural history phase (baseline 3.2 ± 1.4 vs month 6 3.6 ± 2.2 ng/mg creatinine, $p = 0.98$). 6MWT total distance did not change over the 6-month natural history assessment.[15] Using a combined distribution and anchor-based method, the MCID for 6MWT distance was determined to be 25–83 m for *RYR1*-RM–affected individuals.

NAC randomized controlled trial

There was no significant effect of NAC treatment on 15-F2t isoprostane concentration (least squares means difference 0.1 [95% confidence interval [CI] −1.4 to 1.6] ng/mg creatinine, $p = 0.88$) (table 2). Following intervention, 6MWT distance increased by 25 m after controlling for 6-month (pre-intervention) distance and treatment group only. After controlling for the identified significant covariate of height, the increase in the NAC treatment group was 24 m. This improvement was borderline clinically meaningful but did not reach statistical significance (least squares means difference 23.9 [95% CI −5.5 to 53.4] m, $p = 0.11$) (table 2).

Results for additional secondary endpoints are shown in table 3. Quantification of MRI results is ongoing; however, we have reported qualitative findings in cases with novel *RYR1* variants.[20] Exploratory endpoints were not affected by treatment with NAC.

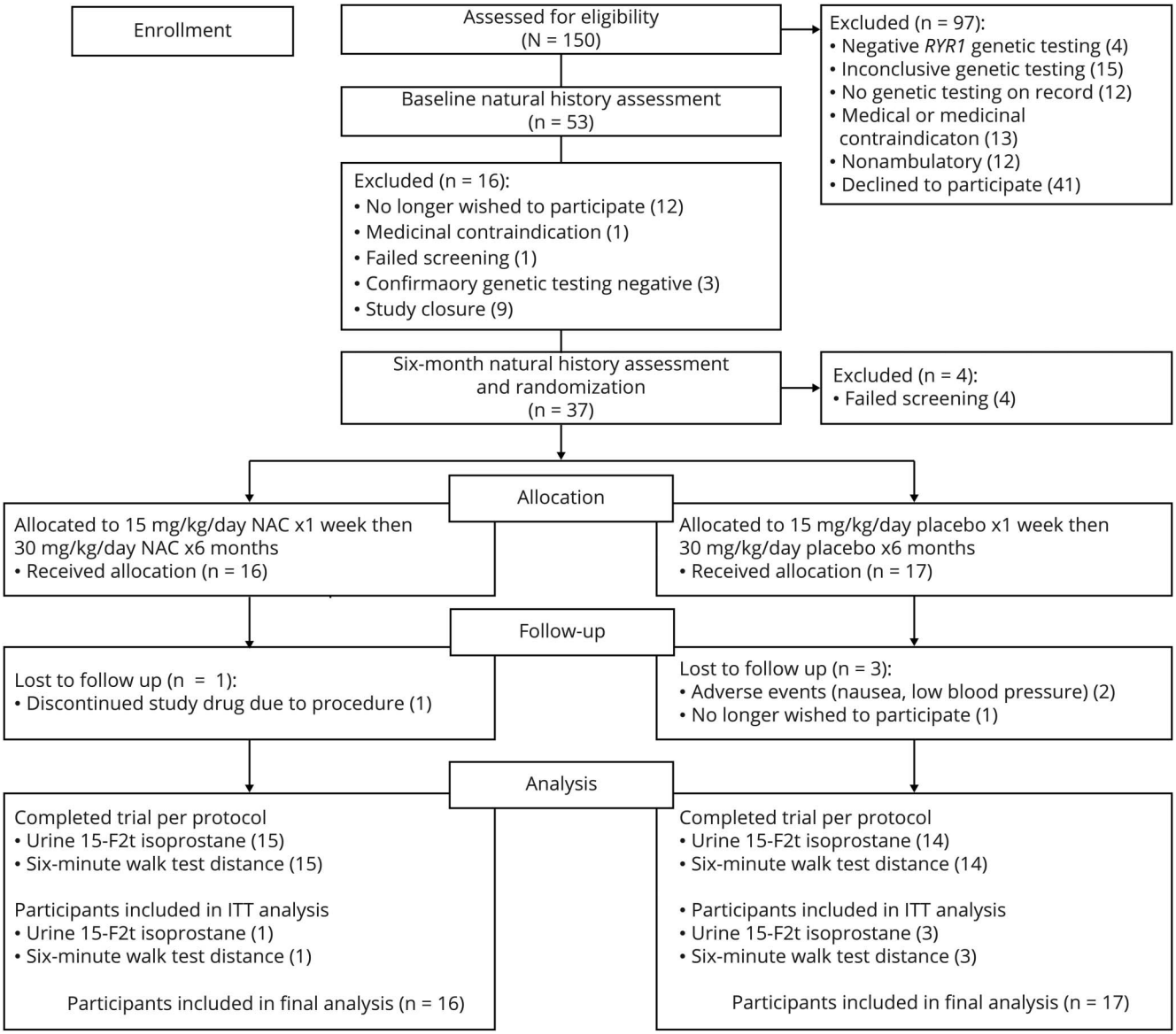
Safety and tolerability

The most frequent AEs, observed in analysis of all randomized participants, were fall (n = 7; 21% of participants), followed by diarrhea and nausea (both n = 5; 15% of participants). There was no difference in AEs observed between the NAC and placebo groups, with a higher frequency of events on placebo (table 4). No events were >3 (out of 5) per Natioanl Cancer Institute Common Criteria severity grading. Based on system organ class, AEs under the gastrointestinal disorders category were most frequent (n = 19; 58% of participants), followed by injury, poisoning, procedural complications category (n = 8; 24% of participants), and investigations category (9%; 27% of participants). A total of 61 AEs were reported with 36 in those assigned to placebo and 25 in those assigned to NAC. A total of 9 serious AEs (SAEs) were observed during the intervention phase of the study. Overall, 5 SAEs occurred on placebo and 4 on NAC. Of the 4 on NAC, 1 child was found to have malrotation of bowel by his local provider (considered unrelated to NAC) and 1 male participant had chest tightness (probably drug-related because this is a known side effect of NAC in the inhaled and IV formulations). One child was diagnosed with postural orthostatic tachycardia syndrome (possibly related to NAC), but the participant had symptoms before intervention. One female participant had an incidental finding of ovarian cyst (unlikely related to NAC due to lack of biological plausibility).