A PILOT TRIAL OF RNS60 IN AMYOTROPHIC LATERAL SCLEROSIS

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ABSTRACT: *Introduction:* RNS60 is a novel immune-modulatory agent that has shown neuroprotective effects in amytrophic lateral sclerosis (ALS) preclinical models. RNS60 is administered by weekly intravenous infusion and daily nebulization. The objective of this pilot open-label trial was to test the feasibility, safety, and tolerability of long-term RNS60 administration in ALS patients. *Methods:* The planned treatment duration was 23 weeks and the primary outcomes were safety and tolerability. Secondary outcomes included PBR28 positron emission tomography (PET) imaging and plasma biomarkers of inflammation. *Results:* Sixteen participants with ALS received RNS60 and 13 (81%) completed 23 weeks of RNS60 treatment. There were no serious adverse events and no participants withdrew from the trial due to drug-related adverse events. There were no significant changes in the biomarkers. *Discussion:* Long-term RNS60 administration was safe and well-tolerated. A large, multicenter, phase II trial of RNS60 is currently enrolling participants to test the effects of RNS60 on ALS biomarkers and disease progression.

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease. Loss of motor neurons leads to progressive paralysis of voluntary muscles and death, usually within 3–5 years after symptom onset.[1] Although there are 2 drugs approved by the United States Food and Drug Administration (FDA) to treat ALS, riluzole, and edaravone, the availability of new treatments for ALS continues to be an unmet medical need.[2,3]

Neuroinflammation is increasingly implicated in ALS pathogenesis,[4–9] including activation of circulating monocytes,[9–13] lymphocytic infiltration,[14] and microglial activation in the central nervous system (CNS).[7,8] Mouse models of ALS show that activation of microglia and influx of T-lymphocytes into the CNS occur before symptom onset[4,9,15] and brain imaging in ALS patients reveals that neuroinflammation localizes to the motor regions.[7,8,16] Emerging evidence points to T-lymphocytes as key players in ALS progression. Among different lymphocytic subpopulations, regulatory T-lymphocytes (Tregs) are $CD4^{+}CD25^{high}FOXP3^{+}$ cells that normally suppress proinflammatory responses and hold neuroinflammation in check.[5] In ALS patients, Treg suppressive function is impaired and the Treg transcription factor FOXP3 is reduced. Reduction in Treg function and FOXP3 expression correlates with faster ALS progression.[17–19]

RNS60 is a novel immune-modulatory agent with neuroprotective properties in several *in vitro* and *in vivo* models of neurodegeneration,[20–26] including preclinical models of ALS.[27] In $SOD1^{G93A}$ transgenic mice, RNS60 treatment resulted in upregulation of FOXP3-expressing Tregs and activation of protective astrocytes and microglia, which rescued the motor neurons and ameliorated disease progression.[27] These findings prompted translational efforts to human disease.

The preliminary safety of RNS60 was previously established in a series of phase I trials of healthy volunteers and those with asthma where it exhibited a robust safety profile (studies were conducted by Revalesio Corp. between 2010 and 2015 [unpublished data]; see NCT01511302, NCT01057498, NCT01264783, and

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Additional supporting information may be found in the online version of this article.

Abbreviations: AE, adverse event; ALS, amyotrophic lateral sclerosis; ALSFRS-R, ALS Functional Rating Scale—Revised; ATLIS, Accurate Test of Limb Isometric Strength; CNS, central nervous system; CSF, cerebrospinal fluid; cDNA, complementary DNA; Ct, cycle threshold number; FDA, Food and Drug Administration; FOXP3, Forkhead box P3; FEW, familywise error; IL-17, interleukin-17; IRCCS, Istituto di Ricovero e Cura a Carattere Scientifico; IV, intravenous; LPLV, last patient, last visit; MGH, Massachusetts General Hospital; MND, motor neuron disease; MR-PET, magnetic resonance positron emission tomography; mRNA, messenger RNA; ROI, region of interest; RT-PCR, real-time polymerase chain reaction; SUV(r), standardized uptake value (ratio); Treg, regulatory T cell; TSPO, translocator protein; SVC, slow vital capacity; UMN-B, Upper Motor Neuron Burden

