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 ALS pre-clinical 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 people with ALS.

METHODS: The planned treatment duration was 23 weeks and the primary outcomes were safety and tolerability. Secondary outcomes included PBR28 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, multi-center, phase II trial of RNS60 is currently enrolling participants to test the effects of RNS60 on ALS biomarkers and disease progression.

Keywords

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

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease. Loss of motor neurons leads to progressive paralysis of voluntary muscles and death, usually within three to five years after symptom onset.[1] Although there are two drugs approved by the US 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 prior to symptom onset[4,9,15] and brain imaging in ALS patients reveals that neuro-inflammation localizes to the motor regions.[7,8,16] Emerging evidence points to T-lymphocytes as key players in ALS progression. Among

different lymphocytic sub-populations, 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 pre-clinical models of ALS.[27] In SOD1^{G93A} transgenic mice, RNS60 treatment resulted in up-regulation 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 people 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; NCT02490865). The administration regimen of RNS60 is unique as it is administered by weekly intravenous (I.V.) 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 towards 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 bio-fluid and neuro-imaging 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 10/25/2017. Partners Human Research Committee approved this study. The trial was 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 orcognitive impairment were excluded per Principal Investigator judgement.

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 positron emission tomography (PET) using the radioligand

[11C]-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 tri-modal 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 [11C]-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. In order to be maximally sensitive to potential treatment-related changes in [11C]-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 immunomodulatory 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 two routes: by intravenous infusion at MGH once a week (infusion dose: 375 mL, infused over approximately 40 minutes) and by nebulization at home the remaining 6 days a 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 prior to 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 (Supplementary Figure 1) 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 four 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 (AE) 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, vital signs including weight and drug accountability were measured weekly. Isometric strength was tested using 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 similar methods 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 on site, 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 (#AL219C) from Perkin Elmer following the manufacturer's procedure. The AlphaLISA signals were detected using an Ensight Multimode Plate Reader (PerkinElmer). FOXP3 mRNA levels were measured by RT-PCR.

Real Time PCR

Total RNA from blood was extracted and purified with PAXgene Blood RNA Kit (Qiagen) following manufacturer's instruction. RNA samples were treated with DNase I and reverse transcription was performed with a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Real-time PCR was performed using the Taq Man Gene expression assay (Applied Biosystems), following the manufacturer's instructions, on cDNA specimens using 1X Universal PCR master mix (Thermo Fisher Scientific) and 1X 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 data for 2- Ct analysis.

Brain imaging

Brain imaging studies included simultaneous magnetic resonance- positron emission tomography (MR-PET) imaging using the radiotracer [11C]-PBR28. [11C]-PBR28 was produced in-house as previously described.[33] The radiotracer was administered as an intravenous 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 to 90 min post 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).

Secondary outcomes included levels of 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 in Loggia et al.[34] Briefly, $SUVR_{60-90min}$ images were fed into a whole brain voxel-wise between-groups analysis (*post*-treatment vs. *pre*-treatment). Non-parametric permutation inference[35] using a paired design was performed using 5,000 permutations and threshold-free cluster enhancement.[36] The resultant statistical maps were family-wise error (FWE) adjusted ($p_{FWE} < 0.05$) to correct for multiple comparisons. In addition to this voxel-wise 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 sub-cortical 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 a Wilcoxon signed-rank test.

Results

Study Population

Twenty-four volunteers were assessed for eligibility; 18 eligible individuals were enrolled due to 6 screen failures (Figure 1). Of the 18 eligible individuals, 2 were never dosed with RNS60 because they developed unrelated medical problems and withdrew consent prior to baseline. Their data from screening was excluded from analysis, leaving a dataset of 16 study participants (Figure 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 Supplementary Table 1 by MedDRA preferred term). No serious adverse events related to RNS60 occurred and no participant withdrew from the trial due to drug-related adverse events. Eighty-one percent of study participants (N=13) completed 23 weeks of RNS60 treatment (Figure 1). Withdrawals were due to disease progression (N=2) and relocation to a different state (N=1). Compliance with I.V. 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 Supplementary figure 2.