[21–23] The cyst was present at baseline but grew over the course of the study.

Discussion

We present results from a natural history assessment and randomized controlled trial of NAC in individuals with *RYR1*-RM. We show that *RYR1*-RM–affected individuals have increased oxidative stress, disrupted redox equipoise, and decreased physical endurance compared to the general population. This is consistent with findings in cell culture and animal models of the disease (mice, zebrafish, and patient-derived myotubes).[6–8] At baseline, all participants had urine 15-F2t isoprostane concentrations greater than the general population mean reference value (1.1 ng/mg creatinine)

Figure Consolidated Standards for Reporting Trial flow diagram



ITT = intent-to-treat; NAC = *N*-acetylcysteine.

despite wide variability in genotype and clinical phenotype, indicating that this may be a promising biomarker of oxidative stress for *RYR1*-RM. Moreover, this is consistent with prior reports of elevated 15-F2t isoprostane in other genetic disorders including Rett syndrome, cystic fibrosis, and sickle cell anemia.[24] We also corroborate a stable or slow disease course of *RYR1*-RM in ambulatory individuals over a 6-month time frame, which has only been reported anecdotally to date.[25] This observation lends support to future *RYR1*-RM clinical trials that focus on detecting improvements in clinical manifestations, such as weakness and impaired motor function, rather than stabilization of disease course over this time frame, especially in ambulatory individuals. However, a longer natural history study that includes both ambulatory and nonambulatory affected individuals is needed in this population.

NAC was safe and well-tolerated at the doses provided in this study, with a total of 9 SAEs observed, and no difference in the frequency of AEs between NAC and placebo groups. In the 33 randomized individuals, a total of 4 SAEs occurred in the NAC group, of which only 2 were considered possibly or probably related to study drug. The probably related event, chest tightness, is a known side effect of inhaled and IV NAC formulations.