Key words: ALS; clinical trial; motor neuron disease (MND); neuroinflammation; PBR28

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NCT02490865). The administration regimen of RNS60 is unique as it is administered by weekly intravenous (IV) infusion and daily nebulization. This raised the question of whether this dosing regimen would be feasible and safe in ALS patients who commonly experience progressive loss of mobility, respiratory compromise, and difficulty traveling. As an important first step toward developing RNS60 as a potential therapeutic agent for ALS, we conducted a pilot clinical trial to test the long-term feasibility, safety, and tolerability of RNS60 treatment in people with ALS. Secondary outcomes included biofluid and neuroimaging biomarkers to test the impact of the drug on inflammatory pathways.

METHODS

This study was an investigator-initiated, open-label, pilot clinical trial that enrolled participants at Massachusetts General Hospital (MGH) between October 2015 and February 2017. The "last patient, last visit" (LPLV) occurred on October 25, 2017. The Partners Human Research Committee approved the study. The trial has been registered on clinicaltrials.gov (NCT02525471).

Participant Selection Criteria. Eligible participants had a diagnosis of possible, probable laboratory-supported, probable, or definite ALS by revised El Escorial criteria.[28] As this was a pilot study, inclusion criteria were broad by design. There were no restrictions in disease duration, vital capacity (VC), use of assisted ventilation, or presence of a feeding tube. Exclusion criteria included active infection, abnormal liver or kidney function, clinically significant unstable medical condition (other than ALS), and pregnancy. People with unstable psychiatric disease or cognitive impairment were excluded per principal investigator judgment.

Two brain imaging studies were planned for each participant: pre- treatment (between screening and baseline) and post- treatment (between the week 18 and week 23 visits). Brain imaging consisted of PET using the radioligand [^{11}C]-PBR28 that binds to the translocator protein (TSPO). Blood samples were collected at screening for genotyping for the Ala147Thr polymorphism in the TSPO gene, which imparts a trimodal pattern of binding affinity to the radioligand.[29] Participants with low-affinity binding (Thr/Thr) were excluded as they tend to have very little detectable [^{11}C]-PBR28 signal. Participants with high- (Ala/Ala) and mixed- (Ala/Thr) affinity binding, who demonstrate high and intermediate signal levels, respectively, were included, and their binding affinity status was modeled as a covariate in the analyses. To be maximally sensitive to potential treatment-related changes in [^{11}C]-PBR28 signal and to avoid floor effects, participants with an Upper Motor Neuron Burden (UMN-B) score of less than 25 were also excluded.[7] Additional exclusion criteria for the brain imaging studies were exposure to immune-modulatory medications within 30 days of the screening visit, current use of tobacco or tobacco products, any contraindication to undergo neuroimaging studies such as presence of cardiac pacemaker, metallic clips or metallic particles in the body, or claustrophobia.

Intervention. Eligible participants received RNS60 through 2 routes: by IV infusion at MGH once a week (infusion dose 375 ml, infused over approximately 40 minutes) and by nebulization at home on the remaining 6 days per week (4 ml/day). Participants were provided with a nebulizer for home use at the beginning of the study. RNS60 for home use was dispensed every 7–8 weeks and participants were asked to return empty study drug containers weekly to determine compliance.

Study Overview. Participants signed the approved informed consent form at screening before any research-related activities. Medical history, detailed ALS history, physical and neurological examinations, medication review, vital signs, and laboratory tests were performed to determine eligibility. All eligible participants received active drug starting with the first infusion at the baseline visit (day 0), which occurred within 42 days of screening (see Fig. S1 in the Supplementary Material available online), and at-home nebulization the following day. Follow-up visits included weekly in-person visits at the infusion center up to week 23 for a total of 24 infusions. A final phone call occurred 4 weeks after receiving the last dose of study drug. Participants who completed the trial (23 weeks of treatment) were offered the option of remaining on RNS60 for a total of up to 47 weeks of treatment (optional open-label extension).