Of the 13 participants who completed the trial, 6 elected to remain on study drug (optional extension) and 4 remained on RNS60 for a total of 47 weeks. The two 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, five were high- and four were mixed-affinity binders. Whole-brain voxel-wise analysis revealed no significant difference in $[^{11}C]$-PBR28 $SUVR_{60-90\ min}$ between *pre*-treatment scans and *post*-treatment scans. ROI analysis did not show statistically significant changes in $[^{11}C]$-PBR28 uptake when *pre*-treatment scans were compared to *post*-treatment results. (Figure 2).

Bio-fluid 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]) (Figure 3).

Discussion

The trial met its primary endpoints, demonstrating the feasibility, safety, and tolerability of administering RNS60 long-term in people 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 U.S. [2] Since then, the ALS community has developed considerable experience with long-term I.V. drug administration though 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 intravenous access as is now done routinely for edaravone therapy in the U.S.

RNS60 treatment was safe and well-tolerated. No serious adverse events related to RNS60 occurred and no participant withdrew from the trial due to drug-related adverse events. While 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.

This study cannot provide any conclusions about the effects of RNS60 on ALS progression since 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 stable over the study period.

Whether this finding reflects the therapeutic effect of the drug or is due to chance is unknown. 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 SOD1[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 CSF of ALS patients compared to 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 non-significant 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 neuro-inflammation in the motor regions. Both the ROI and exploratory whole brain approaches did not reveal a significant difference in the *pre* vs. *post* treatment timepoints 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 to 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 neuro-inflammation 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 neuro-inflammation.

In conclusion, we demonstrated the feasibility, safety, and tolerability of long-term administration of RNS60 in people with ALS. This study builds on promising data in ALS pre-clinical models[27] and represents the first translational step in the development of RNS60 as a potential therapeutic for ALS. A large, multi-center, 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).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

Abbreviations

AEs	adverse events
ALS	amyotrophic lateral sclerosis
ALS-FRS-R	ALS Functional Rating Scale – Revised
ATLIS	Accurate Test of Limb Isometric Strength
CNS	central nervous system
CSF	cerebrospinal fluid
(c)DNA	(complementary)Deoxyribonucleic acid
Ct	cycle number
FDA	food and drug administration
FOXP3	forkhead box P3
FWE	family-wise error
IL-17	interleukin 17
IRCCS	Istituto di Ricovero e Cura a Carattere Scientifico
I.V.	intravenously
LPLV	Last Patient Last Visit
MGH	Massachusetts General Hospital
MND	motor neuron disease
(MR)-PET	(magnetic resonance) - positron emission tomography
(m)RNA	(messenger) ribonucleic acid
ROI	region of interest
RT-PCR	real time polymerase chain reaction
SUV(r)	standardized uptake value (ratio)

T regs	regulatory T cells
TSPO	Translocator protein
(S)VC	(slow) vital capacity
UMN-B	upper motor neuros - burden