Despite increased 15-F2t isoprostane concentrations at baseline, NAC provided in effervescent tablet formulation over a 6-month period did not ameliorate increased 15-F2t isoprostane concentrations at the doses provided. This is contrary to a recent study of otherwise healthy individuals stratified by baseline erythrocyte glutathione concentrations, in which those with low erythrocyte glutathione concentrations

Table 2 Effect of *N*-acetylcysteine (NAC) treatment on the primary endpoints

Measure	NAC (n = 16)	Placebo (n = 17)	Postintervention difference	*p* Value
Aim 1: Oxidative stress				
Postintervention urine 15-F2t isoprostane concentration (ng/mg creatinine)	2.7 (1.7–3.7)	2.6 (1.5–3.6)	0.1 (−1.4 to 1.6)	0.88
Aim 2: Physical endurance				
Postintervention 6MWT total distance (m)	495.8 (475.8–515.9)	471.9 (451.1–492.7)	23.9 (−5.5 to 53.4)	0.11

Abbreviation: 6MWT = 6-minute walk test.
Data are least squares means (95% confidence interval).

had a 22% decrease in 15-F2t isoprostane concentration with oral NAC treatment (2,400 mg total daily dose for 30 days).[26]

The ability of NAC to benefit systemic redox equipoise has been reported previously, but a relevant question may be whether NAC can elicit a beneficial effect in skeletal muscle by crossing the sarcolemma and targeting intracellular redox imbalance. In otherwise healthy male patients undergoing an exercise challenge, IV infusion of NAC (125 mg/kg for 1 hour) resulted in increased cysteine and glutathione availability in skeletal muscle tissue.[27] However, the route of NAC administration may influence skeletal muscle bioavailability, especially as NAC is rapidly deacetylated in the gastrointestinal tract, and it undergoes extensive first-pass metabolism.[28] Following oral administration, NAC is distributed to skeletal muscle, albeit at lower concentrations than other tissues such as the kidney, liver, adrenal gland, and lung.[28] It is possible that the absence of a treatment effect with NAC in our study may have been due to the route of administration. Also, all adults received the same dose, but adult weights ranged from 50 to 105 kg, thus dose may have contributed to the lack of effect.

It is worth considering whether the intervention actually did make a clinical difference, but the low sample size or imperfect oxidative stress biomarker simply did not allow for detection of this. However, we did not observe a treatment effect on muscle DCFH, a biomarker of general oxidative stress at the target tissue. Of note, DCFH was only assayed in those who underwent a muscle biopsy, which limited the sample size (n = 10). A longer natural history study evaluating isoprostane levels and other measures of oxidative stress in more individuals, including those with greater disease severity, would be beneficial to address the conundrum identified in this study of borderline improvements in 6MWT, descend stairs, supine to stand, but not in oxidative stress levels. Nevertheless, 15-F2t isoprostane was substantially elevated over a period of 1 year in *RYR1*-RM–affected individuals, supporting further investigation of oxidative stress biomarkers in this population.

Secondary outcomes with a p value ≤0.05 included graded functional tests (time to descend 4 steps and move from the supine to sitting position). Interestingly, these endpoints measure changes in motor function rather than physical endurance. However, it should be noted that these p values were not corrected for multiple testing. Finally, several other secondary outcomes and all exploratory outcomes did not improve post-NAC treatment, suggesting that the borderline clinically meaningful improvement seen in the 6MWT and graded functional test results should be interpreted with caution.

We established an *RYR1*-RM MCID for 6MWT distance of 25–83 m, which fell within the MCID range for Duchenne muscular dystrophy (29 m) and a broad range of other diseases (range 14–31 m).[29] The relatively wide 6MWT MCID range for *RYR1*-RM likely reflects the clinical heterogeneity of the disease. Obtaining an *RYR1*-RM-specific MCID for 6MWT is of value for future studies using this outcome measure to test the effects of other interventions. However, 6MWT is known to have limitations, including a large SD and a volitional component, that can decrease objectivity of the measure. We intentionally used 6MWT as a measure of physical endurance in this study based on findings of increased swim endurance post-NAC in *ryr1b* mutant zebrafish.[6] Similarly, future trial outcomes should not be limited to 6MWT but, rather, should also be based on observations identified in preclinical work testing the drug compound of interest. To that end, the data obtained in this study, including descriptive statistics on >20 other outcome measures throughout the 6-month natural history component, will contribute to powering future *RYR1*-RM trials. Despite being a rare disease, this study has demonstrated that recruitment of approximately 50 *RYR1*-RM–affected individuals is feasible at a single site. However, our data indicate that sample sizes of approximately 80–100 would be required to detect treatment effects on several motor function-related endpoints (including 6MWT, descend stairs, supine to stand), and 182 for 15-F2t isoprostane. Future phase 2 efficacy trials in *RYR1*-RM should consider a multicenter design to maximize the likelihood of reaching sufficient participant accrual in a timely manner to a detect potential treatment effects in this clinically heterogenous population.