Clinical Measurements. Clinical measurements included safety labs, slow vital capacity (SVC), the ALS Functional Rating Scale—Revised (ALSFRS-R)[30] questionnaire, collection of adverse events (AEs) and concomitant medication use, and measurements of muscle strength. Clinical measurements were done at baseline, week 11, and week 23, and were repeated at week 35 and week 47 for participants who opted for extended treatment. AEs, concomitant medication changes, and vital signs, including weight and drug accountability, were measured weekly. Isometric strength was tested using the Accurate Test of Limb Isometric Strength (ATLIS) designed to precisely measure strength of 12 muscle groups in the arms and legs using an adjustable chair and frame with a fixed wireless load cell.[31] The average arm and leg scores are presented by converting raw force values to percent of predicted values using methods similar to those used to calculate SVC.[32]

Biofluid Biomarker Assays. Blood draws for biomarker assays were performed at baseline, week 11, and week 23 in the subset of 11 participants who also underwent brain imaging studies. Samples were processed onsite, aliquoted, cryopreserved at −80°C, and shipped to the IRCCS Mario Negri Institute for Pharmacological Research for analysis. The following markers were measured: IL-17 in plasma and intracellular FOXP3 mRNA expression in whole blood. IL-17 plasma concentration was measured by Alpha technology using an AlphaLISA kit (No. AL219C; PerkinElmer, Waltham, Massachusetts), following the manufacturer's procedure. The AlphaLISA signals were detected using an Ensight Multimode Plate Reader (PerkinElmer). FOXP3 mRNA levels were measured by real-time polymerase chain reaction (RT-PCR).

RT-PCR. Total RNA from blood was extracted and purified with the PAXgene Blood RNA Kit (Qiagen, Venlo, The Netherlands) following the manufacturer's instructions. RNA samples were treated with DNase I and reverse transcription was performed with a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Waltham, Massachusetts). Real-time PCR was performed using the TaqMan gene expression assay (Applied Biosystems, Foster City, California), following the manufacturer's instructions, on cDNA specimens using 1× Universal PCR master mix (Thermo Fisher Scientific) and 1× mix containing Forkhead box P3 (FOXP3; Hs01085834_m1; Thermo Fisher Scientific) probe. Relative quantification was calculated from the ratio between the cycle number (Ct) at which the signal crossed a threshold set within the logarithmic phase of FOXP3 gene and that of the reference β-actin gene (Hs01060665_g1; Thermo Fisher Scientific). Each sample was run in triplicate and the mean value was used as individual datum for $2^{-\Delta Ct}$ analysis.

Brain Imaging. Brain imaging studies included simultaneous magnetic resonance positron emission tomography (MR-PET) imaging using the radiotracer $[^{11}C]$-PBR28. $[^{11}C]$-PBR28 was produced in-house as previously described.[33] The radiotracer was administered as an IV bolus (mean administered dose was 473.6 MBq at baseline and 468.4 MBq at follow-up). Standardized uptake value (SUV) computed from data collected 60–90 minutes after time of injection were normalized to whole-brain mean to create an SUV ratio ($SUVR_{60–90min}$).[7,34] This whole-brain normalization approach accounts for global difference in PET signal across participants, thereby allowing increased sensitivity to detect regional effects.[7,34]

Statistical Analysis. Primary outcomes were safety, as measured by the number of and severity of all adverse events, and tolerability, defined as the ability to complete the 23-week treatment period on study drug. Adverse events (AEs) were coded to preferred terms from the MedDRA Library (version 16.1). As an additional safety measure, disease progression measured by ALSFRS-R was evaluated with a Wilcoxon signed rank test. All analyses were carried out using R version 3.4.3 (R Foundation for Statistical Computing, Vienna, Austria).