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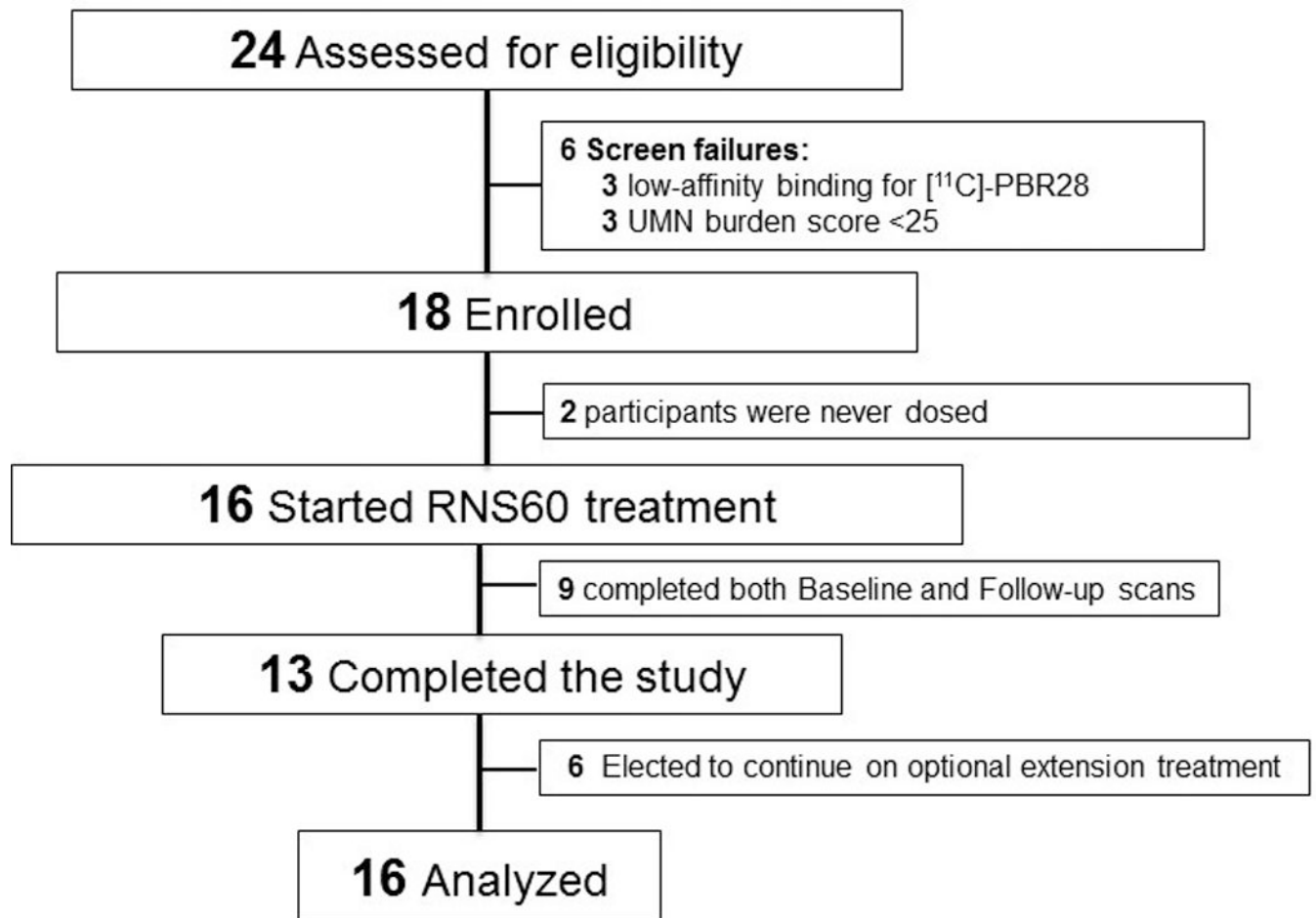
Figure 1. CONSORT diagram: participant enrollment and follow-up for the trial.