Table 3 Effect of *N*-acetylcysteine (NAC) treatment on secondary endpoints

Measure	No.	NAC	No.	Placebo	Postintervention difference	*p* Value
DCF fluorescence intensity, AU[a]	6	1.9 (0.7–3.1)	4	3.4 (1.7–5.1)	−1.5 (−3.6 to 0.7)	0.14
Ascend steps, s	15	3.2 (2.9–3.5)	14	3.3 (3.0–3.6)	−0.1 (−0.5 to 0.3)	0.62
Descend steps, s	15	1.9 (1.5–2.3)	14	2.4 (2.1–2.8)	−0.5 (−1.1 to 0.0)	0.05
Walk/run 10 meters, s	15	5.2 (4.3–6.1)	14	5.9 (5.0–6.9)	−0.8 (−2.1 to 0.6)	0.25
Supine to stand, s	15	7.4 (6.6–8.1)	14	8.4 (7.7–9.2)	−1.1 (−2.1 to 0.0)	0.05
MFM-32 D1, % of max score	15	74.9 (72.1–77.6)	14	71.5 (68.6–74.3)	3.4 (−0.6 to 7.3)	0.09
MFM-32 D2, % of max score	15	97.0 (95.9–98.1)	14	96.7 (95.5–97.9)	0.3 (−1.3 to 2.0)	0.69
MFM-32 D3, % of max score	15	95.5 (93.9–97.1)	14	96.7 (95.0–98.3)	−1.2 (−3.5 to 1.1)	0.31
MFM-32 total score, % of max	15	84.1 (82.8–85.4)	14	83.0 (81.7–84.3)	1.1 (−0.8 to 3.0)	0.22
Hand grip strength, kg	15	17.8 (16.1–19.5)	14	17.9 (16.1–19.7)	0.1 (−2.6 to 2.4)	0.93
Hand pinch strength, kg	15	4.7 (4.0–5.5)	14	4.9 (4.2–5.7)	−0.2 (−1.3 to 0.8)	0.66
Peak torque flexion, nM	14	24.7 (23.0–26.3)	12	22.6 (20.8–24.3)	2.1 (−0.4 to 4.6)	0.09
Peak torque extension, nM	14	42.9 (39.7–46.1)	12	38.8 (35.3–42.2)	4.2 (−0.7 to 9.0)	0.09
Adult PROMIS fatigue, *t* score	9	49.5 (43.9–55.1)	7	55.0 (49.5–60.6)	−5.5 (−13.4 to 2.4)	0.15
Adult NeuroQoL fatigue, *t* score	9	45.1 (40.0–50.3)	7	51.5 (46.3–56.6)	−6.4 (−13.7 to 1.0)	0.08
Pediatric PROMIS fatigue, *t* score	2	34.8 (18.5–88.2)	5	51.1 (30.9–71.3)	−16.27 (−80.1 to 47.5)	0.39
Pediatric NeuroQoL fatigue, *t* score	2	43.1 (0.7–85.5)	5	53.1 (40.4–65.9)	10.0 (−59.5 to 39.5)	0.57
MFI-20 general fatigue score	14	11.6 (9.7–13.5)	12	13.7 (11.7–15.8)	−2.1 (−5.0 to 0.6)	0.12
MFI-20 physical fatigue score	14	11.5 (9.3–13.6)	12	11.9 (9.7–14.2)	−0.5 (−3.6 to 2.7)	0.76
MFI-20 reduced activity score	14	9.2 (7.2–11.2)	12	8.7 (6.6–10.8)	0.5 (−2.5 to 3.5)	0.72
MFI-20 reduced motivation score	14	7.8 (6.2–9.4)	12	7.4 (5.7–9.1)	0.4 (−1.8 to 2.7)	0.70
MFI-20 mental fatigue score	14	8.8 (6.7–10.9)	12	8.6 (6.4–10.8)	0.2 (−2.8 to 3.3)	0.88
FACIT-F total score	9	76.1 (67.7–84.5)	7	73.6 (64.1–83.2)	2.5 (−10.3 to 15.2)	0.61
FACIT-F trial outcome index	9	37.6 (32.3–42.8)	7	37.0 (31.0–42.9)	0.6 (−7.4 to 8.5)	0.87
Peds-FACIT-F total score	6	29.8 (12.0–47.6)	6	42.3 (30.4–54.3)	−12.5 (−38.9 to 13.9)	0.28

Abbreviations: DCF = dichloro-dihydro-fluorescein; FACIT-F = Functional Assessment of Chronic Illness Therapy–Fatigue; MFI-20 = Multidimensional Fatigue Inventory 20; MFM-32 = Motor Function Measure 32; NeuroQoL = quality of life in neurologic disorders; Peds-FACIT-F = Pediatric Functional Assessment of Chronic Illness Therapy–Fatigue; PROMIS = Patient-Reported Outcomes Measurement Information System; VO_2 peak = peak oxygen uptake.
Data are least squares means (95% confidence interval).
[a] Secondary endpoint of oxidative stress.