Secondary outcomes included levels of interleukin IL-17 in plasma and FOXP3 mRNA expression in whole blood. Linear mixed models were used for the plasma and whole-blood measures as the outcome. Fixed effect for time (baseline or week 23), random slope, and intercept for each participant were used to estimate a 23-week change.

Neuroimaging analyses were similar to those previously reported by Loggia *et al.*[34] Briefly, $SUVR_{60–90min}$ images were fed into a whole-brain voxelwise between-group analysis (post- treatment and pre- treatment). Nonparametric permutation inference[35] using a paired design was performed using 5,000 permutations and threshold-free cluster enhancement.[36] The resultant statistical maps were familywise error (FWE) adjusted ($p_{FWE} < 0.05$) to correct for multiple comparisons. In addition to this voxelwise analysis, we performed a region of interest (ROI) analysis. The ROI was defined based on FreeSurfer cortical reconstruction of the standard template MNI152. The ROI encompasses the precentral and paracentral cortex and underlying subcortical white matter (motor region) bilaterally.[37] $[^{11}C]$-PBR28 $SUVR_{60–90min}$ was then computed within this ROI. Pre- to post- change scores for $[^{11}C]$-PBR28 $SUVR_{60–90min}$ extracted from this ROI was compared using the Wilcoxon signed rank test.

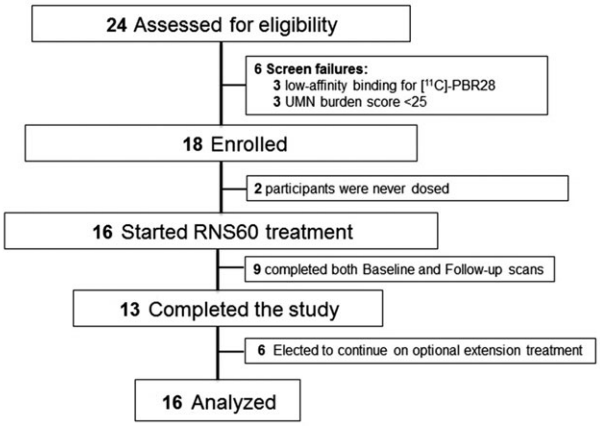

FIGURE 1. CONSORT diagram: participant enrollment and follow-up for the trial. Twenty-four volunteers with ALS were assessed for eligibility. Eighteen enrolled in the trial but 2 were never dosed as they withdrew consent between screening and baseline due to unrelated medical problems. The remaining 16 participants received RNS60 and contributed data.

RESULTS

Study Population. Twenty-four volunteers were assessed for eligibility; 18 eligible individuals were enrolled due to 6 screen failures (Fig. 1). Of the 18 eligible individuals, 2 were never dosed with RNS60 because they developed unrelated medical problems and withdrew consent before baseline. Their data from screening were excluded from analysis, leaving a data set of 16 study participants (Fig. 1). Demographic and clinical characteristics of the study participants are listed in Table 1.

Safety and Tolerability. The trial met its primary endpoints for safety and tolerability. The most common AEs included falls (75%), headaches (50%), nasopharyngitis (38%), and contusions (31%) (all AEs that occurred in at least 12% of participants [*n* = 2] are summarized in Table S1 online by the MedDRA preferred term). No serious AEs related to RNS60 occurred and no participant withdrew from the trial due to drug-related AEs. Eighty-one percent of study participants (*n* = 13) completed 23 weeks of RNS60 treatment (Fig. 1). Withdrawals were due to disease progression (*n* = 2) and relocation to a different state (*n* = 1). Compliance with IV infusion visits and daily home nebulization were very high (Table 1). There were no clinically significant changes in vital signs or standard laboratory tests. As expected, ALSFRS-R and muscle strength declined over the 23 weeks of observation (Table 2). There were no statistically significant changes in SVC during the study period (Table 2). Individual ALSFRS-R and SVC trajectories are shown in Figure S2 (online).