Twenty-four volunteers with ALS were assessed for eligibility. Eighteen enrolled in the trial but two 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.
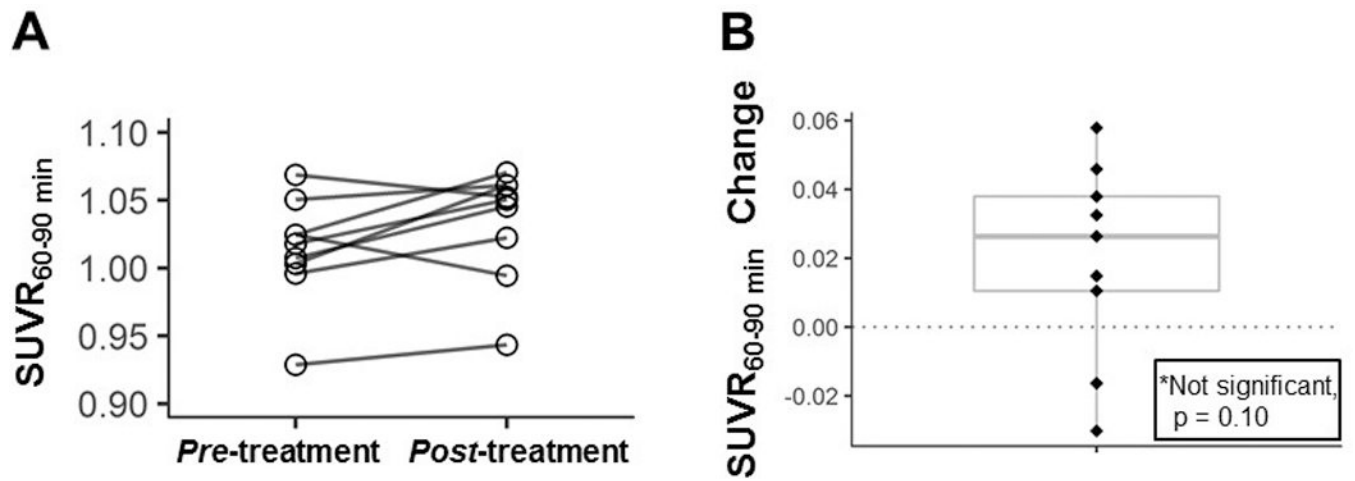
Figure 2. [^{11}C]-PBR28 uptake within the Region of Interest (ROI).
Panel A: [^{11}C]-PBR28 uptake (expressed as SUVR$_{60-90min}$) for each individual participant.
Panel B: individual changes in [^{11}C]-PBR28 uptake (expressed as SUVR$_{60-90min}$) from *pre*-treatment to *post*-treatment scans. There 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*-treatment and *post*-treatment scans with boxplot showing median and interquartile range (Wilcoxon signed-rank test; mean change in SUVR$_{60-90min}$ was 0.02 [p= 0.10]).
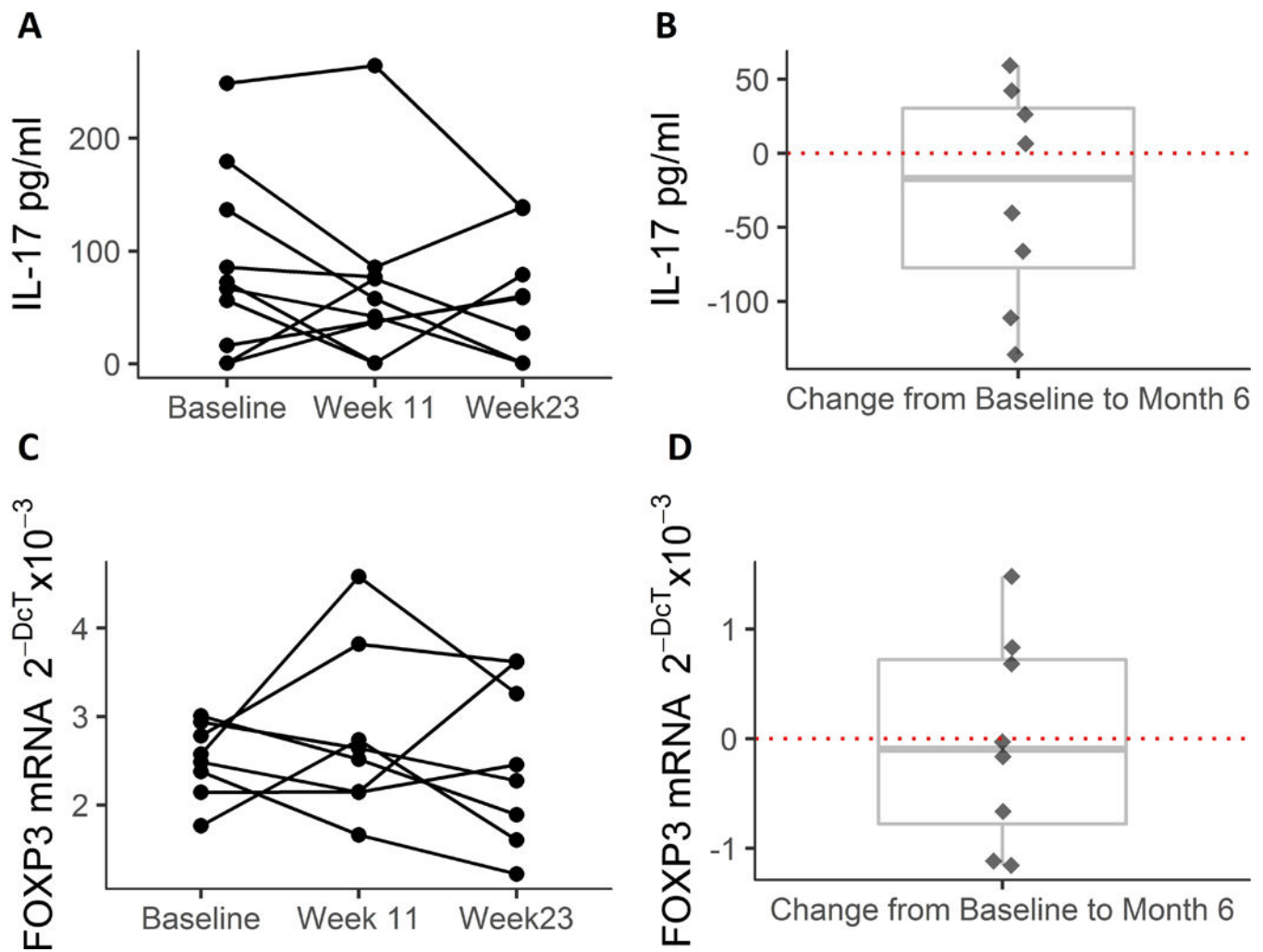
Figure 3. Exploratory inflammatory biomarkers.
Panel A: Individual levels of IL-17 in plasma (N=10).
Panel B: individual changes in IL-17 from *pre*-treatment to *post*-treatment. There were no statistically significant changes over the course of the study with boxplot showing median and interquartile range (Wilcoxon signed-rank test).
Panel C: Individual levels of FOXP3 mRNA expression (a marker of Tregs) in whole blood (N=8).
Panel D: individual changes in FOXP3 mRNA expression from *pre*-treatment to *post*-treatment. There were no statistically significant changes over the course of the study with boxplot showing median and interquartile range (Wilcoxon signed-rank test).