Our observation of elevated 15-F2t isoprostane concentrations, and hence lipid peroxidation, in *RYR1*-RM–affected individuals raises the question of whether dietary intervention with omega-3 polyunsaturated fatty acids (n-3 PUFAs) could represent a low-risk therapeutic approach to alleviate the pathologic sequalae of RyR1 dysfunction.[30] This is plausible given that n-3 PUFAs have been shown to target ion channels, may inhibit RyR activity, and can alter skeletal muscle lipid composition, favoring an increase in % total n-3 PUFA/total fatty acid content, in as little as 2 weeks in humans.[31–33] In support of this, lipid peroxidation has been shown to compromise the integrity of muscle mitochondrial membranes in *RYR1* mutant Y522S mice, and n-3 PUFA supplementation decreases lipid peroxidation byproducts in other genetic disorders.[7,24,32] Future trials may also consider investigating mitochondria-targeted antioxidants such as the coenzyme Q_{10} analog, mitoquinol mesylate, and the tetrapeptide, Szeto-Schiller-31.[34] Indeed, both compounds can permeate the mitochondrial membrane and therefore hold the prospect of addressing redox imbalance at the source. Alternatively, the Rycal RyR stabilizer (S48168) acts directly on RyR1 by restoring ligand binding of calstabin1 (FKBP12) to RyR1. This serves to improve channel integrity and decrease RyR1-mediated Ca^{2+} leak.[5] It is therefore foreseeable that a combination of channel stabilization and targeted antioxidant therapy could yield the greatest clinical benefit in future trials.

Table 4 Summary of safety data by treatment allocation

Safety event	NAC (n = 16)	Placebo (n = 17)	Total events	*p* Value
Any AEs	25	36	61	0.16
AEs related to study drug	6	14	20	0.07
SAEs	4	5	9	0.74
SAEs related to study drug	2	2	4	1.00
Unanticipated problems	6	4	10	0.53
Deaths	0	0	0	1.00

Abbreviations: AE = adverse event; NAC = *N*-acetylcysteine; SAE = serious adverse event.
Data are shown as counts. Each AE was assessed for its relationship to drug treatment prior to unblinding by categorizing each event as likely, probably, possibly, unlikely, or unrelated (to drug treatment). Only the possibly, probably, or likely events were included in the 2 rows above indicating related to study drug.

Although compliance with intervention was assessed by means of a pill count, this was not verified using a biomarker approach and may be considered a study limitation. The well-established instability of redox analytes, such as glutathione, systemic reduced-to-oxidized ratio for glutathione, cysteine, and reduced-to-oxidized ratio for cysteine, make these challenging as clinical trial endpoints. Indeed, at 6 months, GSH:GSSG values were unreliable due to methodologic issues, and the protocol was amended to switch the primary endpoint to 15-F2t isoprostane. Switching the primary endpoint could be considered a limitation, however, this practice is not infrequent in clinical trials.[14] Importantly, in this study, the change was performed before unblinding and was reviewed by the IMC, IRB, and Food and Drug Administration. 15-F2t isoprostane may represent a more appropriate endpoint for assessing oxidative stress in clinical trials as it is not affected by collection time, sample volume, or long-term storage and can be reliably quantified.[11] Nevertheless, there is evidence that 15-F2t isoprostane concentration may be influenced by age, sex, and exercise, as well as dietary and lifestyle factors.[24] In this study, age and sex did not affect 15-F2t isoprostane concentration and were therefore not included in the final statistical model. To address potential confounding, future trials that assess 15-F2t isoprostane should consider monitoring for habitual changes in diet and exercise, prior to and during intervention. Nonambulatory individuals were excluded from this study, owing to the requirement of 6MWT completion. Future studies assessing oxidative stress in *RYR1*-RM–affected individuals should consider enrolling nonambulatory individuals to determine the potential benefits of antioxidant therapy in those with greater clinical severity. The absence of a statistically significant treatment effect may also have been due to the limited sample size.