Of the 13 participants who completed the trial, 6 elected to remain on the study drug (optional extension) and 4 remained on RNS60 for a total of 47 weeks. The 2 withdrawals from the extension treatment were due to difficulties traveling to the study site (*n* = 1) and scheduling difficulties at the time of initiation of edaravone treatment (*n* = 1).

Brain Imaging. Of the 11 eligible participants, 9 completed both scans (2 did not return for the post- treatment scan due to interval development of orthopnea). Of the 9 participants who had both baseline and follow-up scans, 5 were high- and 4 were mixed-affinity binders. Whole-brain voxelwise analysis revealed no significant difference in $[^{11}C]$-PBR28 $SUVR_{60–90\ min}$

Table 1. Baseline demographics and clinical characteristics of study participants.

Demographics and clinical characteristics	All participants (n = 16)
Male gender	8 (50%)
White race	14 (88%)
Age (years)	52.9 ± 11.3 (24.5–69.8)
Bulbar onset	3 (18.8%
Riluzole use	16 (100%)
Months since symptom onset	30.3 ± 17.8 (7.1–68.4)
ALSFRS-R total score	34.1 ± 6.2 (23–44)
SVC	63.8 ± 27.6 (28–99)
ATLIS arm measures	35.9 ± 21.8 (0–64)
ATLIS leg measures	53.9 ± 31.0 (4–95)
Compliance with infusion visits at study site	99 ± 1% (96–100)
Compliance with home nebulization	96 ± 6% (76–100)
EEC definite	7 (43.75%)
EEC probable	3 (18.75%)
EEC probable lab-supported	4 (25%)
EEC possible	2 (12.5%)

Data expressed as either number (percent), mean ± standard deviation (range), or percent (range). ALSFRS-R, ALS Functional Rating Scale—Revised; ATLIS: Accurate Test of Limb Isometric Strength (results are presented as percent of predicted values)[32]; BMI: body mass index (kg/m^2). EEC: El Escorial criteria; SVC: slow vital capacity (results are presented as percent of predicted values for age, gender, and height).

Table 2. Disease progression (expressed as change per month) at week 23.

Outcome of disease progression	All participants (n = 16)
ALSFRS-R total score	−1.1 ± 1.72* (−2.3 to −0.2)
SVC	−0.6 ± 4.41 (−4.9 to 1.9)
ATLIS arm measures (%)	−1.7 ± 3.12* (−4.2 to 0)
ATLIS leg measures (%)	−1.7 ± 5.61* (−8 to 1)

Data are presented as mean ± standard deviation (expressed as change per month). Minimum–maximum shown in parentheses. ALSFRS-R: ALS Functional Rating Scale—Revised. ATLIS: Accurate Test of Limb Isometric Strength (results presented as percent of predicted values)[32]; SVC: slow vital capacity (results presented as percent of predicted values for age, gender, and height).

*Denotes statistically significant difference on Wilcoxon signed rank test for change at week 23 compared with baseline ($P < 0.05$).

between pre- and post- treatment scans. ROI analysis did not show statistically significant changes in [^{11}C]-PBR28 uptake when pretreatment scans were compared with post- treatment results (Fig. 2).

Biofluid Biomarkers. Biomarkers included IL-17 plasma levels (n = 10) and FOXP3 mRNA expression in whole blood, a marker of Treg function (n = 8). There were no statistically significant changes in IL-17 levels or FOXP3 expression through the course of the core study (estimated mean change in IL-17: −25.4 pg/ml [P = 0.3]; estimated mean change in FOXP3: −0.02 [P = 0.9]; Fig. 3).

DISCUSSION

The trial met its primary endpoints, demonstrating the feasibility, safety, and tolerability of administering RNS60 long-term in patients with ALS.