Table 1.

Baseline demographics and clinical characteristics of study participants

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

Data are presented as either percentages or means (±SD). Ranges are indicated in parenthesis as applicable.

Abbreviations: 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 (measured in kg/m^2). EEC: El Escorial Criteria. N= number of subjects. SD=standard deviation. 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 Mean ± SD (Min-Max)	−1.1 ± 1.72[†] (−2.3, −0.2)
SVC Mean ± SD (Min-Max)	−0.6 ± 4.41 (−4.9, 1.9)
ATLIS Arm Measures (%) Mean ± SD (Min-Max)	−1.7 ± 3.12[†] (−4.2, 0)
ATLIS Leg Measures (%) Mean ± SD (Min-Max)	−1.7 ± 5.61[†] (−8, 1)

Data are presented as means (±SD) (expressed as change per month). Minimum and maximum changes are shown in parenthesis.

Abbreviations: ALSFRS-R= ALS functional rating scale revised. ATLIS: Accurate Test of Limb Isometric Strength (results are presented as percent of predicted values)[32]. N= number of subjects. SD=standard deviation. SVC= slow vital capacity (results are presented as percent of predicted values for age, gender and height).

[†]Denotes statistically significant Wilcoxon for change at Week 23 compared to Baseline ($p < 0.05$).