NAC was safe and well-tolerated in the *RYR1*-RM population at the dose provided. *RYR1*-RM–affected individuals had elevated lipid peroxidation and disrupted redox equipoise; however, this was not rescued following 6 months of NAC treatment at the stated doses. This study provides Class I evidence that, for individuals with *RYR1*-RM, treatment with oral NAC does not decrease oxidative stress as measured by 15-F2t isoprostane. *RYR1*-RM–affected individuals also had decreased physical endurance, as measured by 6MWT distance. Although this did not significantly change with NAC intervention, 6MWT distance did approach a clinically meaningful improvement in NAC-treated participants. This study, comprising a natural history assessment and randomized controlled trial, provided information on feasibility of recruitment for trials in this rare disease population, stability of disease course in ambulatory individuals over a 6-month time frame, descriptive statistics of a broad range of endpoints in *RYR1*-RM–affected individuals to power future trials, and identification of a biomarker of oxidative stress suggesting lipid peroxidation as a promising therapeutic target.

Acknowledgment

The authors thank all study participants for their participation; BioAdvantex for supplying the study drug and matching placebo free of charge; the following individuals and groups: Karez Hawkins, NCMA: patient care coordinator; Nicol Voermans, MD, Grace Yoon, MD, Sheila Muldoon, MD, Pierre Fequiere, MD, Meganne Leach, PPCNP-BC, Livija Medne, MS, CGC, and the RYR1-Foundation: participant referral; Mary Blake, MD(c) and Michaela Cortes, BSN: data entry and cleaning; Suzanne Wingate, PhD, ANP-BC, Tanya Lehky, MD, A. Reghan Foley, MD, Diana Bharucha, MD, Etsuko Tsuchiya, BS, and Kim Amburgey, MS, CGC: nursing, medical and scientific expertise; Joan Austin, PhD, RN, Kenneth Fischbeck, MD, Hiroko Matsumoto, PhD(c), and Gina Norato, ScM: review of manuscript; Joshua Woolstenhulme, PhD, Carmel Nichols, MD(c), and Ruhi Vasavada, MS: rehabilitation medicine expertise; Ronald Cohn, MD, Andrew Mammen, MD, and Joan Austin, PhD, RN: independent monitoring committee; and the NIH pharmacy and the NIH outpatient pediatric and 9th floor units for their support.

Study funding

This study was funded by the Intramural Programs of the National Institute of Nursing Research, National Institute of Neurologic Disorders and Stroke, the NIH Clinical Center, and Bench to Bedside Award (10–2013/Office of Rare Disease/NINR).

Disclosure

J.J. Todd and T.A. Lawal report no disclosures relevant to the manuscript. J.W. Witherspoon has received support from the RYR-1 Foundation. I.C. Chrismer, M.S. Razaqyar, M. Punjabi, J.S. Elliott, F. Tounkara, A. Kuo, M.O. Shelton, C. Allen, M.M. Cosgrove, M. Linton, D. Michael, M.S. Jain, M. Waite, B. Drinkard, and P.G. Wakim report no disclosures relevant to the manuscript. J.J. Dowling is a member of scientific advisory board of the RYR-1 Foundation and Denature and a member of the scientific council of the Muscular Dystrophy Association.

J.J. Dowling has received support from the RYR-1 Foundation and Muscular Dystrophy Association. C.G. Bönnemann is a member of the scientific advisory board of the RYR-1 Foundation. C.G. Bönnemann has received funding from Cure CMD. M. Emile-Backer reports no disclosures relevant to the manuscript. K.G. Meilleur has received support from the RYR-1 Foundation and an NIH Clinical Center Bench to Bedside Award (10–2013/Office of Rare Disease/NINR). Go to Neurology.org/N for full disclosures.

Publication history

Received by *Neurology* May 5, 2019. Accepted in final form September 9, 2019.

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Jessica W. Witherspoon, PhD	NIH, Bethesda	Author	Designed and conceptualized study, reviewed the manuscript for intellectual content
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Magalie Emile-Backer, PharmD	NIH, Bethesda	Author	Data quality control and database lock, reviewed safety data and the manuscript for intellectual content
Katherine G. Meilleur, PhD	NIH, Bethesda	Author	Designed and conceptualized study, analyzed the data, reviewed the manuscript for intellectual content

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Randomized controlled trial of *N*-acetylcysteine therapy for *RYR1*-related myopathies

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Neurology published online January 15, 2020
DOI 10.1212/WNL.0000000000008872