Tolerability and compliance were high, demonstrating the feasibility of this regimen in a broad ALS population. Of note, our trial started before the approval of edaravone in the United States.[2] Since then, the ALS community has developed considerable experience with long-term IV drug administration, although this route of administration was already used successfully in a previous trial.[38] Future drug development efforts of RNS60 should include consideration of home infusions and long-term IV access as is now done routinely for edaravone therapy in the United States.

RNS60 treatment was safe and well-tolerated. No serious AEs related to RNS60 occurred and no participant withdrew from the trial due to drug-related AEs. Although this trial did not include a placebo group, the frequency of AEs recorded over a long-term observation period was consistent with what is expected in a cohort of patients with a progressive neurodegenerative disease.

Our findings cannot lead to any conclusions about the effects of RNS60 on ALS progression, because this pilot trial was limited by the small sample size and lack of placebo group. This trial did not have the statistical power to detect changes in disease progression measured by the ALSFRS-R or muscle strength analyses. We used ATLIS to measure muscle strength for the first time in the context of an ALS drug trial. This novel outcome measure may be valuable in future trials. The vital capacity was relatively

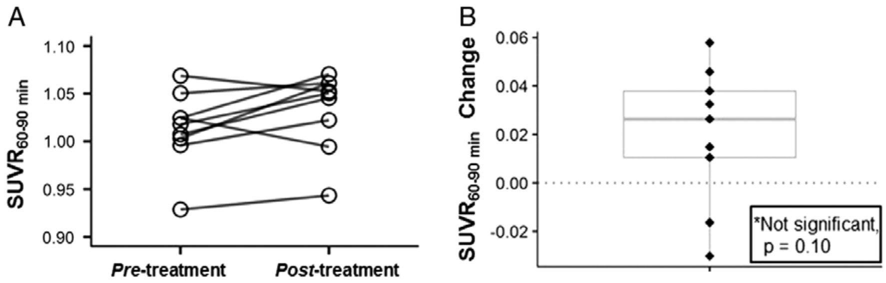

FIGURE 2. [^{11}C]-PBR28 uptake within the region of interest (ROI). **(A)** [^{11}C]-PBR28 uptake (expressed as $SUVR_{60–90min}$) for each individual participant. **(B)** Individual changes in [^{11}C]-PBR28 uptake (expressed as $SUVR_{60–90min}$) from pre- to post- treatment scans. There were no statistically significant changes in [^{11}C]-PBR28 uptake over the course of the core study in the 9 participants who had both pre- and post- treatment scans with boxplot showing median and interquartile range (Wilcoxon signed rank test; mean change in $SUVR_{60–90min}$ = 0.02 [P = 0.10]).

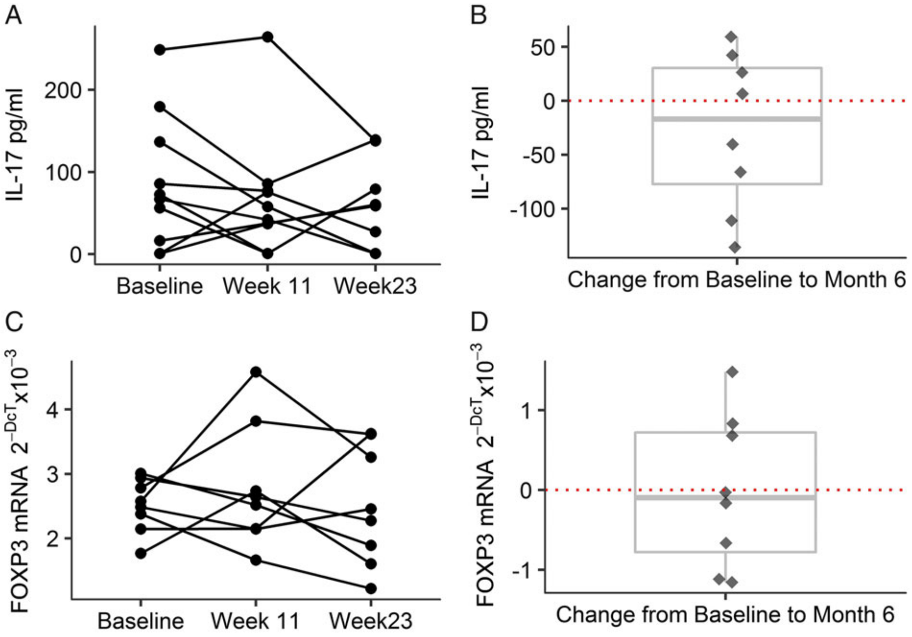

FIGURE 3. Exploratory inflammatory biomarkers. **(A)** Individual levels of IL-17 in plasma (*n* = 10). **(B)** Individual changes in IL-17 from pre- to post- treatment. There were no statistically significant changes over the course of the study with the boxplot showing median and interquartile range (Wilcoxon signed rank test). **(C)** Individual levels of FOXP3 mRNA expression (a marker of Tregs) in whole blood (*n* = 8). **(D)** Individual changes in FOXP3 mRNA expression from pre- to post- treatment. There were no statistically significant changes over the course of the study with the boxplot showing median and interquartile range (Wilcoxon signed rank test). [Color figure can be viewed at wileyonlinelibrary.com]

stable over the study period. It is unknown whether this finding reflects the therapeutic effect of the drug or is due to chance. A larger study is needed to evaluate the clinical efficacy of RNS60 for the treatment of people with ALS.

The addition of biomarkers to track inflammation in this pilot trial was uninformative. In mouse models of autoimmune encephalomyelitis, RNS60 upregulated FOXP3 (a marker of Tregs), reduced IL-17 levels, and ameliorated disease phenotype.[21,25] Recently, RNS60 treatment was shown to prevent the downregulation of FOXP3 mRNA in $\text{SOD1}^{\text{G93A}}$ transgenic mice, and this was associated with slowing of disease progression.[27] The number of Treg cells inversely correlates with the rate of progression in ALS patients. Furthermore, increased levels of IL-17 have been detected in serum and cerebrospinal fluid (CSF) of ALS patients compared with healthy controls.[19,39–41] In the present study, there was no change in blood levels of FOXP3 mRNA or IL-17 during the treatment period. The small sample size may have contributed to these nonsignificant findings and a larger, placebo-controlled trial of RNS60 is underway to test the effects of the drug on these biomarkers (NCT03456882). We used neuroimaging to track neuroinflammation in the motor regions. Both the ROI and exploratory whole-brain approaches did not reveal a significant difference in the pre- vs. post-treatment time-points of this small pilot study (*n* = 9). The ROI approach focused on motor areas that were previously shown to exhibit increased [^{11}C]-PBR28 uptake in ALS compared with healthy controls and to correlate with upper motor neuron burden severity.[7,16] This study successfully demonstrated that longitudinal PET imaging is feasible in the context of ALS clinical trials, however, this trial was underpowered for the imaging outcome as well. Our longitudinal data suggest that a sample size of 30 ALS subjects has 80% power to detect a 2% reduction in PBR28 PET signal after a successful treatment.[42] This imaging technology could be used to increase the efficiency of the drug development by detecting target engagement and guiding dose selection. It could also be used to select participants who are more likely to respond to RNS60 (i.e., patients with higher neuroinflammation at baseline). A larger study is needed to investigate whether treatment with RNS60 leads to significant differences in [^{11}C]-PBR28 uptake, reflecting changes in neuroinflammation.

In conclusion, we have demonstrated the feasibility, safety, and tolerability of long-term administration of RNS60 in patients with ALS. This study builds on promising data in ALS preclinical models[27] and represents the first translational step in the development of RNS60 as a potential therapeutic for ALS. A large, multicenter, international, placebo-controlled phase II trial of RNS60 is currently enrolling participants to test the effects of RNS60 on ALS biomarkers and disease progression (NCT03456882).

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Ethical Publication Statement: We (the authors) confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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